Contact allergen-specific T cell responses and the involved T cell receptor repertoires

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department

of Biology, Chemistry, Pharmacy

of Freie Universität Berlin

by

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from Ulm

Berlin, 2022

The experimental work for this thesis was carried out at the German Federal Institute for Risk Assessment (BfR) in Berlin from June 2018 to August 2022 under the supervision of Prof. Dr. Dr. Andreas Luch.

1st Reviewer: Prof. Dr. Dr. Andreas Luch

2nd Reviewer: Prof. Dr. Gerhard Wolber

Date of defence: 23.05.2023

Declaration of Independence

I hereby declare that I have independently prepared this thesis entitled

“Contact allergen-specific T cell responses and the involved T cell receptor repertoires”

without the use of any other than the approved resources. All citations are marked as such. The submitted work has not been accepted or found to be unsatisfactory in any previous doctoral proceedings.

Berlin, 12.12.2022

Acknowledgement

At this point, I would like to express my gratitude to all the people who have supported me during my thesis.

First of all, I would like to thank Prof. Dr. Dr. Andreas Luch for giving me the opportunity to conduct my thesis in the Department of Chemical and Product Safety at the Federal Institute for Risk Assessment. As my supervisor, he always offered advice as well as support and gave me the freedom to develop my work independently. Furthermore, I would like to thank Prof. Dr. Gerhard Wolber for acting as the second reviewer for this thesis.

I am extremely grateful to Dr. Katherina Siewert, without whom this work would not have been possible. Dr. Siewert supported me with her immense knowledge and practical experience. Special thanks to her for proofreading all my publications and this thesis several times. In addition, I am very thankful for the advice, practical help, and emotional support of the members of the Allergy Group, especially Dr. Marina Aparicio Soto and Dr. Caterina Curato.

I could not have accomplished this journey without my colleagues, who always listened, supported, and encouraged me. A special thanks must go to Dr. Josephine Kugler and Dr. Thomas Tietz for their valuable discussions, moral support, and proofreading of this thesis.

To conduct my thesis, I needed more than academic support, and I have to thank many people who have made the last few years entertaining and sometimes had to put up with me. Thank you for supporting me with optimism, sarcasm, and your friendship, and for getting me through the Corona period, Charlotte Kromer and Konstantin Simon. Without you guys, I would never make it to the bouldering hall and would have a lot less fun at work. In addition, I would like to acknowledge the current and former doctoral students in Department 7, especially the GZSZ office, for a fun and supportive work environment. I also would like to thank the (former) members of the doctoral students' representation for the interesting exchange across departmental boundaries and the exciting challenges that have challenged us beyond our scientific work.

Outside of my scientific work, I am extremely grateful to my life companion Tony Bewersdorff for his moral support, for constantly motivating me, and for always putting up with me during my doctoral thesis. Thank you for being my test listener for presentations, proofreading the thesis and giving me scientific input. A huge thank you goes to my friends in the far south, especially Ayla Schulz for her advice and friendship.

Finally, I would like to express my gratitude to my parents for always supporting and encouraging me to finish my thesis. I am grateful for the lessons you have taught me that have helped shape me into the person I am today.

Zusammenfassung

Die allergische Kontaktdermatitis (*allergic contact dermatitis*, ACD) ist eine T-Zell-vermittelte Erkrankung. Etwa 20 % der Bevölkerung in Industriestaaten sind von mindestens einer Kontaktallergie betroffen[1]. Bislang wurde eine *in vitro* T-Zellaktivierung aufgrund technischer Limitationen jedoch nur für einige wenige Kontaktallergene beschrieben.

Im Rahmen dieser Dissertation wurde ein Assay entwickelt, der mittels Aktivierungs-induzierten Oberflächenmarkern (*activation-induced marker*, AIM) in Kombination mit Durchflusszytometrie die T-Zellaktivierung nach Stimulation mit chemischen Allergenen detektiert. Für diese Arbeit wurden die Metallsalze $NiSO_4$, $CoCl_2$ und $PdCl_2$ sowie das organische Modellallergen 2,4,6-Trinitrobenzolsulfonsäure (TNBS) als chemische Allergene ausgewählt. Die mRNA der an der Aktivierung beteiligten T-Zell-Rezeptoren (TZR) wurde mittels Hochdurchsatzsequenzierung (*high-throughput sequencing,* HTS) sequenziert.

Der AIM-T-Zell-Assay ermöglicht eine schnelle, sensitive, quantitative und umfassende Analyse Allergen-spezifischer, naïver und Gedächtnis CD154+CD4+- oder CD137+CD8+ T-Zellen. Parallel können weitere assoziierte Oberflächen- und intrazelluläre Marker, exprimierte Zytokine und das TZR-Repertoire untersucht werden. Darüber hinaus sind keine vorherigen Kenntnisse über die beteiligten TZR-Bindungspartner nötig. Mögliche Bindungspartner sind der Haupthistokompatibilitätskomplex (*major histocompatibility complex*, MHC), das präsentierte Selbstpeptid und, im Falle der ACD, das chemische Allergen. Die Durchführung des AIM-Assays erlaubt die Verwendung hoher Konzentrationen des chemischen Allergens, die es ermöglichen den gesamten reaktiven T-Zell-Pool zu erfassen.

Ungewöhnlich hohe Frequenzen metall- und TNBS-spezifischer CD154+ CD4+ Gedächtnis-T-Zellen wurden in mononukleären Zellen des peripheren Blutes (*peripheral blood mononuclear cells*, PBMC) von allergischen und nichtallergischen Spendern beobachtet. Nur einige Nickel- und/oder Kobaltallergiker konnten aufgrund deutlich höherer Frequenzen von Nichtallergikern unterschieden werden. Die Spezifität der aktivierten T-Zellen wurde durch die Analyse zusätzlicher Marker bestätigt. Weiterhin ergaben sich erste Hinweise auf die *in vivo* Relevanz der identifizierten Metall-spezifischen T-Zellen. Die Aktivierung war TZR-vermittelt und MHC-abhängig, wie die Restimulation einzelner Klone zeigte.

Zusammenfassung

Mehrere Mechanismen der Allergenerkennung von T-Zellen, die der ungewöhnlich starken T-Zellaktivierung zugrunde liegen, wurden durch die Analyse des TZR-Repertoires aufgeklärt. Im TZR Chemikalien-spezifischer T-Zellen wurden spezifische Bindungsstellen für Metallionen und TNBS identifiziert. Metall-spezifische TZR exprimierten signifikant häufiger bestimmte variable Gensegmente. Im Fall von Ni^{2+} wurde damit die Relevanz eines früheren Berichts aus der Literatur über einen Ni^{2+}-spezifischen TRAV9-2+ T-Zell-Klon bestätigt[2].

Ein zweiter, unabhängiger Mechanismus der Chemikalien-spezifischen T-Zellaktivierung ist die Bindung von Allergenen an Aminosäuren in der komplementaritätsbestimmenden Region 3 (*complementary determining region 3,* CDR3), der variabelsten Bindungsstelle des TZR. Für Metallionen war ein Histidin die bevorzugte Bindungsstelle in der CDR3. Die exakte Position des Histidins innerhalb der CDR3-Sequenz war dabei allerdings variabel. Die Bindung von TNBS an CD154+CD4+ und CD137+CD8+ T-Zellen erfolgte im Gegensatz dazu spezifisch an ein Lysin oder Tryptophan an einer bestimmten Stelle der CDR3 der TZR β-Kette.

Die Bindung von verschiedenen Metallionen an ein CDR3-Histidin deutet auf eine Kreuzreaktivität der beteiligten TZR hin. Kreuzreaktivität ist die Erkennung verschiedener Metallallergen-induzierter Epitope oder Metallionen durch dieselbe T-Zelle. Eine solche Kreuzreaktivität kann nur schlecht durch Patch-Tests beurteilt werden. Eine Alternative zum Patch-Test stellt die Kombination aus AIM-Assay, TZR HTS und bioinformatischer Datenanalyse für die intra- und interindividuelle TZR-Kongruenzanalyse dar, die in dieser Arbeit weiterentwickelt wurde. Es wurde eine umfangreiche Kreuzreaktivität der metall-spezifischen TZR beobachtet, die mit den identifizierten Repertoire-Merkmalen einhergeht. Diese Kreuzreaktivität wurde für einzelne T-Zell-klone bestätigt.

Die Herausforderungen bei der Entwicklung neuartiger *in vitro* T-Zell-Assays zum Nachweis Chemikalien-spezifischer T-Zellen sind unter anderem die Bewertung der Toxizität und der Fluoreszenz-Interferenzen der eingesetzten Chemikalie. Zusätzlich muss die Art der Chemikalien-induzierten T-Zell-Epitopbildung für jede neue Chemikalie analysiert werden. Der AIM T-Zell Assay bietet eine effiziente Lösung für diese Herausforderungen. Daher hat der AIM T-Zell Assay das Potenzial, die nächste Generation von *in vitro* Tests für die Allergiediagnose und die regulatorische Risikobewertung zu begründen.

Abstract

Allergic contact dermatitis (ACD) is a T cell-mediated disease. Approximately 20% of the population in industrialised countries is affected by at least one contact allergy[1]. However, due to technical limitations, *in vitro* T cell activation has only been described for a few contact allergens so far.

Within the scope of this thesis, an activation-induced marker (AIM) assay in combination with flow cytometry was established to detect T cell activation following stimulation with chemical allergens. The metal salts $NiSO_4$, $CoCl_2$ and $PdCl_2$ and the organic model allergen 2,4,6-trinitrobenzene sulfonic acid (TNBS) were selected as chemical allergens. mRNA of T cell receptors (TCR) involved in the activation were sequenced via high-throughput sequencing (HTS), and TCR repertoires were analysed. The AIM T cell assay enables the fast, sensitive, quantitative, and comprehensive analysis of allergen-specific, naïve and memory CD154+CD4+ or CD137+CD8+ T cells, respectively. The assay setup allows cell sorting for downstream experiments. In parallel, other associated surface and intracellular markers and expressed cytokines can be examined. In the AIM T cell assay, high chemical concentrations are tolerated, which capture the complete reactive T cell pool. In addition, no prior knowledge of the TCR binding partners is required. TCR epitopes comprise proteins of the major histocompatibility complex (MHC), the presented (self-) peptide, and the chemical allergen in the case of ACD.

Unusually high frequencies of metal- and TNBS-specific CD154+CD4+ T cells were observed in peripheral blood mononuclear cells (PBMC) from allergic and non-allergic donors. Due to significantly higher frequencies, some nickel and cobalt allergic individuals could be distinguished from non-allergic individuals. The specificity of the activated T cells was confirmed by analysing additional activation markers. Furthermore, the first evidence for the *in vivo* relevance of the identified metal-specific T cells emerged. The metal-specific T cell activation was TCR-mediated and MHC-dependent, as shown by single clone restimulation.

Several mechanisms of chemical allergen recognition by T cells underlying the unusually strong T cell activation were elucidated by analysis of the TCR repertoires and improved bioinformatic data processing. Thereby, specific binding sites for metal ions and TNBS were identified in the TCR of chemical-specific T cells.

Metal-specific TCR more frequently contained individual variable gene segments. Among metal-specific TCR, the protein segments encoded by the gene segments TRAV9-2, TRAV2 and the TRBV4 family were involved in the binding of Ni^{2+}, Co^{2+} or Pd^{2+} ions, respectively. In the case of Ni^{2+}, the results confirmed the relevance of a previous literature report on one Ni^{2+}-specific TRAV9-2+ T cell clone[2].

As a second, independent mechanism of chemical-specific T cell activation, the binding of allergens to amino acids in the complementary determining region 3 (CDR3) was described for the first time. The CDR3 is the most variable binding site of the TCR. For metal ions, histidine was the preferred binding partner. The interacting histidine residue within the CDR3 was not restricted to a particular position. In contrast, binding of TNBS to CD154+CD4+ and CD137+CD8+ T cells occurred selectively to a lysine or tryptophan residue at a specific position in the CDR3 of the TCR β-chain.

The shared binding of different metal ions to a certain CDR3 histidine residue suggests the cross-reactivity of the TCR involved. Cross-reactivity is the recognition of different metal allergen-induced epitopes by the same T cell, e.g. by interchangeable metal ion binding. Cross-reactivity can hardly be assessed by patch testing. An alternative is the combination of the AIM assay, TCR HTS, and bioinformatic data analysis for intra- and interindividual TCR congruence analysis, which was further developed in this work. Extensive cross-reactivity of metal-specific TCR associated with the identified repertoire features was also observed and confirmed on a single T cell clone level.

Challenges in developing novel *in vitro* T cell assays to detect chemical-specific T cells include assessing the toxicity and fluorescence interferences of the chemicals used. In addition, the nature of chemical-induced T cell epitope formation must be considered for each new chemical. AIM T cell assays offer an efficient solution to these challenges, as this thesis demonstrates. Therefore, AIM T cell assays can potentially establish the next generation of *in vitro* assays for allergy diagnosis and regulatory risk assessment.

Abbreviations

A

AIM	activation-induced marker
AIRR	*adaptive immune receptor repertoire*
APC	*antigen-presenting cell(s)*

B

B cell	*bone marrow lymphocyte*
BB	*bandrowski's base*
BCR	*B cell receptor(s)*
bp	*base pair(s)*

C

CCR	*C-C chemokine receptor*
CDR	complementarity determining regions
C-*gene segment*	constant *gene segment*
CLA	*cutaneous lymphocyte-associated antigen*
CMV	*cytomegalovirus*
Co^{2+}	*cobalt ion(s)*

D

DAMP	*damage-associated molecular pattern(s)*
DC	*dendritic cell(s)*
D-*gene segment*	*diversifying gene segment*
DNA	deoxyribonucleic acid

F

Fab	*fragment antigen binding*
Fc	*fragment crystallisable*

H

HLA	*human leukocyte antigen*
HTS	*high-throughput sequencing*

I

IFN	*Interferon*
Ig	*immunoglobulin*
IL	*interleukin*
ILC	*innate lymphoid cell(s)*
IMGT	*international immunogenetics information system*

J

J-*gene segment*	*joining gene segment*

L

LFA	*lymphocyte function-associated antigen*
LLNA	*local lymph node assay*
LTT	*lymphocyte transformation test(s)*

M

MHC	major histocompatibility complex

N

Na_2PdCl_4	*sodium tetrachloropalladate*
Ni^{2+}	*nickel ion(s)*

P

PAMP	*pathogen-associated molecular pattern(s)*
PBMC	*peripheral blood mononuclear cell(s)*
Pd^{2+}	*palladium ion(s)*
PPD	p-phenylenediamine
PRR	*pattern recognition receptor(s)*

R

RAG	*recombination activating gene*

S

SEB	*staphylococcal enterotoxin B*

T

T cell	*thymus lymphocyte*
TCR	T cell receptor(s)
T_{FH}	*follicular helper T cell(s)*
TGF	*transforming growth factor*
Th	*T helper cell(s)*
TLR	*toll-like receptor(s)*
TNBS	*2,4,6-trinitrobenzenesulfonic acid*
TNP	*2,4,6-trinitrophenol*
Treg	*regulatory T cell(s)*
TT	*tetanus toxoid*

U

UMI	*unique-molecular identifier*

V

V-gene segment	*variable gene segment*

Table of content

1. Introduction

The human immune system is a complex decentralised network composed of a multitude of tissue structures, effector cells and molecules. The immune system evolved as the body's defence against infections with pathogenic bacteria, viruses, fungi, and parasites. Additionally, it is also capable of destroying cancerous cells. At the same time, the immune system must maintain tolerance to harmless foreign substances (e.g. food) and natural microbial flora of wide variety (e.g. on surfaces such as the skin). If tolerance fails, allergies and autoimmune diseases may develop. The mammalian immune response may be divided into primary innate defence mechanisms, induced innate responses, and adaptive immunity[3]. Together, innate- and adaptive immunity efficiently protect the body from most harmful substances, viruses and bacteria and enable biological life as we know it[4]. To understand what happens during an aberrant reaction of the immune system, e.g. during allergic contact dermatitis (ACD), the processes involved in maintaining a healthy immune system must first be understood. The three parts of the immune system and the possible adverse immunologically mediated hypersensitivity reactions are discussed below.

1.1 Innate immunity and its link to adaptive immunity

Several tissue structures, as well as cellular and molecular players, are considered to contribute to innate immunity. Of these, epithelia are the first major physical barrier against pathogens. Epithelial layers are located on all contact surfaces of the body with the environment (skin, respiratory tract, gastrointestinal tract) and are specialised depending on the area[5, 6, 7]. A microorganism or chemical may enter the body by simply crossing or upon binding to an epithelium. The physical barrier function of epithelial layers is supported by phagocytes and chemical defences[8].

High quantities of small molecules can pass through the epithelium, depending on their size and physicochemical properties[9, 10]. Most molecules are not harmful to the body as they may not be present in quantities that pose toxicological risks, such as acute or specific organ toxicity. However, allergic reactions may occur if the immune system overreacts to these compounds. To act as an allergen, molecules must react with the body's proteins to form chemically modified (“haptenated”) proteins. If the allergic reaction is characterised by eczema, it is called ACD. In contrast, if chemicals directly damage the skin, it is referred to as irritant contact dermatitis. The skin is a frequent entry route for contact allergens, especially if it is damaged by scratches or, for example, piercings[11]. Possible pathways for penetration into the skin are straight through cells (intracellularly), through gaps between cells (intercellularly), or through appendages such as hair follicles and ducts of perspiratory

glands[12]. In addition, Langerhans cells could contribute to chemical uptake by passing their dendrites through the tight junction barriers during tissue inflammation[13]. Langerhans cells represent a subpopulation of the dendritic cells (DC) which, among others, act as antigen-presenting cells (APC). APC are part of the interconnection between innate and adaptive immunity as they can present chemical-induced T cell epitopes to cells of the adaptive immune response[14]. A distinction is made between conventional body cells that act as APC and primarily express the cell surface protein major histocompatibility complex (MHC) I and professional APC that express MHC II along with certain co-stimulatory signals[15]. Professional APC such as B cells and DC are essential for adaptive immunity. The diverse group of DC can be found in all body tissues where they are involved in *de novo* adaptive immune responses. DC take up microorganisms that are processed by immunoproteasomes into peptide antigens containing a hydrophobic C terminus. The expression of immunoproteasome proteins is induced by oxidative stress and proinflammatory cytokines[16, 17]. The produced peptide fragments then may optimally fit into the binding grooves of MHC and are eventually presented on the cell surface. The detailed mechanisms of antigen presentation of DC and the recognition by T cells are further discussed in sections 1.3 and 1.4.

Besides their function as APC, DC and macrophages are sensor cells that detect infections and produce inflammatory mediators that trigger responses from the innate immune system. Most sensor cells are also phagocytes that are located beneath the epithelial barrier. They can ingest and destroy invading microorganisms. In addition to macrophages and DC, monocytes and granulocytes do also exert phagocytic activity. Macrophages are found in most body tissues, especially connective tissue[18, 19].

Monocytes develop in the bone marrow and circulate in the blood. During infection, they migrate into tissues and develop into activated inflammatory monocytes or macrophages. Granulocytes include neutrophils, eosinophils, and basophils. In a healthy organism, they are found in large numbers only in the blood and are short-lived. These sensor cells of the innate immune system express pattern recognition receptors (PRR) that recognise pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). PRR are very conserved in their evolution and have their roots in prokaryotic genes. PAMP enable the recognition of microbial surfaces as they are molecules that are present in many pathogens but not in cells of the human body. DAMP are endogenous molecules that are released upon cell damage, or infection of cells and thereby induce cellular stress[20, 21]. One example for PRR are the toll-like receptors (TLR). TLR recognise PAMP and DAMP and induce proinflammatory and antiviral cytokines[22]. The cytokines then attract other phagocytotic cells to the site of infection and can trigger other systemic effects such as fever.

Sensitising chemicals, for example, metal ions, may bind to PRR and, therefore, functionally mimic PAMP. Nickel ions (Ni^{2+}) can bind to two chelating histidine residues on the human TLR4 to induce

a proinflammatory response. The binding of Ni^{2+} to TLR4 triggers co-stimulatory signals through the induction of proinflammatory gene expression. The co-stimulatory signals seem to contribute to the promotion of sensitisation and subsequent elicitation in ACD[23]. A similar effect has also been described for other transition metals like cobalt (Co^{2+}) and palladium ions (Pd^{2+})[24]. Reactive chemicals can also activate the innate immune response by triggering the release of DAMP through the induction of reactive oxygen species (ROS) and the activation of the inflammasome[25, 26].

Besides their sensor function, DC produce cytokines that trigger innate lymphoid cells (ILC), which are some sort of effector cells of the innate immune system[27, 28]. ILC arise in the bone marrow from the same common lymphoid progenitors as B and T cells. Various types of ILC (ILC1-3) secrete various cytokines, contributing to the defence against intracellular and extracellular bacteria, viruses, and fungi. In addition, they support the physical barrier. Overall, the ILC amplify the immune response through specialised effector activities[29].

Another defence mechanism supporting the combat against pathogens is the complement system. It plays a major role in the innate immune response, but it also interacts with antibodies, B and T cells of the adaptive immune system. Additionally, the complement system influences tumour growth, tissue regeneration and several pathological conditions. The complement system comprises over 30 different plasma proteins produced primarily in the liver and circulate in an inactive form in the blood and other body fluids[30]. Three pathways can activate the complement, which aim to opsonise pathogens, lyse infected or transformed cells, and attract other immune cells through chemokines (i.e., chemoattraction)[31, 32]. In addition, the system can facilitate phagocytosis, attack membranes, or trigger inflammatory responses to facilitate the fight against the pathogen. The complement system also affects cytokine production by APC, which in turn influences adaptive immunity[31, 33].

In summary, one of the major tasks of the innate immune system is to recognise infection or cellular disturbances by chemical irritation and to fight the progress of associated diseases in the body. If the infectious agents override these initial control mechanisms, the innate immune response may be able to keep the pathogen at bay until the adaptive immune response is fully triggered. In addition, the innate immunity is crucial for the subsequent development of adaptive immune responses.

1.2 Adaptive immunity

In addition to the innate immune response, the adaptive immune response acts as a second highly effective layer of protection against pathogens. The adaptive immune response is characterised by its high adaptability to new or changed pathogens. However, for the *de novo* expression of antigen-specific antibodies and the clonal expansion of antigen-specific T cells, the adaptive immune response requires significantly longer reaction times of up to 14 days.

The basis of adaptive immunity are the highly specialised and highly variable receptors of the cells of the lymphatic system, the thymus (T cell) and bone marrow (B cell) derived lymphocytes[34]. In addition, B cells express antibodies, also called soluble B cell receptors (BCR). The T cell receptors (TCR), BCR, and antibodies are genetically highly variable, as they arise from the process of variable (V-), diversifying (D-), and joining (J-) gene segment recombination. Once an infection has been overcome by the adaptive immune system, long-lived antigen-specific plasma cells, memory B, and memory T cells remain to protect the organism from re-infection. These cells can respond quickly with a powerful effector response to the same pathogen even month and years after the first contact. Several subsets of memory cells, including tissue-resident populations, have been described[34, 35].

Since T cells, and especially its TCR, are essentially involved in the pathogenesis of ACD, the development of this T cell surface receptor protein will be discussed in more detail below. B cell development will also be shortly described to understand an immune response comprehensively. In addition, the interaction of the different adaptive immune cells will be considered in conjunction with the innate immune response.

1.2.1 V(D)J gene recombination underlies immune cell receptor diversity

Two major factors contributing to the high diversity of the antigen binding sites of BCR (antibodies) and TCR are the V(D)J gene recombination and the junctional insertion and deletion of nucleotides (indels) that occur during this process[36]. Although the human genome consists of about 3 billion nucleotide base pairs (bp), this space is not nearly large enough to encode the observed diversity of antigen receptors[37]. Of the major receptor binding sites, the complementarity determining regions (CDR), two (CDR1 and 2) are encoded in gene segments that are randomly rearranged during somatic deoxyribonucleic acid (DNA) recombination. The third, CDR3, spans the indel junction where an individual coding sequence arises.[38].

In the following, V(D)J recombination will be explained using the T cell receptor with an α and β protein subunit. For the T cell, the different gene segments are recombined in the thymus. The TCRα gene locus consists of approximately 70 V- and 61 J-gene segments. The TCRβ gene locus consists of 52 V-,

2 D- and 13 J-gene segments. In addition, both gene loci express only one or two constant (C-) gene segments (**Figure 1**)[3].

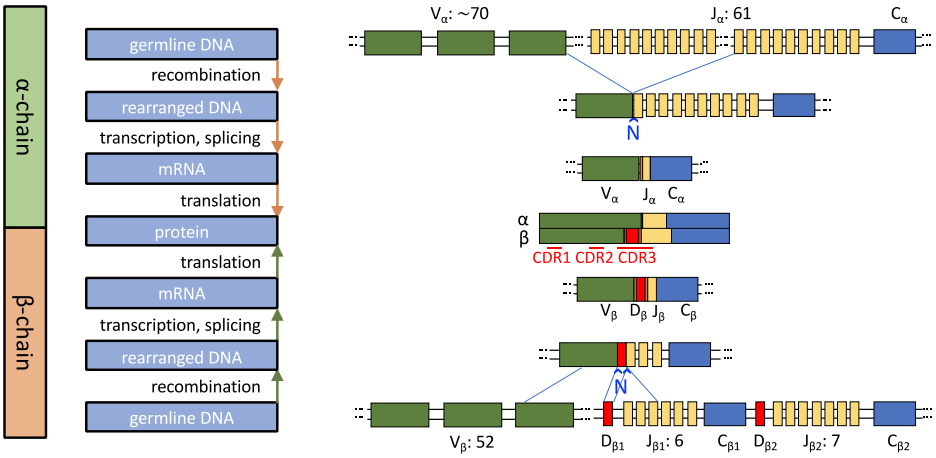

Figure 1: T cell receptor (TCR) variability is mainly achieved through V(D)J gene recombination.

During the somatic recombination of different TCR gene segments, additional insertions and deletions of nucleotides occur at the junction sites (indels, N, blue). Because of this diversifying process, the most variable antigen binding site, the complementarity determining region (CDR) 3 (in red), arises. V – variable segment, D – diversifying segment, J – joining segment, C – constant segment. The graph depicts the assembly of αβ TCR as an example. Generation of γδ TCR or immunoglobulins ("antibodies") occurs in a similar process. Modified from Janeway's Immunobiology[3].

The *recombination activating gene* 1 (*RAG*1) and *RAG*2 encode the lymphocyte-specific recombinase (*RAG* proteins), which initiate the rearrangement of the gene segments. For a complete TCR chain to be expressed, DNA rearrangements must be regulated so that a V-gene segment is only connected to one D- and/or J-gene segment. Therefore, rearrangements are controlled by conserved non-coding DNA sequences (conserved recombination signal sequences). During V(D)J recombination, *RAG* proteins bind to and cleave DNA, resulting in double-strand breaks between the V-, D-, and J-gene segments and the flanking recombination signal sequences[39]. Afterwards, the ends of the recombination signal sequences are joined by a large group of DNA repair enzymes. However, the joining of the coding ends is imprecise, resulting in the random insertion and deletion of nucleotides at the ends of the coding sequences. This further increases the variability drastically[36, 40].

The messenger ribonucleic acid (mRNA) is obtained by rearranged DNA transcription and the subsequent splicing and is translated into protein. Theoretically, 10^{15} to 10^{61} individual αβTCR can be generated, but the body only contains ~10^{12} T cells, leading to different TCR repertoires in each individual[41, 42, 43, 44].

In antibodies and BCR, different gene segments are recombined in the heavy and light chains in a similar process[3]. As a result, the variable region can consist of an almost infinite number of different amino acid sequences, which can recognise just as many antigens. Due to the greater variability of functional properties of the antibody compared to the TCR, diversification of its constant region is also important[45].

1.2.2 Structure and function of adaptive immune cell receptors

The highly specialised antigen receptors expressed by B- and T cells, BCR (antibodies) and TCR are responsible for antigen-specific recognition. Because of the V(D)J recombination, each individual B and T cell ("clonotype") expresses only one particular BCR and TCR, respectively, which gets passed down during cell division.

Antibodies bind pathogens in the extracellular space, facilitating antigen recognition by phagocytic cells. In this process, antibodies can have a neutralising or opsonising effect and activate the complement system[46, 47, 48]. Antibodies consist of a constant fragment crystallisable (Fc) region and a variable fragment antigen binding (Fab) region (**Figure 2**). The Fc fragment can occur in five forms, i.e. immunoglobulin (Ig) A, IgD, IgE, IgG and IgM. The suffix specifies the kind of heavy chain that the antibody contains. The parts of the heavy chains inside the hinge and Fc region determine the effector function of the antibody[47, 49]. The variable region can consist of an almost infinite number of different amino acid sequences, which can recognise just as many antigens. The antibody is composed of two identical heavy and light chains containing a variable and constant region (**Figure 2**). There are two different types of light chains (Lambda-(λ-) and Kappa-(κ-)chain), but only one type occurs in an antibody at a time. There is no functional difference between the two light chains[47].

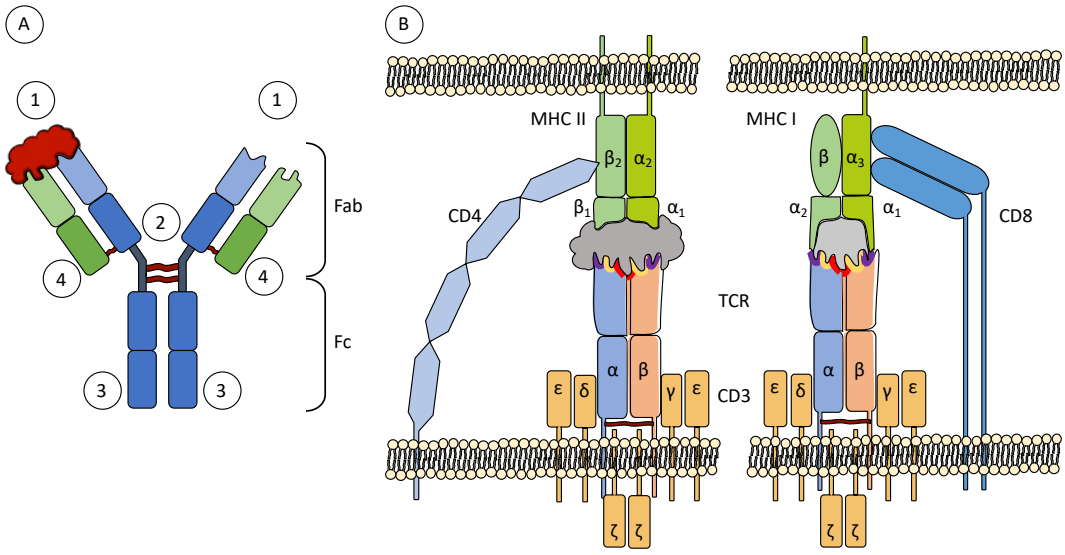

Figure 2: Structure and antigen recognition modes of adaptive immune cell receptors.

A) B cell receptor/antibody: 1) The antigen-binding site (paratope) interacts with a conformational antigen structure (epitope), e.g. on a protein (red), 2) hinge region, 3) heavy chains (blue), 4) light chains (green), brown lines: disulphide bridge. B) T cell receptor (TCR): TCR recognise T cell epitopes that are classically formed by peptides (grey) presented by proteins of the major histocompatibility complex (MHC) (green). The MHC I binding groove accommodates peptides with usually 8-11 amino acids in length. The MHC II proteins present longer peptides (typically 13-17 residues). Most TCR consist of an α- and β-chain, which are connected through a disulphide bridge. Each TCR α - or β -chain consists of a constant and a variable region from which the three main antigen binding regions (complementary determining regions (CDR)1-3, red, yellow, purple) protrude. Additional proteins (δ, ε, γ, ζ) are associated with the TCR, most importantly surface proteins of the CD3 signalling complex (T cell lineage marker). The co-receptors CD4 and CD8 designate distinct T cell subpopulations and support TCR binding to MHC II (CD4) or MHC I (CD8), respectively. Therefore, CD4 and CD8 are mandatory for the successful recognition of T cell antigens.

The BCR structure is identical to the one of antibodies but is located on the surface of B cells. The BCR recognises conformational proteins and surface structures. On the other hand, TCR recognise linear peptide sequences presented on proteins of the MHC complex. Each TCR consists of two polypeptide chains, the TCR α- and β-chains (**Figure 2**). A T cell can carry either an αβ TCR or a γδ TCR on its surface[50]. In peripheral blood, ~95% of the total T cell population are T cells with αβ TCR[51]. T cells express roughly 30,000 identical TCR molecules on their surface[52]. The α- and β-chains of the TCR are connected by a disulphide bridge in the constant region. Each TCR chain consists of an amino- (N-) terminal variable region (consisting of the V(D)J-gene segments), a constant region, a transmembrane region, and a short cytoplasmic region (all encoded by the C-gene segment). The variable region of each TCR chain thus also contains the three CDR that form the antigen binding site of the receptor (**Figure 1**, **Figure 2**)[53].

On the cell surface, the TCR is associated with the T cell lineage marker CD3, which identifies the cell as a T cell. The CD3 antigen forms a signal transduction complex with the TCR. The TCR interacts with the CD3 complex through opposing charges on basic and acidic amino acids on the receptor subunits in the membrane (**Figure 3**)[54]. Besides the CD3 antigen, αβTCR are associated with either CD4 or CD8 coreceptors, by which the CD3 positive T cells are further subdivided. These coreceptors bind to either MHC II (CD4) or MHC I (CD8) proteins at an invariant site, thereby contributing to the stabilisation of the connection. Due to the binding of CD4, the affinity of TCR to antigens is about 100-fold higher[55]. MHC I and MHC II molecules, termed human leukocyte antigen (HLA) in humans, have some structural differences within their subunits but are very similar in their overall structure. MHC I molecules consist of two polypeptide chains. The α chain forms three domains and spans the membrane. The β2-microglobulin only contributes to one (extracellular) domain. The peptide binding groove is highly polymorphic and is formed by the α_1 and α_2 domains. It can bind short peptides of 8 to 10 amino acids in length. MHC II proteins are heterodimers, i.e., they consist of an α- and β-chain. Both span the membrane, and each consist of two domains. The α_1 and β_1 domains form an open peptide binding cleft. Therefore, the length of the bound peptide is not constrained but usually accommodates peptide sequences made of 13-18 amino acids[56, 57].

In the classical αβTCR binding mode, the TCR is diagonally aligned over the peptide-MHC complex[58]. The variable region of the α-chain is located over the N-terminal end of the peptide, the α2 helix of the MHC I, and the β1 helix of the MHC II. The variable region of the β-chain lies over the carboxy-terminal end of the peptide and the α1 helix of both MHC complexes. The binding points of the CDR 1 and 2 to the MHC are mostly conserved[59]. The αβCDR3 sequences encompass the primary contact points interacting with the antigen peptide. The exact binding conformation of TCR CDR loops, the peptide and the MHC may differ for various TCR (Section 1.5.4).

1.2.3 Maturation of adaptive immune cells

Like T cells, B cells are a part of the white blood cells. They develop in the bone marrow in several stages from hematopoietic stem cells. In the first step, V(D)J gene recombination (Section 1.2.1) of the heavy chain locus occurs in pro B cells. The recombination results in the formation of a pre-BCR, which consists of the final heavy chain and a surrogate light chain. Through this receptor, the B cell is getting stimulated, and proliferation starts.

Further gene rearrangements of the BCR light chain result in an immature B cell expressing a complete IgM molecule at the cell surface. Autoreactive B cells are removed from the repertoire during negative selection in the bone marrow as they bind to auto-antigens. The mature B cells can express IgD and IgM on their surface and eventually become activated in secondary lymphoid organs via contacts with foreign antigens[47, 60, 61].

Lymphoid progenitors, the precursors of T cells, also develop from multipotent hematopoietic stem cells in the bone marrow. From there, they migrate via the blood to the thymus. Most steps of T cell development occur in the thymic cortex. The thymus is a primary lymphoid organ and an essential immune system component. In the body, it is located in the upper front of the chest, behind the sternum and in front of the heart. It comprises several lobes, each consisting of a central medulla and an outer cortical region. Upon birth, the human thymus is fully developed. Thus, most T cells are produced until puberty. After that, the thymus recedes and mostly stops producing new T cells (thymic involution)[3].

T cell progenitors receive a signal in the thymus from thymic stromal cells that is mediated via the neurogenic locus notch homolog protein 1 (Notch 1) receptor. This receptor activation determines that cells will develop into T cells rather than B cells[62]. Thereupon, the progenitor cell starts rearranging the TCR genes. In cells that express neither the CD4 nor CD8 co-receptor (double-negative CD4-CD8- cells), V(D)J gene rearrangement of the TCR β-chain begins. Once the genes are rearranged, the β-chain is expressed and paired with a surrogate α-chain. This pre-TCR is expressed on the cell surface and induces cell proliferation in combination with CD3 signals. As a result, the gene rearrangement of the β-chain is completed. Once the following gene rearrangement of the α-chain is completed, a low level of the αβ TCR with the associated CD3 complex is expressed. Additionally, CD4 and CD8 co-receptors are expressed on the cell surface[63]. The double-positive CD4+CD8+ T cells with expressed TCR interact with cortical epithelial cells, expressing a high density of MHC I and II molecules with bound self-peptides. The T cells must bind to a self-peptide-MHC complex during the selection process. If this does not happen, or if the binding is too weak to initiate the intracellular signalling cascade responsible for T cell survival, death by neglect occurs (~90% of CD4+CD8+ T cells, positive selection). If T cells bind too well to self-peptides, apoptosis is also initiated since these cells are potentially autoreactive

(negative selection, ~5% of T cells). The suitable mean level of TCR signalling initiates effective maturation. The optimal binding causes αβ T cells to mature into single positive CD8+ or CD4+ T cells, depending on whether the TCR binds to MHC I or MHC II[63, 64].

In addition to the binding to MHC I or II proteins, a number of transcription factors determine whether single CD4+ or CD8+ T cells are generated[65]. Despite the extensive selection, the thymus releases a vast repertoire of naïve T cells into the bloodstream for further circulation to the lymphoid organs. The mature T cells are continuously exposed to interleukin (IL)-7 to survive and may bind with low affinity to self-peptide-MHC complexes in secondary lymphoid organs. Despite the contact of the TCR with the self-peptide-MHC complex, the T cell remains naïve[66]. If the T cell pool in the blood is depleted, the missing T cells are replaced through homeostatic proliferation[67].

1.3 T cell-mediated immune responses

1.3.1 Priming of naïve T cells

Once activated by innate immune stimulation, professional APC, especially DC, mature and migrate to the draining local lymph nodes. The process of initial cognate antigen recognition (naïve T cell priming) takes place in the draining lymph node, where mature DC accumulate. Naïve T cells and DC are directed to the lymphoid organs by the chemokine (C-C motif) ligand (CCL)21, which binds to the C-C chemokine receptor (CCR)7[68, 69]. The innate signal leads to the upregulation of co-stimulatory molecules (CD40, B7) and the expression of proinflammatory cytokines[28, 70]. DC present short peptide antigens on their MHC complex. Naïve T cells that recognise a peptide-MHC complex with their specific TCR are primed in the presence of appropriate co-stimulatory molecules (**Figure 3**), i.e. they proliferate and differentiate into various possible T effector and memory cell subsets. DC are, therefore, along with cytokines, one of the most critical connection points between innate and adaptive immunity[34, 71].

T cells bind weakly to APC, so the TCR has time to scan the numerous MHC molecules loaded with different antigen peptides. Because of the weak binding, the TCR can also quickly separate if a receptor does not find a suitable specific antigen. This mechanism is enabled by a connection of lymphocyte function-associated antigen (LFA)-1 (CD18 and CD11) and LFA-2 (CD2) cell surface molecules on the side of T cells and intercellular adhesion molecule (ICAM)-1 (CD54), -2(CD102) and LFA-3 (CD58) on the APC[72]. The encounter of antigen-specific T cells and antigen-presenting DC leads to the formation of an immunological synapse[70]. In addition, when a TCR recognises a specific peptide-MHC ligand, a signal triggers a conformational change of LFA-1 and increases its binding affinity[73].

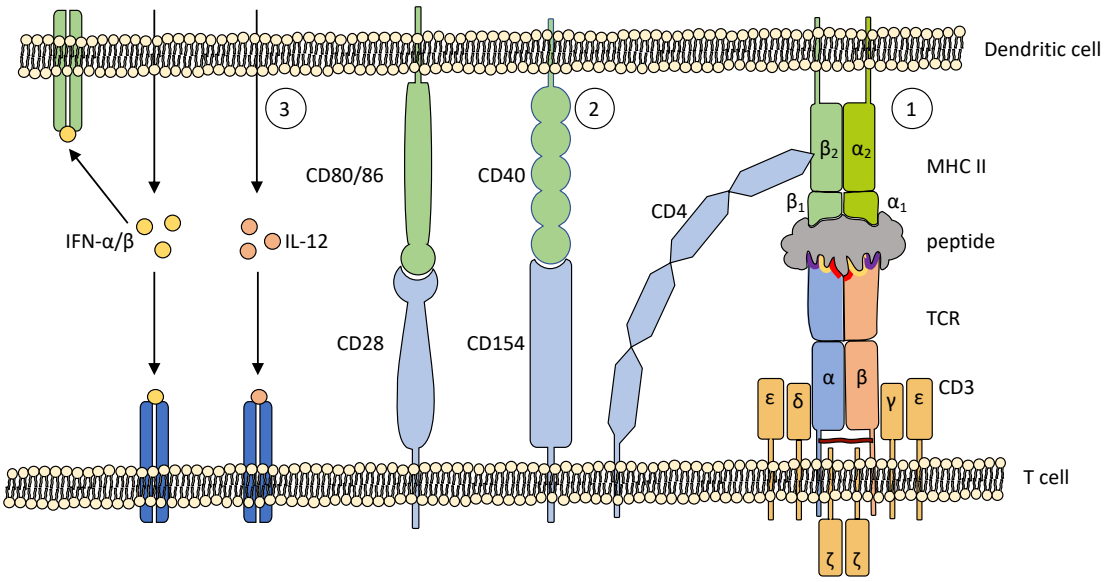

Figure 3: Signals involved in naïve T cell priming.

The binding of the T cell receptor (TCR) to a cognate peptide-major-histocompatibility-complex (MHC) complex alone (signal 1) is not sufficient for a complete activation of naïve T cells. On the T cell membrane, the expression of co-stimulatory molecules like CD154 (CD40L) or CD28 enhance signalling. On the APC side, the respective CD40 and CD80/86 ligands are expressed (signal 2). Cytokine signalling (e.g. Interleukin (IL)-12 or interferon (IFN)-α/β) further completes T cell activation and differentiation (signal 3).

The binding of the TCR to cognate peptide-MHC complexes and the binding of the coreceptor CD4 or CD8 (signal 1, **Figure 3**) is insufficient for clonal expansion and differentiation of naïve T cells. In addition to signal 1, the naïve T cell requires co-stimulatory signals (signal 2, **Figure 3**) that prolong cell survival and guide the differentiation into different subsets of effector cells. For example, the binding of CD28 on T cells to CD80 (B7-1) and CD86 (B7-2) on DC mediates and enhances the immune response and activates T cells to express further co-stimulatory molecules and cytokines[70, 74, 75]. In contrast, there are also cell surface receptors like CD152, the antagonist of CD28, that attenuate the activation signals and thereby prevent an excessive immune response or induce the formation of regulatory T cells (Treg)[75, 76]. Another co-stimulatory signal is the binding of CD40 and CD154 (CD40L). It supports T cell activation and cytokine production as well[77].

Antigen recognition by the TCR and coreceptors causes tyrosine residues in the cytoplasmic domains of CD3, known as immunoreceptor tyrosine-based activation motifs, to be phosphorylated by Src family kinases, thereby triggering TCR signalling[54, 78]. The activation of a series of signal proteins causes the transcription of many specific genes, e.g. the gene for the cytokine IL-2, which is essential for the proliferation and differentiation of T cells.

Signal 3 consists of inflammatory cytokines, for example, IL-12 and Interferon (IFN)-α/β, which provide additional signals for expansion, differentiation, and migration[34, 79]. Once a naïve T cell is activated, it proliferates for four to five days bound to the DC. During this binding, clonal expansion and differentiation occurs.

1.3.2 Effector and memory T cell subpopulations

Naïve CD8+ T cells differentiate mainly into cytotoxic T cells after priming, eliminating endogenous cells infected by viruses or bacteria[80, 81]. Cytotoxic CD8+ T cells kill target cells to fight intracellular pathogens, especially viruses. CD8+ effector T cells are characterised by the surface marker CD45RA but do not express CCR7 and CD45RO on their surface. They develop in several steps from naïve CD8+ T cells via central memory and memory precursor effector to short-lived CD8+ effector T cells[82].

Naïve CD4+ T cells primarily develop into various effector T helper (Th) cells with different immunological functions and regulate the stimulation of other immune cells by secreting cytokines that activate macrophages and B cells, for example[83]. The innate immune system provides the cytokine milieu, which regulates the development of the different effector cell subsets[84]. The differentiation of Th cells is controlled by characteristic transcription factors induced through soluble cytokines. Th cells then support other immune cells by releasing cytokines[85]. To answer the question of which part of the immune system overreacts during an ACD, it is necessary to know the different subtypes of Th cells and to be able to distinguish them in their individual role. Therefore, a brief description of the origin of the different Th cell types and their secreted cytokines follows (**Figure 4**).

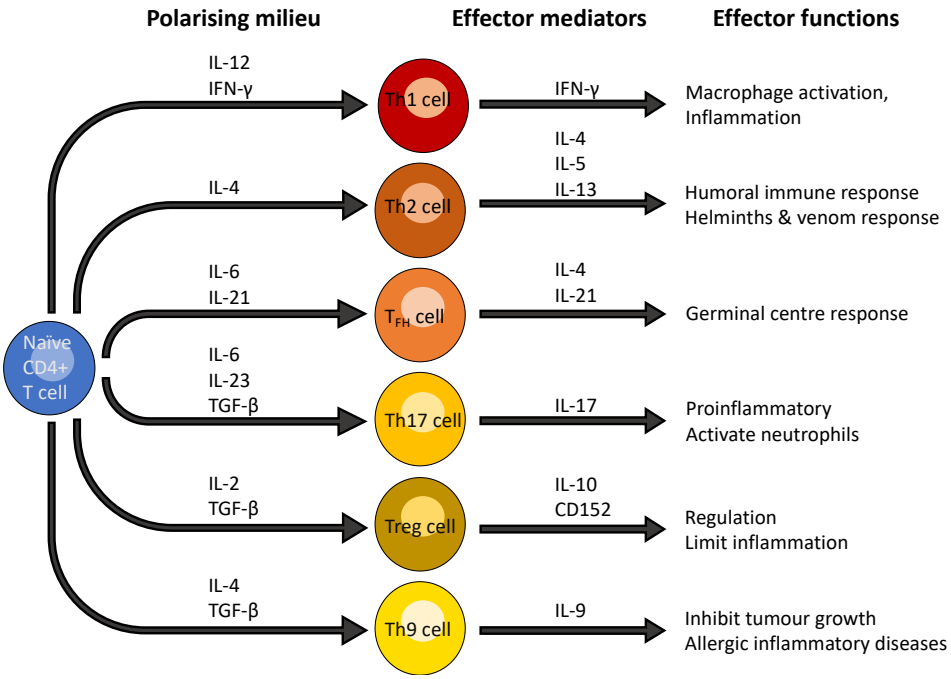

Figure 4:Differentiation of CD4+ T cells into varying effector and memory populations

Naïve CD4+ T cells recognise an antigen presented by an antigen-presenting cell, e.g., dendritic cells. The naïve T cell is thereby activated and divides several times. During this process, the cells are polarized into different effector T helper (Th) cells. The polarization of CD4+ T cells is controlled by various cytokines. Differentiated Th cells in turn express characteristic cytokines that mediate various effector functions. Regulatory T (Treg) cells and follicular helper T cells (T_{FH}) are named after their effector functions as opposed to the other subtypes. Further subpopulations and/or plasticity among cell types are currently discovered. Interleukin – IL, interferon – IFN, tumour growth factor – TGF, cluster of differentiation – CD. Adapted from Swain, et al. 2012[85].

Th1 cells help in the clearance of viruses and intracellular bacteria that proliferate in macrophages and are not destroyed by phagocytosis. Th1 cells are induced by IL-12 and IFN-γ produced by DC and ILC in the early phase of infections. In addition, Th1 cells then produce IFN-γ by itself to stimulate the antimicrobial activity of macrophages, which then destroy the pathogens[86].

Th2 cells support the humoral immune response and help fight extracellular parasitic helminths and venoms. IL-4 stimulates their generation, and they produce IL-4, -5 and -13, which support the release of toxic mediators from eosinophils, basophils and mast cells[87]. Additionally, they promote tissue repair and mucus production[86].

Th17 arise in the presence of IL-6, IL-23 and transforming growth factor (TGF)-β and are named after their secreted cytokine, IL-17. They support epithelial and stromal cells in the release of antimicrobial peptides and in expressing chemokines that guide neutrophils to the sites of infection. As a result, they are able to help fight extracellular bacteria and fungi[86].

Treg suppress other T cell responses, thereby constraining immune responses and preventing autoimmunity. Treg are induced by harmless antigens which do not induce co-stimulatory DC signals, e.g. food antigens and TGF-β and IL-2. Treg express IL-10 and CD152, limit inflammation and regulate DC and effector Th cells[86].

Follicular helper T cells (T_{FH}) interact with naïve B cells via coupled recognition of antigens. They promote germinal centre responses in B cell follicles, supporting the formation of antigen-specific antibodies. In addition, T_{FH} secrete cell lineage-determining cytokines and develop with either Th1, Th2, or Th17 cells. Thus, they influence naïve B cells to form different antibody groups (IgG2a by Th1 cytokines, IgE by Th2 cytokines)[86].

Recently, a new subset, Th9 cells, has been described, which seem to be mainly residential to skin tissues[88]. Plasticity between Th subsets and heterogeneity at the clonotype level are subjects of current research[89].

1.3.3 Activation of effector and memory T cells

Mature effector or memory T cells can be activated by an antigen peptide presented on an MHC complex even without co-stimulation by B7 or other surface receptors. This enables efficient targeting of pathogen-infected cells and renders most body cells capable of serving as APC[34]. The second function of selectins and integrins, such as LFA-1, is that these molecules mediate rolling adhesion and cellular arrest for extraversion from blood vessels into lymph nodes or inflamed tissue that expresses the appropriate counter receptors[90, 91].

Once a pathogen has been successfully eliminated, effector T cells undergo apoptosis while memory T cells remain to protect the body from re-infection with pathogens effectively[35].

1.4 Orchestration of adaptive immune responses by CD4+ T cells

CD4+ T cells interact with innate and adaptive immune systems through their multiple effector functions, including cytokine production. Two prominent examples are the support of Th cells in B cell and CD8+ T cell activation.

During the humoral immune response, the BCR binds to soluble or membrane-bound antigens, an interaction that triggers a signalling cascade in the B cell (signal 1). Next, the protein antigens are then transported into the cytosol of the B cell, where they are processed (degraded) by proteasomes. The resulting peptides bound to MHC II are finally presented on the surface of the B cell. The initial signalling of the antigen-presenting BCR is mediated by cytokine signals and amplified by the coreceptors CD19, CD21 and CD81, which may further get into contact with opsonised surfaces of microorganisms (**Figure 5**).

The second co-stimulatory signal is provided by T_{FH} cells in the germinal centre of lymphoid tissues. T_{FH} recognise a T cell epitope consisting of the MHC-presented peptide sequence generated by the B cell and express surface markers and cytokines. This process is called linked recognition[47, 92]. CD154 on T_{FH} cells binds TO CD40 on B cells, which then initiates further signalling cascades, promoting B cell proliferation and survival (**Figure 5**)[34].

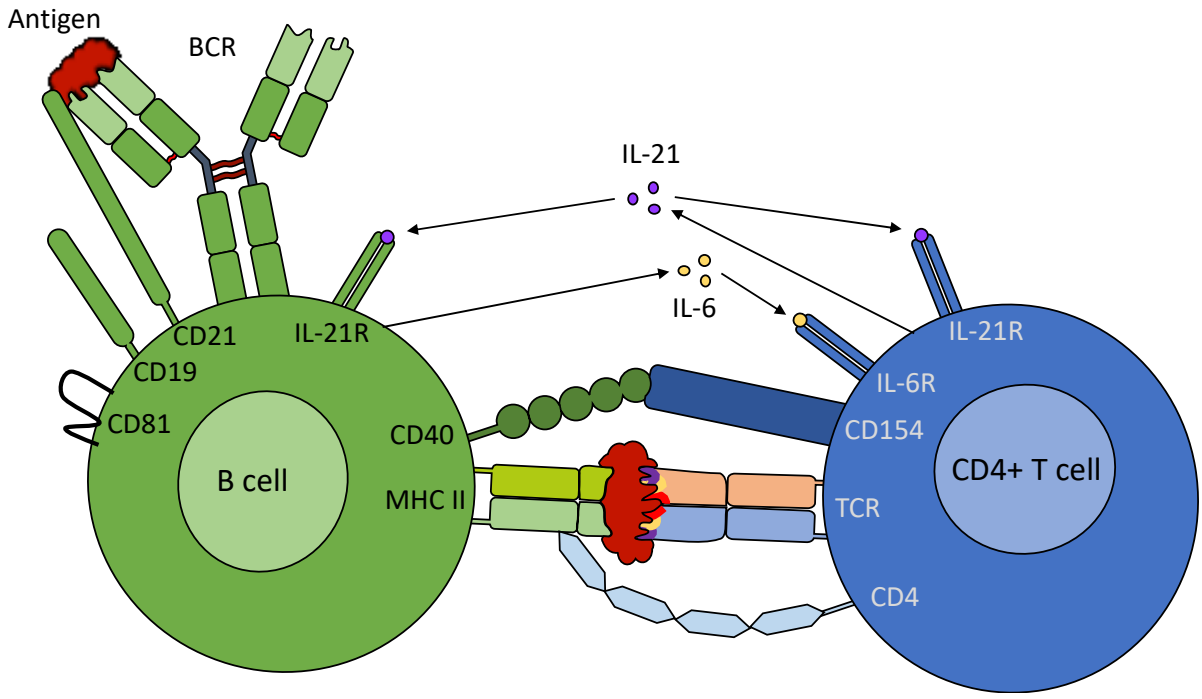

Figure 5: Molecular linkage between B cell and T cell responses.

The first signal of B cell activation occurs via cognate antigen recognition by the B cell receptor (BCR) and requires the co-stimulatory signals from CD19, CD21 and CD81. Additionally, co-stimulation by CD4+ T cells is required for full activation, class switching and somatic hypermutation. For this T cell help, the recognition of an antigen-derived major histocompatibility complex (MHC) II-presented peptide by the T cell receptor (TCR) is required. In addition, various cytokines (e.g. Interleukin (IL)-21) are required for costimulation. Adapted from den Haan, et al. 2014[34].

Affinity maturation of the BCR to a specific antigen is achieved during the germinal centre reaction. Maturation occurs through the diversification of immunoglobulin genes through somatic hypermutation of the variable region and isotype switching. The process results in antibodies with the same antigen specificity but a different heavy chain and therefore results in the formation of IgG, IgA or IgE antibodies. The proliferation of activated naïve B cells results in plasma blasts and the differentiation in antibody-producing plasma cells and memory B cells. This process is also regulated by Th cells through the release of additional cytokines[47].

During the neutralisation, antibodies bind to pathogens and their degradation products, inhibiting the toxic effects and infectivity by preventing penetration into the host cell. Moreover, the binding of antibodies to pathogens supports their uptake by macrophages and neutrophils through binding of the Fc region of antibodies to Fc receptors on their surface. In addition, particular antibody heavy chains can activate C1, the first protein in the classical pathway of complement activation. The activation of C1 helps to mobilise further phagocytotic cells to the site of infection[47].

Antibody binding and ensuing effector responses can also be misdirected, e.g. when a person prone to allergies encounters normally harmless allergen protein antigens contained in food, pollen, or house dust (with allergenic mite proteins). A *de novo* immune response leads to the differentiation of B cells into plasma cells and a class switch to IgE. Plasma cells produce large amounts of allergen-specific IgE antibodies that bind to the high-affinity IgE receptor (FcεRI) on mast cells and basophils. If renewed contacts with the allergen occur, a hypersensitivity reaction with clinical symptoms may be induced by IgE cross-linking. Mast cells then release huge amounts of inflammatory mediators, for example, histamine for acute inflammation and cytokines and chemokines for late reactions[93, 94, 95]. After an infection, antigen-specific, long-lived plasma cells and memory B cells remain. The latter continue to transform into antibody-secreting plasma cells[96, 97]. In addition, large numbers of tissue-resident memory T cells survive to fight a new infection quickly and extensively[34].

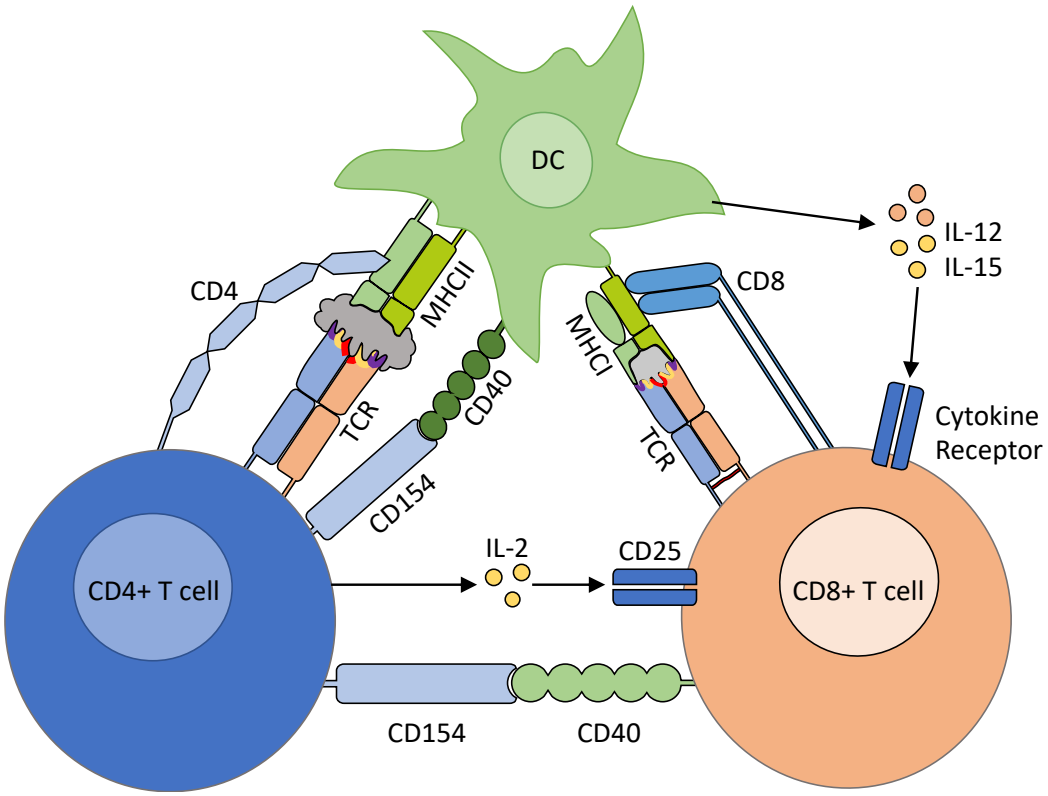

Figure 6: Cluster of differentiation (CD)4+ T cells support CD8+ T cell activation.

In a first step, CD4+ T cells are activated through the recognition of peptides presented on major histocompatibility complex (MHC) II complexes of dendritic cells (DC) by their T cell receptor (TCR). The successful formation of an immunological synapse between CD4+ T cell and DC does not only lead to full T cell activation (see **Figure 3**) but also signals back to the DC. The DC, in turn, expresses higher levels of MHC and co-stimulatory surface markers (e.g., CD4 and CD8) and cytokines (e.g. Interleukin (IL)-2 or IL-12). This supports CD8+ T cells that recognise a peptide bound to MHC I on the same DC. CD8+ T cell activation and proliferation are additionally supported by CD4+ T cells, as depicted in the scheme. Adapted from Laidlaw et al. 2016[98].

Besides B cell activation, CD4+ T cells also support DC-mediated activation of CD8+ T cells (**Figure 6**). The activation by DC is necessary, as only they can deliver strong co-stimulatory signals and produce IL-2 to overcome the activation threshold of naïve T cells[99, 100]. Co-stimulation with CD4+ T cells is essential for CD8+ T cell priming in many viral infections. CD4+ T cells bind to peptide antigen-MHC complexes on APC and subsequently express IL-2 and CD154. IL-2 contributes to the differentiation of CD8+ T cells. CD154 binds to CD40 on DC, providing additional co-stimulation to naïve CD8+ T cells. Other CD154 molecules on CD4+ T cells may bind directly to CD40 on CD8+ T cells to further facilitate the differentiation of CD8+ T cells (**Figure 6**)[79, 98].

1.5 Allergic Contact Dermatitis

Allergies are abnormal immune system reactions where a person reacts to substances in the environment that are usually harmless. Allergies, in general, and contact allergies, in particular, are diseases that are frequent in industrialised societies. People are exposed to thousands of chemicals that may have sensitising or allergenic properties[101]. The first description of allergies dates back to 1819 when John Bostock described the first case of hay fever[102]. An increase in hay fever was recognised in the following years resulting in the publication of the first case series about grass and ragweed pollen in the 1870s[103, 104]. During this time, heavily pollinated grasses were introduced to make hay for dairy herds in Europe and an increased ragweed growth in the USA was observed around fields. Atopic dermatitis, which may be linked to allergic immune responses, was recognised as an epidemic in the 1960s[105]. Nowadays, it is observed more frequently that the immune system reacts to usually harmless environmental antigens. Possible reasons for the increasing occurrence are an improved diagnosis, changes in the lifestyle (e.g. better hygiene) and food habits of people[106]. Since the early 2000s, the prevalence of food allergies has increased significantly[107]. A significant increase in the prevalence of allergen-specific IgG and IgE was observed in the same time frame. While hardly any allergen-specific IgG antibodies were detected in the 1970s, in 2018, IgG antibodies against milk and egg proteins were found in almost all infants at the age of one year[108, 109]. Additionally, due to the ageing society, there is a growing demand for implants and prostheses, which results in systemic contacts of the body with metal alloys.

An ACD is characterised by skin inflammation and rashes caused by the contact of an allergen with the skin. About 20 to 27% of European adults are sensitised to at least one contact allergen[1, 110, 111]. The most common entry route of contact allergens is through skin penetration. In addition, sensitisation via the respiratory tract or systemic sensitisation by implants or drugs has been described. The elicitation of the allergy towards eczematous lesions can then be triggered via skin penetrating compounds, airborne substances, or food ingestion, independent of the original sensitisation pathway. While there are regulations for allergens, such as the EU Nickel Directive, the prevalence of nickel allergies is still high in the general population[112]. Nickel remains the most common contact allergen worldwide and affects 9 and 26% of the population[1, 113, 114]. Nickel is widely present in metal alloys used in industrial and everyday items such as coins, zippers and phones, as well as in food, air, soil and water[115]. In addition, nickel allergies are more common in women than men because they wear earrings and other jewellery more often[111, 116, 117].

The regulation of nickel content in alloys led to its replacement by other metals in consumer products. As a result, allergic contact dermatitis to other metals has increased, such as cobalt, the second most

common metal allergen. Contact allergies are clinically diagnosed through the patch test, during which the allergen is applied to the patient's skin and the potential development of eczema is observed. The most common allergens tested as a standard are summarised in the European baseline series. In patients tested with the European baseline series, the prevalence of individuals allergic against cobalt was 5%[117]. Cobalt is used in paints and dental alloys, often in combination with nickel[118]. Another metal ion released from dental appliances is palladium. Palladium is not included in the standard test baseline series, although it is increasingly used in the car industry, among others, because of its resistance to corrosion[11]. Among ACD patients in the USA, 12% tested positive for palladium[119]. The frequent occurrence of palladium allergy along with nickel allergy is commonly explained by cross-reactivity against both metals[120, 121].

Apart from metals, other important sensitisers are organic chemicals such as fragrances, preservatives, drugs, and many other substances of synthetic or natural origin[122, 123]. One of the best investigated chemical model allergens is 2,4,6-trinitrobenzenesulfonic acid (TNBS). *In vitro*, TNBS covalently binds to free amino groups by nucleophilic aromatic substitution ("trinitrophenylation"). The resulting antigens are 2,4,6-trinitrophenyl (TNP) determinants at accessible lysine residues or free N-terminal amino groups of proteins[124].

1.5.1 Pathomechanism of allergic contact dermatitis

An ACD can be divided into two distinct stages. During the sensitisation phase, chemical or metal allergens penetrate the skin (**Figure 7**). The innate immune response is accountable for the irritant capabilities of sensitising chemicals with possible support from heterologous signals such as wounds, infections, or the presence of additional chemicals. The activated APC travel to the local draining lymph nodes, where they can present the peptide-bound allergen on their MHC receptor to lymphocytes. Metal ions and chemical allergens are presented through allergen-induced T cell epitopes. Naïve T cells recognise this complex, are activated, and differentiate into effector and memory T cells. In the following T cells leave the lymph nodes and circulate in the blood vessels until they reach non-lymphoid tissues, including the skin, where some eventually remain as tissue-resident memory T cells. During the elicitation phase of ACD, the skin of an allergic person reacts upon direct contacts with otherwise harmless substance concentrations (**Figure 7**). The initiators of symptomatic contact allergic reactions are the previously formed CD4+ or CD8+ memory T cells[3]. The mechanism of ACD is thought to be similar to conventional pathogen-directed immune responses, involving the clonal expansion of naïve T cells.

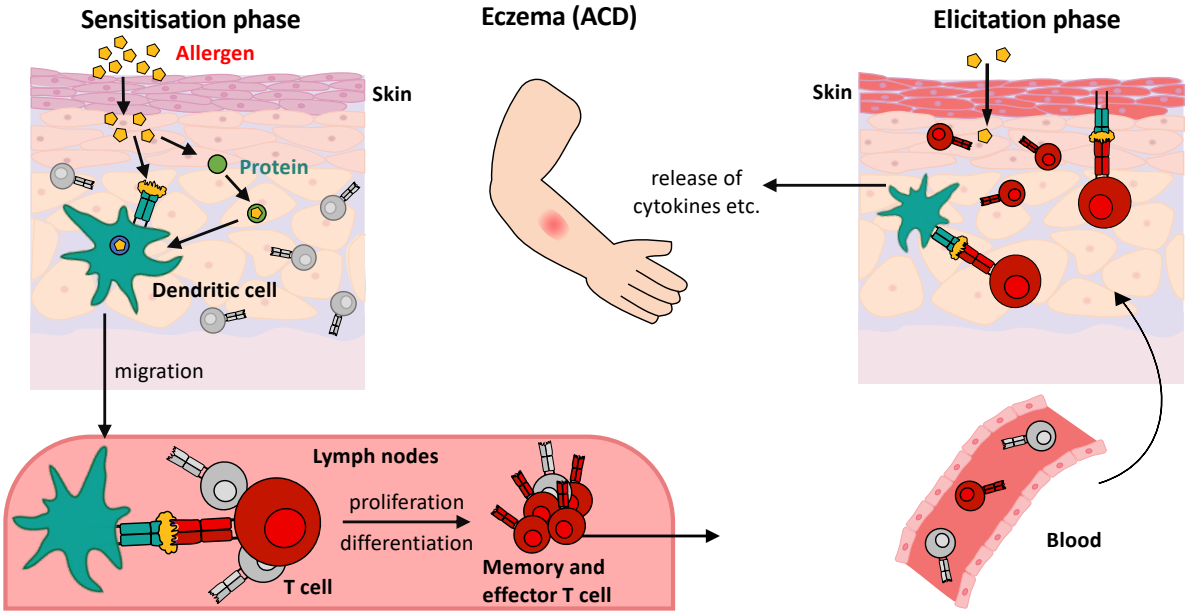

Figure 7: Pathomechanism of allergic contact dermatitis (ACD).

During the clinically silent sensitising phase, naïve T cells recognise chemical-induced T cell epitopes on dendritic cells (i.e., antigen presenting cells). The specific T cells proliferate and differentiate int effector and memory T cells, which are then released into the blood. During the elicitation phase, local tissue-resident memory T cells recognise the antigen. Over the course of several hours to days, they initiate an inflammatory cascade involving multiple molecular and cellular players. This ultimately leads to massive T cell infiltration from the blood followed by clinical symptoms (e.g., eczema). The time between the sensitisation phase and the elicitation phase can range from days to years.

A detailed explanation of the sensitisation and elicitation phase can be found in our detailed review paper on the immunological mechanisms of metal allergies in section 1.5.4.

1.5.2 Possible binding mechanisms of contact allergens to the T cell receptor

No pathogen-derived antigen peptide is present in chemical allergies on the surface of MHC protein complexes. Instead, chemically induced T cell epitopes are formed with MHC-presented self-peptides. Immune cell recognition of organic chemical allergens occurs upon covalent binding to self-proteins (haptenisation) or complex formation (coordinative binding). In contrast, metal ions only form complexes non-covalently via coordinative binding to free electron pairs of amino acids in proteins. Usually, the binding of TCR to self-peptide MHC complexes does not lead to T cell activation because of the initial selection process in the thymus (Section 1.2.3)[116]. However, the new allergen-induced T cell epitopes may provide enough interactions between the self-peptide-MHC complex and the TCR to exceed the activation threshold. Therefore, in most cases, the activation of allergen-specific T cells requires a metal- or chemical-mediated bridge between the TCR and the self-peptide-MHC complex. Metal ions and chemical allergens may modify the MHC-presented peptide directly from the extracellular space. In the case of metal ions, the possible transfer from a specialised metal-binding (transport) protein has been shown[125]. The APC may also process metal-binding proteins, and a metal-

binding peptide may be presented on the MHC complex (Section 1.5.4 and 1.6.1). Another possibility is the complex formation of chemicals and metal ions with amino acid residues in conserved regions of the TCR-peptide MHC interface or with the CDR3 regions of the TCR α- or β-chain. Histidine, tyrosine and glutamic acid have been shown to bind especially to Ni^{2+} [2, 126, 127, 128] (Section 1.5.4 and 1.6.1). Allergens can also cause the activation of T cells without their direct involvement in the binding. Metal ions can alter the antigen processing of a self-peptide, resulting in a metal-free cryptic self-peptide (Section 1.5.4). Alternatively, allergens can bind between the MHC protein and the antigen-peptide. For chemical allergens, non-covalent binding is termed pharmacological interaction (p-i interaction) and is often described in association with specific MHC alleles. One example of this mechanism is the HIV reverse transcriptase inhibitor abacavir, which binds HLA-B*57:01. Binding results in the presentation of an altered peptide repertoire (Section 1.6.1).

An example of atypical metal binding to HLA-DP2 and the presented antigen-peptide are beryllium (Be^{2+}) ions. The binding of Be^{2+} leads to conformational changes in the peptide and creates a metal-free neo-antigen, which is then recognised by the TCR (Section 1.5.4). As another example, lipid ligands on the MHC-like molecule CD1a can be displaced by haptens. The displacement results in polyclonal αβTCR activation through the “empty” CD1a surface (Section 1.6.1).

Overall the prediction of the exact configuration of T cell epitopes is difficult because of the diversity of binding mechanisms and the wide variety of possible target proteins or presented self-peptides.

A detailed discussion of currently known or hypothesised binding mechanisms for metal ions allergens and organic chemical allergens to the TCR can be found in the review on immunological mechanisms of metal allergies in section 1.5.4 and the review on skin sensitising chemicals in section 1.6.1.

1.5.3 Cross-reactive allergen-specific T cell receptors

For most binding mechanisms of TCR to chemical-induced T cell epitopes discussed in section 1.5.2, cross-reactivity is conceivable. For example, metal ions may be replaced by other metal ions with the same ion charge and similar binding properties[116]. The spatial orientation during recognition of the antigen-peptide-MHC complex by the TCR is only partially conserved and follows some general rules. Two general concepts are discussed as mechanisms of cross-reactivity, which are not mutually exclusive. First, cross-reactivity can occur due to conserved binding sites, i.e. a small, conserved amino acid motif stabilises the interaction between the TCR and MHC complex. Only a few mutations are tolerated within the recognised amino acid motive to maintain TCR activation. Outside of this motif, many changes can be tolerated to identify a vast number of antigens. Complementary to this concept, cross-reactivity can be made possible by “induced fit”. During antigen binding, the CDR loops of the

TCR and the peptide-MHC complex cooperatively adjust and subsequently undergo significant conformational changes. In this process, the CDR3 loops change their conformation the most[116].

TCR cross-reactivity to chemical and metal allergens has been studied *in vivo* in various epidemiological patch testing studies in different time frames and locations worldwide. The results show that nickel-allergic individuals can also react to cobalt and palladium. However, the exact percentage of concomitant allergy occurrence varies widely. For example, between 2% and 72% of nickel-allergic patients also react to Co^{2+} and between 11% and 40% respond to Pd^{2+} [116]. Conversely, up to 83% of cobalt-allergic and 95% of palladium-allergic individuals also react to Ni^{2+} [116]. The considerable differences in the detection of co-allergic individuals show that it is challenging to study T cell cross-reactivity using the patch test. In addition, the patch test has a low reproducibility, and different metal salts and concentrations are used in different countries. Most importantly, it is unclear whether co-sensitisation through immunologically relevant co-exposure or cross-reactivity of TCR is detected.

In vitro cross-reactivity assays are more reliable, as the T cell reactivity of T cell clones with defined specificity to different metal salts can be analysed directly. Nevertheless, these tests also have some limitations. The T cell activation is concentration-dependent. Therefore the current literature results depend on the initially used metal ion concentrations. T cell clones can show high activation variability to cross-reactive metal ions, complicating the interpretation of the rarely available data[116].

A more detailed consideration of the advantages and disadvantages of *in vitro* and *in vivo* testing and the existing literature data on this can be found in the review on immunological mechanisms of metal allergies in section 1.5.4.

1.5.4 Review paper: Immunological mechanisms of metal allergies and the nickel-specific TCR-pMHC interface

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Published online on 15 October 2021 in:

International Journal of Environmental Research and Public Health 2021, 18, 10867

DOI: 10.3390/ijerph182010867

Link: https:// doi.org/10.3390/ijerph182010867

Involvement of the author within this publication: Conceptualisation, literature research, writing and visualisation of the manuscript.

Author contributions as published: Conceptualisation, F.R., M.A.-S., C.C., H.-J.T., K.S. and A.L.; investigation, F.R., M.A.-S., C.C., H.-J.T. and K.S.; writing—original draft preparation, F.R., M.A.-S., C.C., H.-J.T. and K.S.; writing—review and editing, F.R., M.A.-S., C.C., K.S. and A.L.; visualisation, F.R. and K.S. All authors have read and agreed to the published version of the manuscript.

International Journal of
Environmental Research and Public Health

MDPI

Review

Immunological Mechanisms of Metal Allergies and the Nickel-Specific TCR-pMHC Interface

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Abstract: Besides having physiological functions and general toxic effects, many metal ions can cause allergic reactions in humans. We here review the immune events involved in the mediation of metal allergies. We focus on nickel (Ni), cobalt (Co) and palladium (Pd), because these allergens are among the most prevalent sensitizers (Ni, Co) and immediate neighbors in the periodic table of the chemical elements. Co-sensitization between Ni and the other two metals is frequent while the knowledge on a possible immunological cross-reactivity using in vivo and in vitro approaches remains limited. At the center of an allergic reaction lies the capability of a metal allergen to form T cell epitopes that are recognized by specific T cell receptors (TCR). Technological advances such as activation-induced marker assays and TCR high-throughput sequencing recently provided new insights into the interaction of Ni^{2+} with the αβ TCR-peptide-major histocompatibility complex (pMHC) interface. Ni^{2+} functionally binds to the TCR gene segment TRAV9-2 or a histidine in the complementarity determining region 3 (CDR3), the main antigen binding region. Thus, we overview known, newly identified and hypothesized mechanisms of metal-specific T cell activation and discuss current knowledge on cross-reactivity.

Keywords: immunotoxicology; metal allergens; allergic contact dermatitis; T cells; T cell receptor; T cell epitopes; cross-reactivity

Citation: Riedel, F.; Aparicio-Soto, M.; Curato, C.; Thierse, H.-J.; Siewert, K.; Luch, A. Immunological Mechanisms of Metal Allergies and the Nickel-Specific TCR-pMHC Interface. *Int. J. Environ. Res. Public Health* **2021**, *18*, 10867. https://doi.org/10.3390/ijerph182010867

Academic Editors: Jörg Lehmann and Robert Landsiedel

Received: 31 August 2021
Accepted: 11 October 2021
Published: 15 October 2021

1. Introduction

Metals are present in many areas of industrialized life. People are exposed to metals through the environment, consumer products, workplaces and medical appliances such as implants or drugs. One of the most common immunotoxic effects of metals is their ability to act as allergens, often affecting the skin causing allergic contact dermatitis (ACD) [1–3]. ACD represents the characteristic example of a T cell-mediated delayed-type hypersensitivity response (type IV allergy). A multitude of metallic elements has been associated with allergic reactions, among them nickel (Ni, Ni^{2+} ions), cobalt (Co, Co^{2+} ions) and palladium (Pd, Pd^{2+} ions) (Figure 1) [4–6]. Metal allergens may interact with both the innate and adaptive immune system. Concerning the latter, the underlying mechanisms of allergic reactions include the formation of allergen-induced T cell epitopes recognized by specific T cell receptors (TCR) [7,8]. Since reactive chemicals are too small to be recognized by TCR or antibodies, binding to self-proteins by a process called "haptenization" is mandatory for the interaction with the adaptive immune system [9]. Thus, chemical allergies can be viewed as misguided adaptive immune responses to otherwise relatively harmless chemical exposures. Despite preventive regulations, metal allergies, especially Ni allergy, remain a significant public health burden [1,2].

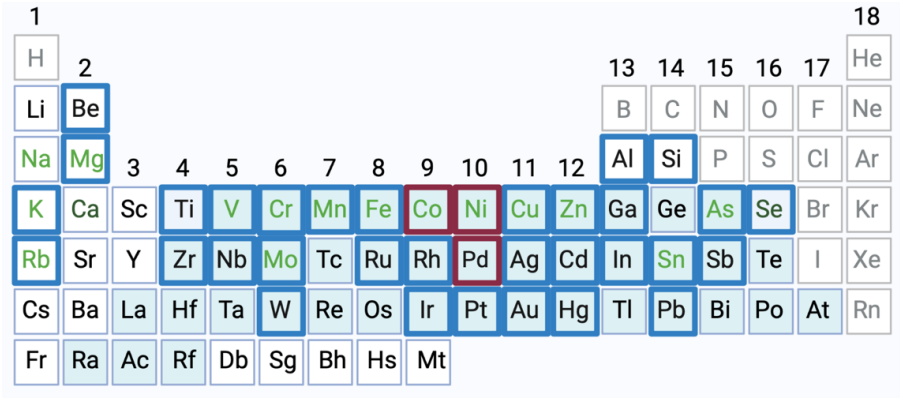

Figure 1. Metals implicated in allergic reactions. Metallic elements related to allergic reactions are highlighted in this periodic table of the elements (blue boxes, without lanthanides and actinides). Most of the metal allergens belong to the group of heavy metals (blue background) and some are (likely) essential trace elements (light green letters) [6,10]. Non-metallic elements are depicted in grey letters. This review focuses on general adverse and immunotoxic effects of nickel (Ni, Ni^{2+} ions), cobalt (Co, Co^{2+} ions) and palladium (Pd, Pd^{2+} ions) (highlighted in red) [6]. Created with BioRender.com.

In this review, we recapitulate the allergy-triggering mechanisms of Ni, Co and Pd identified from in vivo and in vitro studies. We focus on Ni because it is the most common contact allergen and there is abundant literature on Ni allergy. Further, we have selected the second most common metal allergen, Co, which is often used as a substitute for Ni in metal alloys. Besides, Ni-Co co-sensitization is observed in several epidemiological studies but currently there is little mechanistic evidence of a possible T cell cross-reactivity. In addition, we discuss the relatively rare allergen Pd. The current existing literature reports up to 80% Pd co-sensitization with Ni. Pd allergy is rarely diagnosed without additional Ni allergy and there is mechanistic evidence of cross-reactivity. In this context, we update the current understanding of metal-induced T cell epitopes and TCR cross-reactivity for Ni, Co and Pd.

2. Physicochemical Properties, Physiologic Functions and Toxicity of Ni, Co and Pd Ions

Metals are elements able to form cations via oxidation. The pure elements are solid under normal conditions (with the only exception being mercury) and possess high light reflectivity, hardness, ductility, malleability and an excellent heat and electric conductivity. The majority of the elements of the periodic table of the chemical elements (PSE) are metals (Figure 1, [11]).

Heavy metals are elements with at least a five times higher density than water and a high atomic weight [12]. This definition implies some controversial aspects and therefore some experts classify heavy metals as metallic elements with a density higher than 5 g/cm^3 [13]. Popular belief associates the term heavy metal with some negative connotations linked to their potential toxicity. However, some heavy metals like Co are essential nutrients for humans whereas others, like Ni, are essentials for organisms such as bacteria and plants [14]. Heavy metals can play important roles in biochemical and physiological processes and their deficiency is associated with several diseases [15,16]. The toxic and carcinogenic molecular mechanisms of heavy metals are still not fully elucidated, but appear to be linked to a combination of common and unique features of every metal [12]. Some heavy metals can escape cell control mechanisms and bind cell constituents due to their chemical coordination and redox properties, thereby, for example, displacing original metals from their natural binding sites [17]. Other heavy metals lead to oxidative stress affecting several tissues, with liver and kidney being among the most critical ones [18]. Besides,

the toxic properties of some heavy metals, leading to the formation of free radicals and reactive oxygen species (ROS), induce cell membrane and protein dysfunction linked to a malfunctioning immune system and potentially contribute to autoimmunity in humans [19].

According to the International Union of Pure and Applied Chemistry (IUPAC), a transition metal is an element with partially filled d-orbital (sub-shell) atoms or able to generate cations with an incomplete d-orbital. Metals from the first row of transition metals prefer tetrahedral to a square planar geometry (for example iron and Co). However, ions with incomplete d-orbitals (for example, Ni) prefer a planar conformation [20].

The number of ligands of a metal ion promotes a preferential spatial arrangement and influences its protein binding preferences [21]. The most thermodynamically favorable ligands for metal ions binding to proteins are the imidazole substituents in histidine, thiolate substituents in cysteine residues and carboxylate groups from aspartate or glutamate residues [22–24]. To date, global metal-binding proteins remain unknown while metalloproteomics is an emerging field of research [25].

Ni is a transition metal with the atomic (ordinal) number of 28 (molecular weight (MW): 58, 69) which belongs to group 10 of the PSE. It exists in different oxidation states (−1 to +4) but the most common is +2. Ni binds to proteins through the imidazole nitrogen of histidine and the thiol of cysteine [22]. The biological role of Ni in animals remains unclear, but some studies suggest its implication in reproductive and metabolic processes. Ni is a well-known essential co-factor of at least nine bacterial enzymes therefore indirectly influencing human health as part of the natural microbiome or pathogens [20,26–28]. Besides its high allergenic potential, Ni can also act as an immunotoxic and carcinogenic agent, can contribute to acute and chronic cardiovascular and respiratory diseases and it has revealed embryotoxic and teratogenic properties in animal studies [29–34].

Co is another transition metal with the atomic number of 27 (MW: 58, 93; group 9) and usually present at +2 oxidation state. Like Ni, it preferably binds to free histidine residues [35,36]. Co acts as a cofactor for several enzymes in humans and other organisms [37,38]. Exposure to Co may lead to metal allergy, chronic and acute respiratory diseases, metallosis and increased risk of cancer [35,39,40]. Metal implants made of Co alloys have been found to release high concentrations of Co^{2+} ions into the blood [41–43].

Ni and Co are commonly found together in nature and alloys. Co is placed next to Ni in the PSE (same period) with the same number of outer electrons and inner electron shells but a different number of lacking electrons of the inner shells, resulting in similar coordination properties. Although both metals show specific preferences regarding their amino acid binding, histidine and methionine are largely enriched in both metal binding peptides in bacteria, with a stronger histidine enrichment [44]. At certain metal concentrations, Co and Ni bind to the serum protein albumin, Co mainly at carboxylate and tyrosine residues and Ni at carboxylate groups. Co binding to albumin appears to be competitively inhibited by Ni, but Co did not inhibit Ni protein binding, indicating that Ni may have more binding sites [45].

Pd belongs to the same group of the PSE as Ni with an atomic number of 46 (MW: 106, 42; group 10). The most common oxidation state for Pd is +2 [46]. The catalytic properties of Pd are linked to the square-planar geometry of Pd^{2+} complexes [47]. Exposure to Pd via skin, oral cavity or respiratory tract can cause acute toxicity or hypersensitivity with respiratory and dermal symptoms [48–50]. Pd exhibited low genotoxicity in mammalian organisms and bacteria [51].

3. Metal Allergies

3.1. Exposure, Epidemiology and Regulatory Aspects

Exposure to metals can occur through different routes, such as dermal absorption, inhalation and ingestion. In daily life, the main exposure route by which ACD is triggered is the contact of metals with the skin via jewelry, clothes, consumer goods or the environment [2]. Besides, the surge of some metal nanomaterials in consumer products (mainly silicon, titanium, zinc and aluminum) also contribute to dermal and aerial exposures. The

altered metal physiochemical properties and the unique immune effects of these nanomaterials may have important consequences in allergic processes [52]. The occupational exposure to metals, mainly via dermal or aerial routes, constitute an important health concern due to its involvement in ACD [2,53]. Metals can also encounter the human body systemically via implants and prosthetics. The current growing social demand for orthopedic and dental implants, joint arthroplasty and fixation devices may potentially contribute to the rise of contact and systemic allergies. Those devices are made of different alloys which can contain several metals including titanium, Ni, Co, chromium, iron, aluminum, vanadium and Pd among others [54,55]. The ingestion of metals via food and water is the dominant source of human metal exposure [56,57]. An allergic reaction can be triggered by dietary metal exposure in already sensitized individuals. In contrast, the significance for primary sensitization is still unclear [58,59]. The development of some pharmaceutical formulations with metals (mainly iron, zinc, vanadium, gold (Au), platinum and aluminum, among others) can also increase potential metal exposures and subsequent sensitization [15,60,61].

The number of patients with a metal allergy has recently been growing in general and surgical populations. Depending on the age and geographical location, approximately 20–27% of European adults are sensitized to at least one contact allergen as defined by positive patch test reactions and thus might be on the verge of developing ACD [1,62]. Most common are allergies to Ni (11.4%), fragrances (3.5%) and Co (2.7%) [1,6].

Allergen avoidance is the key to preventing sensitization, and ACD or other clinical symptoms. However, this may be difficult due to the ubiquity of the allergens, lack or shortcomings of identifying the substance in the environment, possible cross-reactions and low individual elicitation thresholds [52].

Since it is mainly dependent on exposure, ACD can affect all ages and may start at a very young age [63]. Some haptens (including Ni) have a prevalence peak in early adulthood and then show a decreased prevalence with aging [64]. The age-related variation may be attributable to different exposure conditions during the life span and a waning or reduced immune response with aging.

Although the use of allergens in consumer products, such as Ni in earrings, has been successfully regulated in recent years, the prevention of metal exposure needs further improvement given the severe socio-economic implications.

Ni is the most common cause of contact allergies in the general population worldwide (11.4% in Europe, 8.8–25.7% in China, 17.5% in North America) [1,65–67]. Ni is widely present in industrial and everyday items such as coins, cell phones, laptops and zippers as well as in some commonly consumed foods, air, soil and water [68]. Ni allergy is more prevalent in women (15.7–22.9% compared to 4.3–6.65% of men), probably due to their increased exposure to Ni through earrings and other jewelry [1,69,70]. Over time, Ni-induced ACD evolved from an occupational disease to a common form of ACD among both adults and children [71]. Several endogenous (e.g., genetics) and external factors are involved in the development of ACD to Ni, but the exact mechanisms that lead to human sensitization remain unclear. Major factors seem to be the accumulated Ni skin dose ($\mu g/cm^2$) along with the type of exposure, skin status, skin area, bioavailability in the skin, duration of the contact, previous dermatitis or other skin diseases, sweat involvement and possible combined irritant effects by Ni itself or associated with other irritants [72,73]. Even very low but increased Ni levels in ambient air have been linked to increased sensitization rates [74]. Because of the high sensitization rate to Ni salts, Ni regulations were first implemented in Denmark and Sweden in 1990 and 1994 by the EU Ni Directive and later the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation [75–78]. Since then, Ni is not allowed in items inserted into pierced parts of the body unless the Ni release is less than 0.2 $\mu g/cm^2$/week or, for articles in direct and prolonged contact with the skin where Ni release is greater than 0.5 $\mu g/cm^2$/week [79]. The European Commission also regulated the content of Ni in ambient air, establishing a target value of 20 ng/mg^3 [80]. Although the prevalence of Ni allergy has decreased since

the implementation of restrictions, Ni is nowadays still the most common cause of ACD in the general population. This can be partly attributed to the lack of restriction regarding the frequent contact of consumers with everyday products containing Ni [81].

Ni regulation promoted the use of other metals in consumer products, causing a rise in the incidence of ACD to other metals [82,83]. Co is the second most common metal allergen. Patients tested with the European baseline series between 2015 and 2018 showed a prevalence of positive tested individuals of 5.4%. Women were more frequently affected than men (6.1% vs. 3.9%), but in contrast to Ni, Co ACD does not display any age pattern [70]. Co is used in various dental alloys, paints and coloring components of porcelain and glass, often in combination with Ni [84]. Although Co allergy is relatively common, the causative exposure remains unknown in 80% of patients [1]. Co content has recently been regulated by EU legislation including a temporary generic concentration limit (GCL) of $\geq$0.1% [85]. Besides, a new restriction proposal from French and Swedish authorities aims to reduce the risk of skin sensitization to chemical substances in textile and leather articles. Thus, the proposed concentration limits for Ni and Co in textile and leather products are, respectively, 130 and 110 mg/kg and 70 and 60 mg/kg [86].

The general population is sensitized to Pd mainly through dental appliances. Additionally, Pd is present in jewelry and industrial catalysts in the car industry because of its resistance to corrosion [2]. Although the use of Pd is increasing, it is not included in any standard test baseline series [87]. Out of 910 ACD patients in the USA, 12.1% tested positive for Pd. Among these patients, mouth mucosal diseases were more common than skin diseases [88]. $PdCl_2$ is the most frequent (3%) allergen of the "dental metal series" detected in dental technicians with occupational ACD [89]. The incidence of Pd allergies has frequently been explained through cross-reactivity since isolated allergy to Pd is rare [87]. Currently there is no EU legislation to limit the amount of Pd in consumer products.

3.2. Case Reports

Nowadays, there are many ways to encounter metal allergens, resulting in different clinical symptoms. We here highlight five interesting metal allergy cases from the recent literature.

Case 1—Systemic Ni allergy syndrome [90].

A 48-year-old woman presented with an inflamed esophagus. Ni allergy was diagnosed through patch testing. A Ni-free diet reversed symptoms while an oral Ni challenge induced adverse gastrointestinal effects. An oral desensitization therapy with slowly increasing amounts of Ni led to a complete reversal of symptoms. This case illustrates how metal-containing foods may cause systemic symptoms in metal allergic patients. It is an indication that oral desensitization may work similarly to hypo-sensitization for protein-related allergies, for example to pollen.

Case 2—Severe implant complications due to tiny amounts of Ni in suture anchors [91].

A 36-year-old woman developed severe local skin hematoma two months after an epicondylitis-related surgical procedure during which titanium suture anchors were inserted. Skin transplant and topical steroid treatment failed while Ni patch testing was strongly positive (+++). Although the dimethylglyoxime (DMG) test was negative, inductively coupled plasma mass spectrometry (ICP-MS) showed a release of Ni from the suture anchors. Removal of the anchors reversed symptoms, impressively showing how small amounts of metals can lead to delayed, severe complications in an implant setting.

Case 3—ACD to Ni from green tattoo ink [92].

A 40-year-old woman presented skin excoriations in the green area of a tattoo, that worsened during Ni patch testing (+++). Laser ablation ICP-MS revealed Ni agglomerates in the dermis, providing support for Ni-induced skin symptoms from tattoo inks. Tattoo inks can cause severe long-term problems if an allergic reaction occurs, which may be many years after the tattoo was placed. Metal allergens can be part of undeclared tattoo ink contaminations while the original inks are often not available at the time clinical symptoms emerge [93].

Case 4—Occupational ACD to Co due to contaminated machine oil [94].

A 24-year-old engineer on a container ship developed severe oozing dermatitis on his hands due to skin contact with machine oil. ICP-MS revealed the presence of a very low amount of Co (2.4 ppm) while other methods (spot test, X-ray fluorescence) failed to detect Co. Patch testing against Co was strongly positive (+++). This case illustrates that Co ACD elicitation thresholds can be extremely low in real life settings, especially on potentially damaged skin. The proof of causative metal allergen exposure can thus be very challenging.

Case 5—Pd-induced skin granulomas from earrings [95].

A 28-year-old woman showed epithelioid granulomas on both earlobes several months after piercing. Patch testing was only weakly positive to Pd (+) but after four weeks the patient developed similar granulomas at the site of the patch test. ICP-MS showed Pd agglomerations at all affected skin sites. Since more specific diagnostic tools are missing (e.g., reliable in vitro tests), no distinction between a foreign body and an allergic reaction is possible at present. Thus, this case illustrates current diagnostic limitations.

4. Immunological Mechanisms and Diagnostic Approaches

4.1. Pathomechanism of ACD

4.1.1. Sensitization Phase

Similar to every allergic reaction, the pathomechanism of metal allergies can be separated into two temporally distinct phases, the sensitization phase and the elicitation phase, which each require the activation of innate and adaptive immunity [9,96,97]. One or several initial encounters of the skin with an allergen may lead to sensitization. The extent to which a chemical needs to penetrate the stratum corneum remains unclear, since chemicals may enter via skin appendages like hair follicles or sweat ducts and Langerhans cells may extend their dendrites through the tight junction barrier and thus contribute to chemical uptake [98–100].

Irritant effects of chemical allergens initiate an inflammatory milieu in the skin, leading to dendritic cell maturation and migration to the skin-draining lymph nodes [101–103]. In the lymph nodes, dendritic cells then present allergen-induced T cell epitopes to T cells. Activated, antigen-specific T cells clonally expand and differentiate into effector and memory T cells that distribute globally and may form antigen-specific tissue-resident memory T cells (T_{RM}) [96,104,105]. The irritant power of chemicals seems to correlate with their ability to act as contact sensitizers [106], while the molecular pathways are not yet fully explored. Two main underlying mechanisms have been proposed by which sensitizing chemicals, including metals, modulate innate immune responses. Sensitizers can bind to pattern recognition receptors (PRR) and thereby functionally mimic pathogen-associated molecular patterns (PAMP). In addition, the release of damage-associated molecular patterns (DAMP) can be triggered, e.g., via ROS production or binding to other cellular constituents, leading to inflammasome activation and apoptosis induction [8,107–112]. Ni generates ROS and binds to proteins promiscuously and concentration-dependently, as shown for the formation of T cell epitopes [7,113–115]. Thus, a multitude of cellular effects is observed upon Ni exposure in, e.g., dendritic cells. This includes cholesterol depletion, hypoxic and apoptotic signaling, nuclear factor erythroid 2-related factor 2 (Nrf2) pathway activation and production of inflammatory cytokines like TNFα, IL-1b, IL-6, IL-8, IL-12 or IFNα and -β [116–119]. This leads to further downstream effects, e.g., IL-6 and IL-12 supporting naïve T cell priming.

One important pathway of Ni signaling is it's functional interaction with human TLR4, which depends on the binding to two primate-restricted histidines (H456 and H458) [117]. Ni-sensitized human TLR4 transgenic mice react to intradermal $NiCl_2$ injection in the ear pinna with ear swelling, illustrating the capacity of human TLR4 to contribute to Ni sensitization in vivo. Still, Ni-induced signals differ slightly from those observed with the natural TLR4 LPS ligand, e.g., IL-6 or Il-12p40 secretion is lower [116,117]. Human TLR4 is not expressed on freshly isolated keratinocytes or Langerhans cells, which most likely

form the first site of contact in the epidermis with exogenous agents. Ni, Co and Pd have been shown to bind and signal via TLR4 [120].

Besides the intrinsic irritant capacity of sensitizers, heterologous immune stimulation may play a yet to be determined role in human sensitization to Ni and, in general, to metal allergens. The allergy to Ni is strongly associated with skin injury, e.g., piercing and very low exposure concentrations [74,121]. Likewise, the vaccination to cow pox relies on skin damage [122]. In addition, high metal ion concentrations during patch testing do not sensitize which also argues for a role of heterologous immune stimulation [123].

From a translational point of view, it remains difficult to prevent or interfere with the sensitization process by pharmacological intervention, leaving regulatory restrictions as possible public health measurement.

4.1.2. Elicitation Phase and T Cell Effector Responses

Upon exposure of a sensitized individual to the original or a cross-reactive allergen, memory T cells initiate a faster and more aggressive secondary immune response, which results in a cutaneous inflammatory reaction clinically recognized as ACD (elicitation phase). ACD symptoms appear mainly on the skin (e.g., eczema and redness) but gut or lung symptoms as well as responses in joints or the oral mucosa are possible, e.g., in allergy-associated implant failure [9,124].

In a sensitized individual, dendritic cells, macrophages, Langerhans cells or keratinocytes may take up haptens and present hapten-induced epitopes in situ to effector T and T_{RM} cells developed during the sensitization phase. In order to exert their effector functions at the site of chemical exposure, allergen-specific memory T cells need to enter the tissue. T cell infiltration may be triggered by innate signals resulting from the irritant capacity of a chemical allergen that, as in the sensitization phase, involves a multitude of cellular and molecular players [96,125]. In addition, the activation of local antigen-specific $T_{RM,}$ formed during the sensitization phase or prior elicitation reactions likely contributes. The potent effector functions and important role of T_{RM} have become a major topic in recent years since these cells are considered the main mediators of a plethora of skin diseases such as psoriasis, alopecia areata or mycosis fungoides and they cannot be therapeutically depleted at present [126]. Hence, prevention and regulatory strategies constitute an effective way for ACD management. In mice, CD4+ and CD8+ skin T_{RM} cells protect from infection and antigen-specific clonotypes distribute globally and accumulate at sites of antigen exposure [105,127]. Gaide et al. showed enrichment of individual skin-resident T cell clonotypes during the months following diphenylcyclopropenone (DPCP) exposure in humans [104]. Schmidt et al. illustrated a more rapid recruitment of CD8+ T cells at skin sites of previous ACD reactions which is indicative of local T_{RM} formation [128].

The chemical-induced innate response leads to the release of T cell-attracting chemokines. Kish et al. showed that murine CD8+ Trm produce IFNγ and Il-17A within the first 3 h of allergen exposure, which then triggers CXCL1, CXCL2/MIP-2 production by keratinocytes causing neutrophil infiltration, and, ultimately in this intricate series of events, secretion of T cell attracting chemokines, e.g., CXCL9/10, CCL17, CCL20 and CCL27 [129]. T cell infiltration only starts approximately 24 h after antigen exposure (thus the term "delayed-type hypersensitivity") [96]. The extent of bystander T cell infiltration, i.e., the fraction of T cells that enter the skin but have an unrelated antigen-specificity, remains unknown, but it was shown that antigen-specific T cells proliferate locally, leading to a solidification of the allergic state and T_{RM} formation. In a study with three patients, Kapsenberg et al. found 7–15% of skin infiltrating CD4+ T cells, cloned by limiting dilution cultures, to be Ni-specific in Ni-ACD [130].

Once the inflammatory milieu is established, many cell types may serve as APC for memory T cell activation including keratinocytes that upregulate MHC II upon, e.g., IFNγ exposure [131]. This T cell response is the body's attempt to remove the chemical allergen in a way similar to a pathogen.

Both CD4+ and CD8+ T cells as well as regulatory T cells seem to contribute to the allergic reaction in human metal allergy and murine contact hypersensitivity models [118,132–135]. Metal-specific regulatory T cells have been detected and shown to attenuate Ni-specific immune responses [136,137]. However, many aspects remain unknown, such as the relative contribution of CD4+ and CD8+ T cell subpopulations in humans for different chemical allergens. Here, more research is needed to advance alternative diagnostic and predictive testing. Future in vitro assays, for example, activation-induced marker assays, could track these populations in metal allergies [115,138].

During Ni-associated immune responses, a fraction of Ni-specific T cells expresses the skin-homing marker cutaneous lymphocyte-associated antigen (CLA), MHC II as activation marker (human leukocyte antigen (HLA)-DR) and Ki-67, which indicates an active cell cycle [115,139]. These findings illustrate that relevant metal-specific T cells can be identified by blood-based assays in vivo.

Metal-induced cytokine production by T cells has been studied quite extensively. Most metal-specific T cells produce INF-γ, which likely reflects the general high frequencies of T helper cells (T_H) 1 in human blood [140,141]. Increases in rarer T cell sub-populations, e.g., T_H2, T_H17, T_H9 or IL-10-secreting T cells, may be easier to detect given generally lower background and have been associated with metal allergies [142–146]. Whether there is a common polarization pattern among metal-specific T cells remains unclear. Among a limited number of donors, Ni-specific T cells from allergic donors comprised either a higher percentage of IL-4 or IL-17A producing cells, hence there was no general polarization pattern observed as for T_H1/T_H17A dominated CMV-specific immune response [115].

Given the involved steps as well as cellular and molecular effectors during an elicitation response, several therapeutic options are conceivable, but most have not yet been transferred into practice. Prevention of exposure is the top priority measurement if possible and it may be supported using barrier creams. Once ACD has developed, the first line treatment is the topical use of corticosteroids that dampen the inflammatory immune response. Similar treatments, such as cyclosporine, azathioprine, methotrexate, psoralen or UVA, may also control inflammation and are used as second line approaches upon corticosteroids resistance. So far, biologics (e.g., a-IL17 therapy, [147,148]) or oral tolerance induction have not been included in guideline-based treatment [149,150].

4.2. Diagnosis of Metal Allergies

4.2.1. Patch Testing as the Current Diagnostic Standard

So far, no causative therapy exists for metal allergies. Therefore, a precise diagnosis is crucial to avoid the chemical trigger and to prevent clinical manifestations of the disease. The in vivo method of patch testing was first applied in 1895 and is still the diagnostic standard today [149,151]. Patch testing aims to mirror the elicitation phase of ACD. Due to the high frequencies of positive patch test reactions, both Ni and Co salts are components of the European baseline series of contact allergens, which is used to diagnose contact allergy as a cause of clinically visible skin dermatitis in patients [70,151,152]. Occlusive applications of patch test allergens are deposited on the upper back for 48 h and diagnostic inspection follows after 48 h and 72 h with a standardized scoring of − and + to +++. Late readings, e.g., after 5–7 days, may yield additional positive reactions, especially in the case of metal allergies [153–155]. Patch test reactivity to Ni may decline if tested on other body areas than the upper back, e.g., on palms of the hands, which anatomically possess a thicker epidermis and less antigen-presenting cells (APC) [156].

Patch testing material contains Ni (II) sulfate hexahydrate ($NiSO_4 \times 6H_2O$; 5% concentration (w/w)) and petrolatum (pet.) as a vehicle. For historical reasons, water-soluble Ni sulfate is employed which seems to be less irritant than Ni chloride [157,158].

For Co patch testing, Co (II) chloride hexahydrate ($CoCl_2 \times 6H_2O$; 1% concentration (w/w)) pet. is used (0.4 mg/cm^2). Pd allergy is often diagnosed with 1% Pd (II) chloride ($PdCl_2$ pet.), e.g., in the standard testing series for dental metals in Germany. Interestingly, sodium tetrachloropalladate (Na_2PdCl_4) may represent a better patch test allergen as it reveals

more positive reactions, likely due to enhanced skin penetration [159,160]. This illustrates how the choice of the allergen preparation, including "the stability and purity of the allergen, its physical form, and the homogeneity of its distribution throughout the vehicle" [161] and as well its release from the vehicle, e.g., from petrolatum, influences patch test results.

Patch testing reproducibility is somewhat limited, especially for weakly positive reactions and for metal allergens. For instance, the reproducibility of $CoCl_2$ patch testing is only 35% [162–168]. In the case of patients with a clear history of metal allergy, a skin patch test is recommended before device implantation [169]. Nevertheless, there is no clear consensus regarding when and how to screen metal hypersensitivity before implant or prosthetic interventions. Besides, the correlation between patch test results and the prediction of implant failures is still controversial [55,170]. This could be due to a varying distribution of metal allergen-specific T_{RM} between the skin and target tissue of the implant.

In the case of concomitant diseases, e.g., "angry back" or "excited skin" syndrome, patch testing may not be possible. As another disadvantage of patch testing, the triggered local inflammation due to a positive patch test may enhance subsequent elicitation reactions in previously sensitized individuals as reported for human ACD [128,171].

In summary, patch testing has been proven very useful for over a century, but it is not a perfect tool. Therefore, diagnostic improvements, i.e., the development of reliable additional in vitro tests, are urgently needed.

4.2.2. Challenges Associated with Diagnostic In Vitro Tests

In vitro tests could overcome some of the above-mentioned limitations of patch testing. The aim of in vitro tests is to detect increased frequencies of antigen-specific memory T cells in the blood of allergic individuals similar to assays performed after vaccination to smallpox or yellow fever [172,173]. Yet, in vitro tests have not been officially implemented as diagnostic tools in ACD [149]. The difficulties of in vitro T cell tests for metal allergies seem to be mainly linked to the presence of rather high frequencies of metal-specific T cells in non-allergic individuals. The reactivity in non-allergic donors impedes the distinction of allergy-associated immune responses (Figure 2). In addition, T cell-based assays are labor and cost-intensive.

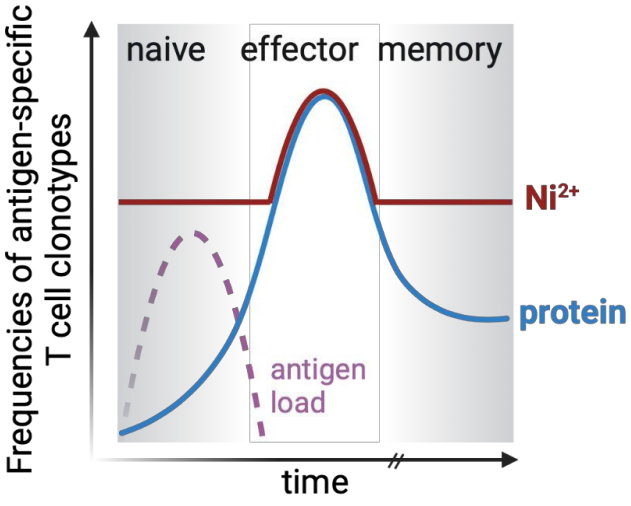

Figure 2. High frequencies of metal-specific T cells in blood interfere with diagnostic blood-based in vitro testing. Following adaptive immune responses, blood frequencies of protein-specific memory T cells are usually higher compared to those in the naïve T cell pool, even decades after antigen exposure (blue line). However, Ni-specific T cells are abundant in non-allergic individuals (red line), due to interactions with certain elements of the TCR repertoire (see Section 5.2) [115]. Therefore, only strongly increased frequencies of Ni-specific T cells may currently be associated with the allergic state. Adapted from [174]. Created with BioRender.com.

Ni is the most intensively studied metal allergen in vitro. Proliferation-based approaches, e.g., lymphocyte transformation or proliferation tests (LTT or LPT), Memory Lymphocyte Immunostimulation Assay (MELISA), or cytokine-secretion assays (e.g., Enzyme-linked-immuno-Spot Assay (ELISpot)) have been employed [6,175]. Our group recently showed that CD154 upregulation identifies the Ni-specific CD4+ naïve and memory T cell pool in a fast, comprehensive and quantitative manner [115]. Thus, the benefits of activation-induced marker assays may also be exploited in the field of chemical allergens, opening new avenues for the investigation of involved T cell responses [138,176].

Regarding Ni-specific T cells, often, similar responses have been observed in non-allergic and allergic individuals, especially for CD4+ T cells [89,115,132,177–179]. The frequency of Ni-specific CD4+ T cells correlates with the used Ni concentrations and varies between ~0.00005%, 0.02% and 0.1% at Ni^{2+} concentrations close to zero, ~40 µM, and 200 µM, respectively [115,132,180]. However, no concentration has been determined which optimally distinguishes allergic from non-allergic individuals [89,177,181]. Local in vivo concentrations in the skin or lymph nodes remain unknown. The use of high but non-toxic Ni^{2+} concentrations in vitro may completely capture the specific T cell pool while unrelated immune responses, e.g., to cytomegalovirus (CMV), do not seem to interfere [115].

Co seems to activate a similar or slightly smaller percentage of T cells compared to Ni, as judged from proliferation-based assays, [181–183] while Pd-specific T cell frequencies have hardly been investigated [89,184]. Unless limited dilution cultures are used, proliferation-based assays do not allow a direct assessment of the initial frequencies of reactive T cells. Cristaudo et al. detected IFN-γ-release by ELISpot after incubation of peripheral blood mononuclear cells (PBMC) with 14 µM $PdCl_2$ and observed strongly increased responses in six allergic donors with ongoing oral symptoms and positive patch test reactions compared to 10 non-allergic controls [185]. This finding indicates that Pd-specific T cells can be detected similar to Ni- and Co-specific T cells in vitro. Of note, cytokine release assays capture only cytokine-secreting T cell subpopulations, not the complete antigen-reactive pool.

In some publications, high frequencies of metal-reactive T cells have been attributed to "unspecific mitogenic effects" [186,187]. Still, the specific restimulation of individual T cell clones and prevention of activation with major histocompatibility complex (MHC) blocking antibodies argues for a mainly TCR-mediated activation [115,188,189].

In summary, only some allergic individuals top background frequencies of metal-specific effector or memory T cells in blood-based in vitro tests (Figure 2). Further research is required to identify more distinctive, allergy-associated T cell subpopulations or, if tissue samples are available, in situ allergy-associated immune responses [175,190].

Future in vitro blood-based allergy tests should optimally detect outgrowth of different cytokine-producing T cell subsets and, if possible, additional markers of T cell activation. For this purpose, activation-induced marker assays are especially promising since they more likely report ex vivo phenotypes of allergen-specific T cells if combined with multiparameter flow cytometry compared to proliferation-based methods [115,176].

4.2.3. Predictive Tests for Sensitizing Properties

Predictive tests aim to determine the sensitization hazard and, optimally, the potency, i.e., the dose ($\mu g/cm^2$) required for sensitization. In mice and guinea pigs, Ni sensitization requires relatively high percutaneous doses or intradermal injection in the presence of costimulatory signals [118,191–193]. One underlying mechanism may be the lack of the primate-restricted histidine duplet (H456/H458) in murine and other rodent TLR4 [117]. Ethically questionable human experiments from the 1960s showed that exposure to $NiCl_2$ sensitized 48% of individuals [194,195]. In real life, Ni allergy has been associated with very low exposure concentrations and skin injury (piercings) [74,196]. This indicates a role for heterologous immune stimulation and illustrates bottlenecks in current regulatory testing. Established in vitro tools do not assess heterologous immune stimulation, T cell activation, or experimentally validated cross-reactivity [197]. Future T cell-based assays may help

to assess T cell activation for either allergen mixtures, metal alloys or both. These tests could possibly be based on activation-induced marker assays which offer many advantages compared to proliferation-based assays [115,138,176].

5. TCR Antigen Recognition, Metal-Induced T Cell Epitopes and Cross-Reactivity

5.1. General Considerations Regarding Antigen Recognition by TCR

Matzinger and Bevan were the first to postulate that a TCR can be activated by a variety of peptides presented by one MHC [198]. In the past, clonal selection theory suggested that each T cell selectively recognizes only one pMHC complex [199,200]. Cross-reactivity, also known as immune polyspecificity, is defined as the ability of a TCR to bind and functionally respond to more than one pMHC complex. Mason further extended the cross-reactivity conceptual framework stating that the TCR repertoire must be able to recognize all foreign peptides presented by MHC proteins [201]. MHC I molecules, which present antigens to CD8+ T cells, may theoretically present more than 12×10^{11} different peptides with a length of 10 amino acids (considering the presence of anchoring residues). MHC II, which present antigens to CD4+ T cells, may present even more peptides because these antigen-presenting molecules have an open-ended binding groove that can accommodate longer peptides [202].

These numbers of possible antigen structures vastly exceed the huge, but still limited numbers of different αβ TCR ($\geq 10^8$, among ~10^{12} T cells) which result from V-(D)-J-recombination and are expressed in each individual [203,204]. Therefore, T cells may only provide complete immune coverage if each TCR detects many (foreign) peptides. Wooldridge et al. showed that a single autoimmune CD8+ T cell clone recognizes more than 10^6 different artificial peptides with a length of ten amino acids on a single MHC I molecule [205]. Once the first multimolecular X-ray diffraction structures of complete TCR-pMHC complexes became available, details on TCR binding and non-mutually exclusive mechanisms underlying cross-reactivity have been discovered [206]. Currently, the STCRDab database lists 68 human TCR-pMHC I and 20 TCR-pMHC II structures (related structures from the same TCR were excluded; http://opig.stats.ox.ac.uk/webapps/stcrdab/, accessed on 13 October 2021 [207]). This still limited but steadily growing database only comprises one metal-specific TCR (beryllium (Be)-specific [208]) and one Ni-specific TCR (ANi2.3), for which a Ni-independent mimotope has been isolated [209]).

5.1.1. TCR-pMHC Contact Points and General Binding Orientation

In the typical αβ TCR binding mode, the TCR is oriented diagonally above the pMHC complex (Figure 3) [210]. The Vα domain lies over the amino-terminal end of the peptide (α2 helix MHC I, β1 helix MHC II) and the Vβ domain lies over the carboxy-terminal end of the peptide (α1 MHC helices). The contact points of the complementarity-determining regions (CDR) 1 and 2 to the MHC are mostly conserved [211]. The CDR3 regions of both, TCR α- and β-chain, are the most variable binding sites and are mainly in contact with the antigen peptide [210]. Details in the binding of the CDR loops to the peptide and the MHC may vary greatly for different TCR.

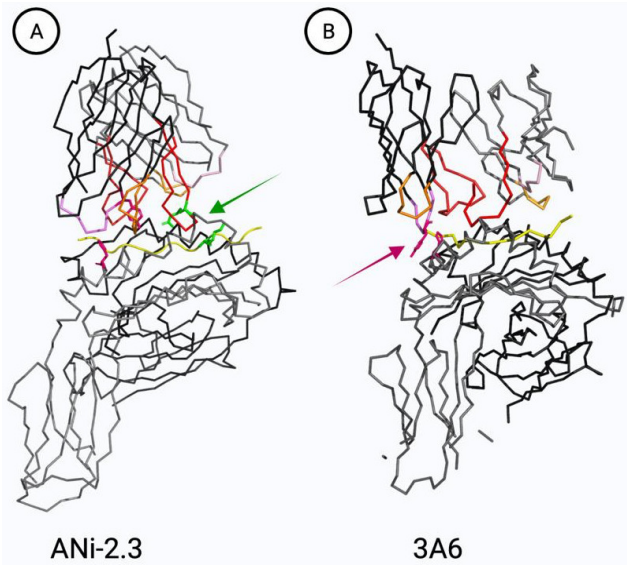

Figure 3. Structure of TCR-pMHC II complexes. TCR α- and β-chains (top) with CDR3 regions (CDR1, pink; CDR2, orange; CDR3, red) are positioned diagonally over MHC II α- and β-chains (bottom) and the presented peptide antigens (yellow). (**A**) Ni-specific TCR ANi-2.3 (TRAV8-TRBV19) in complex with a peptide mimotope (pdb ID code 4h1l). Lys_7 likely replaces Ni in this structure with Asp_{95} being an essential contact point (green stick representation, [209]). (**B**) TCR 3 A6 (MBP 89-101-specific, TRAV9-2-TRBV5) represents the only available TCR-pMHC II structure of a human TRAV9-2 TCR (pdb ID code 1 zgl). His_{81} is a major TCR contact point in the MHC II β-chain and is in close proximity with Tyr_{36} in the TCR α-chain CDR1 of TRAV9-2 (pink stick representation). A recent study found that approximately 35% of Ni-specific TCR expressed TRAV9-2 compared to ~5% in the random repertoire [115]. The depicted TCR do not express a histidine in their CDR3 regions, which constitutes an independent recently discovered major Ni binding mechanism [115] (see Section 3.2). Created with Pymol.

5.1.2. Cross-Reactivity due to Conserved Binding Sites

The interaction between TCR and pMHC is only partly conserved and allows flexibility mainly within the borders of some general orientation and binding rules [202]. Several crystal structures from the same TCR binding to different pMHC complexes formed the concept of conserved interaction. This concept indicates that a small, conserved amino acid motif stabilizes the TCR interaction with the pMHC complexes. Outside of this motif, a large number of changes can be tolerated, allowing a TCR to identify many different binding partners [212–214]. Within the motifs, only a few changes in the amino acids are tolerated. The clonal dependence upon these interactions can vary [215]. Peptide binding to MHC is mainly independent of the TCR engagement and involves different interactions of anchoring amino acids with the MHC binding groove. Thus, it is possible to isolate peptide mimotopes from artificial randomized peptide libraries that may be enriched in MHC-anchoring amino acids at certain positions. Mimotope alignment then reveals the conserved amino acid motif for a given TCR and, by protein database mining, can elucidate the original unknown antigen because biological peptide variability is limited [209,213,216,217]. However, in metal allergies, the coordinative bond of the metal ion poses additional challenges in epitope identification [209].

5.1.3. Cross-Reactivity due to Induced Fit

Another, complementary, concept for cross-reactivity is induced fit [218], also referred to as structural plasticity or conformational melding. During induced fit, the CDR loops and the pMHC complex cooperatively adjust and may undergo large conformational

changes mainly without altering the overall docking orientation [219,220]. In general, the CDR3 loops change their conformation more than the other loops, the peptide or MHC [206,221–224].

An example for cross-reactivity is molecular mimicry. Pathogenic peptides may mimic self-peptides and thus evade immune recognition [225]. In molecular mimicry, ligands share structural and chemical features [226]. The cross-reactivity may be focused on so-called "hot-spot mimicry" where short amino acid stretches are identical between pathogen and self-peptide, showing the connection to the concept of conserved binding sides [227].

Cross-reactive T cells may have advantages and disadvantages. For instance, cross-reactive T cells may recognize antigen peptides from related pathogens and thus provide protection to heterologous infections (this seems limited in the current SARS-CoV-2 pandemic with respect to former common cold coronavirus (SARS-CoV-1) infection [228,229]). On the downside, cross-reactive TCR may contribute to autoimmunity [225].

5.2. Metal-Induced T Cell Epitopes

Non-metallic chemical allergens are considered to bind mainly covalently to proteins via electrophilic-nucleophilic interactions or via the formation of radicals. Alternatively, metal ions as well as some small chemicals bind via complex formation (coordinative binding) at the TCR-pMHC interface [7,230]. In chemical allergies, no pathogen is present. Therefore, self-proteins or peptides are altered to form chemically induced T cell epitopes. The majority of metal-specific T cells seems MHC restricted although exceptions have been reported [231]. Usually, due to the selection process in the thymus, T cells have a low affinity for self-pMHC complexes, which does not lead to activation. However, the presence of a metal ion at the TCR-pMHC interface or cryptic epitopes may provide enough interactions to exceed activation thresholds. The still limited data on individual metal-specific TCR mainly comprise CD4+ T cell clones since metal-reactivity is more abundant on this subset (see Section 4.1.2). Several mechanisms for the generation of metal-induced T cell epitopes have been proposed, which are supported by a varying amount of experimental evidence (Figure 4).

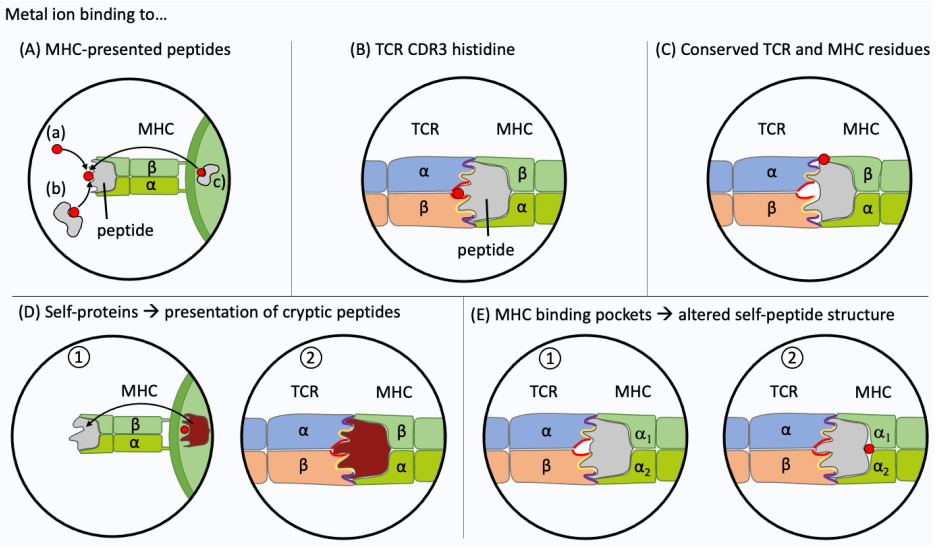

Figure 4. Metal recognition by T cells. Experimental evidence has been obtained for five different interactions between the T cell receptor (TCR), the metal ion (highlighted in red) and the peptide presented at the surface of the major histocompatibility complex (MHC). (**A**) The metal ion haptenizes the presented peptide. Loading can occur directly from the extracellular space (a) or by transfer from a metal-binding protein [232] (b). The metal ion may also be pre-loaded to the MHC-presented peptide, e.g., by antigen-processing of a metal-binding protein (c). (**B**) Metal ion binding at the TCR complementarity-determining region 3 (CDR3). The metal ion may bind to the TCR CDR3. Recently, frequent Ni^{2+} binding via a histidine in the CDR3 of TCR α- or β-chain has been shown [115]. (**C**) Metal ion binding to conserved residues at the TCR-peptide-MHC

interface. Ni^{2+} ions often bind via $tyrosine_{36}$ in the CDR1 of TRAV9-2+ TCR and $histidine_{81}$ in the MHC II β-chain [114,115]. (**D**) Recognition of cryptic epitopes. Metal ions may alter antigen-processing of a self-protein which in turn leads to the presentation of a metal-free cryptic self-peptide. (**E**) Metal ions may bind within the cleft between MHC and antigen peptide. The TCR is not activated by a self-pMHC complex (1). The metal ion binding leads to structural conformation changes of the presented peptide and creates a metal-free neo antigen recognized by the TCR (2). This type of interaction has been shown for beryllium (II) ions in combination with HLA-DP2 [233].

The activation of metal reactive T cells likely requires a metal-mediated bridge between the TCR and pMHC complex in most cases [7]. Metal ions can directly interact with endogenous peptides bound to the MHC (Figure 4A). The TCR can then recognize the metal ion which is present on the pMHC. Ni-binding to the amino acid histidine in an MHC II-presented peptide (CS325-341 EPSDKHIEQYLKKIKNS) has been shown by NMR spectroscopy (Figure 4A(a)). The "empty" peptide activated a CS325-341-specific TCR (clone HM.37), the addition of Ni^{2+} prevented this interaction [234]. Similarly, the addition of Au (III) inhibited the activation of a CST3 (CS378-398 DIEKKICKMEKCSSVFNVVNS)-specific TCR (clone BH26). In this latter case, the peptide does not contain a histidine and the position of Au^{3+} remains speculative [235]. Possibly, Au^{3+} stripped the peptide from the MHC II and this abolished TCR activation [236].

Metals may be transported in human skin and blood by Ni-binding co-mediators towards the vicinity of the MHC molecule. Thierse et al. showed that activation of Ni-specific CD4+ T cells via a Ni-saturated human serum albumin complex can be comparable to activation by metal salts at equimolar concentrations. The human serum albumin was not used as an antigen on the MHC but only served to transfer Ni ions (Figure 4A(b)) [232].

The metal ion may also bind to proteins that undergo antigen processing. Fixation of APC with, e.g., glutaraldehyde, has been used by several groups to distinguish extracellular metal ion binding from intracellular processing of metal-binding proteins (Figure 4A(c)). Moulon et al. studied 42 CD4+ MHC II-restricted T cell clones. They showed that 40% of the clones were unable to recognize Ni^{2+} on the surface of fixed APC [188]. If the epitope is stable after fixation, the metal recognition can be classified as processing-independent and, at the time, the formation of cryptic epitopes can be excluded. Regarding the processing-dependent epitopes, another possible explanation for the lack of T cell activation by fixed APC is the possibility that the conformation of the metal-binding pMHC has been altered and thus external metal ion binding is disturbed. This cannot be ruled out, as no controls were implemented. One example for a CD4+ TCR that recognizes Ni bound to an MHC II-presented peptide is clone "ANi-2.3" (Figure 3A) which reacts to a complex of Ni and an unknown peptide generated in human B cells but not in other cells, e.g., PBMC (Figure 4A(c)) [113]. For clone Ani-2.3, a mimotope was found, that can replace Ni along with the peptide on the surface of MHC. Ni was replaced by the p7 lysine. Antigen recognition of the mimotope and the original Ni-epitope was equally affected when the Ani2.3 TCR was mutated. A CDR3β D95 E mutation abolished recognition, indicating that a geometrically highly defined coordination complex rather than just a negative charged amino acid is required at that position [209]. The exact Ni-binding geometry, however, remains unclear since no structure containing Ni is available. Ani2.3 is already activated by small metal salt concentrations. The concentration required for Ni-induced TCR interactions varies widely as does their dependency on the presence of certain antigenic peptides [115]. Some Ni-specific TCR are activated by very low Ni concentrations, for example by pulsed and washed APC, or even in the absence of professional APC [115,237].

Ni^{2+} may not only be bound by the MHC-presented peptide but also by the TCR itself as recently shown by our group (Figure 4B). Adapting a CD154 upregulation assay to isolate Ni-specific CD4+ T cells, we were able to comprehensively analyze the Ni-reactive TCR repertoire by high-throughput sequencing [115]. In the amino acid composition of the CDR3 of TCR α- and β-chains, a histidine was particularly abundant among Ni-specific TCR (14% of α-chain CDR3 and 29% of β-chain CDR3 at 200 μM $NiSO_4$). As shown for some example clones, one histidine in either TCR α- or β-chain was sufficient for Ni

recognition. Ni-binding to the histidine in the CDR3 may exceed the activation threshold of a non-optimal self TCR-pMHC connection (Figure 4B).

Another major mechanism of Ni recognition was experimentally proven [114,115]. Ni forms a complex with tyrosine$_{36}$ (IMGT nomenclature) in the CDR1 α-chain of the gene segment TRAV9-2 and with histidine$_{81}$ in the MHC II β-chain (Figure 4C). In this case, Ni may be needed as a direct link between the TCR and MHC, with the specified residues being in close proximity (Figure 3B) [114]. Our group showed that 35% of Ni-specific T cells express the variable gene segment TRAV9-2 while only 5% of random T cells contain this segment [115]. The identified TCR repertoire features, i.e., overrepresentation of segment TRAV9-2 or a CDR3 histidine, occurred among naïve and memory CD4+ T cells in allergic and non-allergic individuals. This reflects the high numbers of Ni-specific T cells that are present in non-allergic individuals. Little is known about the association with TCR gene segments for other metal-specific TCR. In a murine BALB/cAJcl metal allergy model, Ni-sensitized and Pd-challenged mice showed a high frequency of TCR with three distinct V-J segment combinations (TRAV7-5/TRAJ56, TRAV8 d-1/ TRAJ49 and TRAV5-1/TRAJ 37) [238]. Takeda et al. studied the pathogenic T cells responsible for Pd allergies. They found that in C57 BL/6 mice CD8+ T cells with the TRAV 7-2*02 segment increased significantly [134]. However, not only murine models but also human TCR repertoires differ, so additional data for humans are needed [105].

Metal ions may be able to alter the processing of self-antigens, causing T cells to respond to cryptic self-peptides. Griem et al. investigated two bovine RNase-specific T cell clones that only reacted upon Au^{3+} pre-treatment but not to the native protein [239]. The addition of Pd^{2+} had the same effect while Ni^{2+} was ineffective. The cellular processing of the proteins modified by metals caused the presentation of metal-free cryptic self-peptides, which could then be recognized by T cells. The altering of the original protein with Au^{3+} and Pd^{2+} results in the same cryptic peptides, which could be a cause for metal-associated TCR cross-reactivity (Figure 4D) [240].

So far, no HLA haplotype associations have been identified for metal allergies except for Be which is linked to HLA-DP2 [233]. Be^{2+} engages in a binding mechanism in which the Be^{2+} ion is not in direct contact with the TCR. Be^{2+} is buried between glutamic acid$_{69}$ in the MHC II β-chain and the presented peptides and causes conformational and biophysical changes on the surface of the complex, generating neoantigens (Figure 4E) [208]. HLA-independent activation has also been described, but only for two Ni-specific CD8+ TCR from two donors. Both clones proliferated even when Ni was presented on allogeneic B cells [231,241].

Cross-reactivity is conceivable for most of the mechanisms presented above since one metal ion may be replaced by another metal with the same ion charge and similar binding properties.

5.3. TCR Cross-Reactivity to Ni, Co, and Pd

TCR cross-reactivity to different metal ions has been investigated in vivo by patch testing and in vitro by restimulation of metal-specific T cell clones with different metal allergens. Regarding the former, various epidemiological patch testing studies from different timeframes and worldwide locations show that Ni-allergic individuals often react to Co^{2+} and Pd^{2+} similarly. Depending on the study, between 2.3 and 72% of Ni-allergic patients also react to Co^{2+} with a positive patch test reaction [82,242,243]. Between 11 and 40% of Ni-reactive individuals respond to Pd^{2+} [87,242,243]. In several studies, up to 83% co-sensitization of Co-allergic patients to Ni^{2+} is also observed in combination with other metals [244,245]. Up to 95% of Pd-reactive individuals co-react to Ni^{2+} [246,247]. Furthermore, 56% of Pd-allergic individuals react to Co^{2+} (vice versa [87]).

A study by Hindsen et al. showed flare-up reactions after oral Ni salt administration at the sites of previous ACD to Pd and, to a lesser extent, ACD to Co, indicating the existence of TCR cross-reactivity, although the extent at the individual T cell level remains to be determined [171]. In a murine metal allergy model, Ni-and Pd-sensitized mice showed

an allergic response after challenge with Ni^{2+} and Pd^{2+} but not with Co^{2+}, chromium (II), copper (II) or Au (II). When the Ni-sensitized mice were challenged with Ni^{2+} and Pd^{2+} simultaneously, the reaction appeared to be additive when compared with the response to each metal alone, indicating Ni-Pd-cross-reactivity [248].

The wide variation in the frequencies of co-sensitized patients between different metal ions indicates that it is difficult to investigate the extend of T cell cross-reactivity by patch testing only. One issue in analyzing cross-reactivity is that it is not known whether the analyzed patients have a history of immunologically relevant co-exposure, i.e., co-sensitization but not cross-reactivity is observed by patch testing. Additionally, the reliability of the patch test is limited, given the low reproducibility and the influence of different metal salts including the used concentrations and varying skin penetration capacities as well as reading protocols for results (See Section 4.2.1).

In vitro cross-reactivity assays may be a more reliable alternative to patch tests. Metal-specific T cell clones can be tested directly with different metals. However, a strong concentration-dependent activation of T cells has been observed [115,132,180]. Therefore, results regarding cross-reactivity should also be interpreted with respect to the metal salt concentrations used. In addition, graded responses may occur, e.g., a distinctive but lower or stronger activation to a cross-reactive metal ion at equimolar concentrations. The high variability between individual T cell clones from one donor and large interindividual donor differences complicate the interpretation of the limited data on T cell cross-reactivity. Given the high frequency of metal-specific T cells in non-allergic patients, analyzed clones may not be allergy-relevant. As a result, comprehensive testing of T cell clones is very time-consuming and has thus been not accomplished. Taking advantage of the CD154+ upregulation assay could speed up this process [115]. Cross-reactivity becomes likely with elements that are listed in close proximity in the PSE. Since Ni^{2+} and Pd^{2+} have physiochemical similarities, they can form similar complexes and thus trigger the same modifications in proteins present in the skin or pMHC. These could then in turn be recognized by the same T cells [249].

Until today, only a few Ni-specific T cell clones were tested for cross-reactivity to Co^{2+} and Pd^{2+} [188,250]. Most of the Ni-specific T cell clones were either specific for Ni^{2+} or cross-reactive only to Pd^{2+}. Moulon et al. and Pistoor et al. each identified only one Ni-specific clone (4.13 and PPN.53), which cross-reacted to Pd^{2+} and to Co^{2+}. To date, for Co-specific T cells, no cross-reactivity to Ni^{2+} could be found [251,252]. Moulon et al. also assessed the cross-reactivity of Pd-specific T cells. Pd-specific clones cross-reacted only to Ni^{2+} and cross-reactivity varied depending on the donor. In one particular donor, all six clones examined, which were established by limited dilution from a line, also responded to Ni^{2+}. In the second donor studied, only one of three clones reacted positively [188]. Cross-reactive T cells showed the same MHC restriction as with the original antigen, indicating exchange of the metal ion at the original epitope site [250].

Ni-specific naïve CD4+ and CD8+ T cells also showed a low rate of cross-reactivity to Co^{2+}. Out of 11 Ni-specific CD4+ naïve T cells, only twocross-reacted to Co^{2+}. Among four Ni-specific CD8+ naïve T cells, no cross-reactivity could be observed [180].

In summary, available experimental results indicate some cross-reactivity among Ni-, Co- and Pd-specific TCR but the exact percentages of cross-reactive T cells and details of the molecular recognition mechanisms remain unclear.

5.4. TCR Cross-Reactivity between Other Metal Allergens

Only very few Ni-specific CD4+ T cell clones have been investigated for cross-reactivity to other metal ions than Co^{2+} and Pd^{2+} and a minority of these proved cross-reactive to various metal ions. These metal ions could utilize the same binding mechanism as Ni^{2+}, by coordinating similarly with the pMHC [113]. The Ni-specific TCR (ANi-2.3) cross-reacted to copper (II) and Au^{2+}. In addition to Ni-Pd cross-reactivity, Moulon et al. and Pistoor et al. found Ni-specific T cell clones that cross-reacted solely with copper

(II) but not with chromium (VI), Co^{2+} or Pd^{2+} [188,250]. The Ni-specific clones 4.13 and PPN.53, which cross-reacted to Co^{2+} and Pd^{2+}, also reacted to copper(II) [188].

In summary, to date there has been only anecdotal in vitro research on the cross-reactivity of metal-specific T cells.

6. Conclusions

Much progress has been made in the understanding of the immunotoxic effects of metals. While some heavy metals like Ni [26] and Co [38] play an important role in biochemical and physiological processes in bacteria or animals, inadequate exposure can constitute a serious risk for human health. One of the most common forms of chronic immunotoxicity in humans is ACD. Adverse effects of metal ions that support the sensitization phase may be mediated by ROS [19] or protein binding. For example, Ni^{2+}, Co^{2+} and Pd^{2+} induce pro-inflammatory gene expression by binding to the human TLR4 receptor [120]. Metal ions form complexes with proteins often via the imidazole moiety of histidine. The binding of metal ions to proteins (haptenization) is of fundamental importance for the development of allergic reactions, as it leads to the formation of allergen-induced T cell epitopes [7].

Although many details of the molecular interactions remain to be resolved, pioneering studies shed light on the way metal ions interact with the TCR-pMHC interface. Several binding mechanisms have been discovered, often using Ni^{2+} as a model allergen. New methods such as activation-induced marker assays combined with high-throughput TCR sequencing may allow a more quantitative analysis of metal-reactive T cell subpopulations as well as the deciphering of common metal-specific TCR repertoire features [115]. The complexation of Ni^{2+} via tyrosine 36 in the CDR1 of segment TRAV9-2 and histidine 81 in the MHC β-chain by a large fraction of TCR among Ni-specific CD4+ T cells was shown [114,115]. In addition, our group discovered that Ni^{2+} may not only bind to the surface of the pMHC complex via various pathways but that it is often complexed by histidine residues in the CDR3 of αβ TCR from CD4+ T cells [115].

Cross-reactivity to peptide antigens was first postulated in 1977 [198] and seems to be ubiquitous, because the huge human αβ TCR ($\geq 10^8$ [204]) is still dwarfed by the number of possible pMHC complexes [202]. The mechanism of cross-reactivity is mainly based on conserved binding sites between TCR and pMHC [213], as well as on induced-fit [224], with no clear separation between both concepts. The diagnostic standard for ACD to date is the patch test, which has clear disadvantages such as limited reproducibility, especially for metal allergens [166]. The patch test is also rather unsuitable for the investigation of cross-reactivity since co-sensitization may be due to unknown previous co-exposure and different skin penetration capacities of the investigated metal salts confound results. In vitro tests could overcome some of the limitations of patch testing. In case of metal allergies, the use of diagnostic in vitro tests is currently limited due to the high frequency of metal-specific T cells in non-allergic individuals and the lack of a possibility to identify the pathogenic populations. Regarding cross-reactivity, to date, there are only anecdotal data from a limited number of donors on the cross-reactivity of Ni-specific T cell clones [188,250]. They point towards a cross-reactivity of Ni-specific T cells to Pd^{2+} and more rarely to Co^{2+} for CD4+ T cells. Here, clearly more research is needed.

The knowledge about the cross-reactivity pattern of metal-specific TCR as the foundation of the adaptive immune response may not only be important for the choice of implant materials to avoid incompatibility reactions. It could also influence the regulation of metal content in consumer products. Nevertheless, the avoidance of allergens, e.g., by a switch to alternative materials such as ceramics for implants is the most effective prevention for the elicitation of metal allergies. Therefore, it is crucial to obtain a deeper mechanistic understanding of the TCR interactions with metal-induced T cell epitopes and the underlying TCR repertoire features. In this context, the in vivo relevance of mechanisms and metal-specific T cells identified in vitro should be further elucidated. Finally, to obtain a deeper insight into ACD, the penetration of metal ions into the skin and the role of antigen-specific T cell subpopulations including T_{RM} should be assessed.

Author Contributions: Conceptualization, F.R., M.A.-S., C.C., H.-J.T., K.S. and A.L.; investigation, F.R., M.A.-S., C.C., H.-J.T. and K.S.; writing—original draft preparation, F.R., M.A.-S., C.C., H.-J.T. and K.S.; writing—review and editing, F.R., M.A.-S., C.C., K.S. and A.L.; visualization, F.R. and K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Federal Institute for Risk Assessment, Grant Number: SFP 1322-718 and SFP 1322-719.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACD: allergic contact dermatitis; Au, gold; Be, beryllium; CDR, complementarity-determining regions; CMV, cytomegalovirus; Co, Co^{2+} ions, cobalt; ELISpot, Enzyme-linked-immuno-Spot Assay; HLA, human leukocyte antigen; ICP-MS, inductively coupled plasma mass spectrometry; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MW, molecular weight; Ni, Ni^{2+} ions, nickel; PBMC, peripheral blood mononuclear cells; Pd, Pd^{2+} ions, palladium; pMHC, peptide-MHC; PRR, pattern recognition receptors; PSE, periodic table of elements; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; ROS, reactive oxygen species; TCR, T cell receptor; T_H, T helper cells; TLR, Toll-like receptor; $T_{RM,}$ tissue-resident memory T cells.

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1.6 *In vitro* detection of chemical-specific T cells

The reliable diagnosis of ACD is increasingly essential for the hazard prediction of chemicals by global regulatory authorities and patient's diagnosis and risk prediction. This would help patients to be aware about the threat enabling them to avoid any contacts with the allergen afterwards. Additionally, the diagnosis could prevent, for example, implant failure in the case of an allergic reaction to the implant alloy. Given the lack of causal therapies, ACD reduces the quality of life and thus can burden on the level of personal health and for the entire public health system[122]. Until today, diagnosing of contact allergies remains challenging. The epicutaneous patch test was first applied in 1895 and still serves as the standard in clinical diagnosis today[129]. For the test, the allergen emulsions, mainly in petrolatum, are applied under occlusion for 48 h, and the visual inspection of eczema follows after 48 and 72 h. However, the reproducibility of the patch test is limited, especially for weak reactions and for metal alloys. For instance, positive patch test results for cobalt are reproducible in only 35% of the cases[130]. Moreover, the triggered local inflammation may enhance subsequent ACD reactions in previously sensitised individuals[131]. In addition, patch test standards do not exist for all sensitising compounds. Therefore, alternative *in vitro* assays, including T cell analysis, are of great value and are thus urgently needed. *In vitro* detection offers the additional opportunity to understand the fundamental mechanisms of allergen binding to the TCR and the subsequent activation of the immune system.

In current regulatory alternative methods to the patch test, such as human cell line activation test (h-CLAT), genomic allergen rapid detection (GARD), direct peptide reactivity assay (DPRA) and KeratinoSens/LuSens, the important aspects of T cell activation are not addressed at all. So far, only activation of the innate immune system is being included. Therefore, there is no substitute for animal tests, such as the local lymph node assay (LLNA) or the guinea pig maximisation test. These tests are *in vivo* assays which naturally include T cell activation as part of the entire pathophysiological trigger cascade. Both assays measure lymphocyte proliferation in draining lymph nodes of animals as part of the initial compound-mediated sensitisation phase or the subsequent T cell-mediated skin eczema formation ("elicitation phase"), respectively.

To directly analyse conventional antigen-specific T cells, it is possible to stain them by applying peptide-MHC multimers. However, this method cannot be applied to chemical allergens. So far, there is only one exception: the T cell clone Ani2.3 was identified by a Ni^{2+} modified peptide tetramer that mimicked its epitope (so-called mimotope)[132]. An alternative to peptide-MHC multimer staining is the assessment of the functionality of the T cells. Historically, mainly the proliferation of T cells has been analysed (e.g. by lymphocyte transformation tests (LTT)), but recently priming assays that include naïve T cell responses have become more popular[133, 134, 135]. As alternative activation markers, cytokines can

be examined (e.g. by enzyme-linked immunosorbent assay (ELISA), or enzyme-linked immune absorbent spot assay (EliSpot)). The disadvantage of cytokine detection is that it only enables the investigation of T cell subpopulations since not all memory T cells express cytokines. New solutions are activation-induced marker (AIM) T cell assays, which measure T cell activation as a result of antigen stimulation by upregulation of surface markers. AIM T cell assays are well described for CD154+ CD4+ T cells and protein antigens[136, 137, 138]. CD154+ CD4+ T cells can be detected either intracellularly or on the T cell surface in the presence of an anti-CD40 blocking antibody. Other antigen-specific T cell subpopulations that can be analysed through AIM assays are CD137+CD4+ Treg or CD137+CD8+ T cells[139, 140]. AIM assays have been recently extensively applied to monitor SARS-CoV-2-directed human T cell immune responses[141]. However, detecting chemical-specific T cells poses challenges of its own, such as a potentially inefficient epitope formation *in vitro*, the insolubility of chemicals, inherent toxicity, or interferences with flow cytometry[124, 127, 142].

Because of the various challenges, T cell activation has been proven only for very few organic chemical allergens *in vitro*, mainly to p-phenylenediamine (PPD), its bandrowski's base (BB) and TNBS. Each new chemical included requires individual assay optimisation at this stage. The current literature on the detection of chemical-specific T cell responses is summarised in the following review on skin-sensitising chemicals in section 1.6.1.

1.6.1 Systemic review paper: *In vitro* monitoring of human T cell responses to skin sensitizing chemicals—A systematic review

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Published online on 28 December 2021 in:

Cells 2022, 11, 83.

DOI: 10.3390/cells11010083

Link: https:// doi.org/10.3390/cells11010083

Involvement of the author within this publication: Literature research, writing and visualisation of the manuscript.

Author contributions as published: K.S., M.A.-S. and C.C. conceived the study; C.C. designed the searching strategy; C.C., M.A.-S. and K.S. carried out the searching strategy; K.S., M.A.-S., C.C. and F.R. wrote the manuscript; visualisation was done by C.C. and F.R.; M.A.-S., C.C. and K.S. interpreted data; H.-J.T. and A.L. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

cells

MDPI

Review

In Vitro Monitoring of Human T Cell Responses to Skin Sensitizing Chemicals—A Systematic Review

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Abstract: Background: Chemical allergies are T cell-mediated diseases that often manifest in the skin as allergic contact dermatitis (ACD). To prevent ACD on a public health scale and avoid elicitation reactions at the individual patient level, predictive and diagnostic tests, respectively, are indispensable. Currently, there is no validated in vitro T cell assay available. The main bottlenecks concern the inefficient generation of T cell epitopes and the detection of rare antigen-specific T cells. Methods: Here, we systematically review original experimental research papers describing T cell activation to chemical skin sensitizers. We focus our search on studies published in the PubMed and Scopus databases on non-metallic allergens in the last 20 years. Results: We identified 37 papers, among them 32 (86%) describing antigen-specific human T cell activation to 31 different chemical allergens. The remaining studies measured the general effects of chemical allergens on T cell function (five studies, 14%). Most antigen-specific studies used peripheral blood mononuclear cells (PBMC) as antigen-presenting cells (APC, 75%) and interrogated the blood T cell pool (91%). Depending on the individual chemical properties, T cell epitopes were generated either by direct administration into the culture medium (72%), separate modification of autologous APC (29%) or by use of hapten-modified model proteins (13%). Read-outs were mainly based on proliferation (91%), often combined with cytokine secretion (53%). The analysis of T cell clones offers additional opportunities to elucidate the mechanisms of epitope formation and cross-reactivity (13%). The best researched allergen was *p*-phenylenediamine (PPD, 12 studies, 38%). For this and some other allergens, stronger immune responses were observed in some allergic patients (15/31 chemicals, 48%), illustrating the in vivo relevance of the identified T cells while detection limits remain challenging in many cases. Interpretation: Our results illustrate current hardships and possible solutions to monitoring T cell responses to individual chemical skin sensitizers. The provided data can guide the further development of T cell assays to unfold their full predictive and diagnostic potential, including cross-reactivity assessments.

Keywords: allergic contact dermatitis; chemical sensitizers; in vitro test; T cell assays; lymphocyte transformation test; antigen specificity

Citation: Aparicio-Soto, M.; Curato, C.; Riedel, F.; Thierse, H.-J.; Luch, A.; Siewert, K. In Vitro Monitoring of Human T Cell Responses to Skin Sensitizing Chemicals—A Systematic Review. *Cells* **2022**, *11*, 83. https://doi.org/10.3390/cells11010083

Academic Editors: Paul V. Lehmann and Greg A. Kirchenbaum

Received: 31 October 2021
Accepted: 23 December 2021
Published: 28 December 2021

1. Introduction

Thousands of chemicals have a sensitizing capability [1,2]. In allergic individuals, skin exposure can trigger allergic contact dermatitis (ACD). Depending on the form of the chemical contact, respiratory, systemic and local symptoms at other body sites may occur [3]. In Europe, approximately 20–27% of the general population is allergic to at least one chemical allergen [4,5]. Nickel remains the most common sensitizer with an

approximate prevalence of 11.4%, while reactions to fragrance mix I (3.5%), cobalt (2.7%), balsam of Peru (1.8%) and *p*-phenylenediamine (PPD, 1.5%) are also frequent [4]. Apart from metals, other important sensitizers comprise preservatives, drugs, excipients and many other substances of synthetic or natural origin [6–8].

Given the lack of causal therapies, reduced quality of life and even forced occupation changes, ACD constitutes a huge burden for personal and public health [9–12]. To tackle these challenges, accurate predictive and diagnostic tests are essential. Nowadays, the available predictive in vivo tests are limited by species differences and ethical considerations. In addition, the huge number of new compounds including nanomaterials that are constantly being developed by the chemical industry renders comprehensive in vivo testing impossible. Similarly, diagnostic epicutaneous patch testing has some disadvantages [13,14]. Patch testing may sensitize, although the risk is small for current standard substances [15] and boost existing allergies, at least locally [16]. Results can be unclear concerning distant skin eczema and patch testing may not be possible in patients with angry back syndrome or some other ongoing skin conditions [17]. For some allergens, suitable test substances are missing, or preparations do not penetrate the skin leading to false negative reactions, as demonstrated for $PdCl_2$ or tattoo inks [18,19].

To overcome the shortcomings of in vivo tests, alternative in vitro tests have been developed and validated by the Organization of Economic Cooperation and Development (OECD). Established in vitro tests cover all pathogenic events of the adverse outcome pathway of skin sensitization, except for the final key event, which is T cell activation [20]. During the sensitization phase, chemical allergens bind proteins (key event 1), resulting in the activation of keratinocytes (key event 2) and dendritic cells (DCs, key event 3). DCs migrate to the draining lymph nodes and present chemical-induced epitopes to activate rare antigen-specific T cells (key event 4) among millions of irrelevant bystander T cells ($\geq 10^8$ different T cell receptor (TCR) clonotypes per individual [21]). DC responses to chemical sensitizers critically determine T cell activation strength and subsequent effector and memory T cell responses, including tissue homing and subset formation with defined cytokine secretion capacities [22,23]. The function of DCs and other cells that may serve as antigen-presenting cells (APC) in the elicitation phase of ACD have been reviewed elsewhere and are also a matter of ongoing research [24–30]. Activated T cells proliferate, differentiate and distribute in the body, preferentially accumulating at tissue sites of previous inflammation as tissue-resident memory T cells (T_{RM}) [16,31–34]. Subsequent encounters with the same allergen lead to the activation of powerful local antigen-specific T_{RM} cells and accompanying innate immune responses. After ~24 h, further (antigen-specific) memory T cells infiltrate from blood [27,35]. This relatively slow process of immune cell egress into the tissue is linked to the slow evolvement of clinical symptoms, thus the term delayed hypersensitivity. In addition, quick antibody-based effects or functions may play a minor role, depending on the experimental system [36].

Taken together, chemical-specific T cells are key players of allergic reactions, but in vitro detection has remained challenging [37]. Here, we review recent original research papers that succeeded in the detection of T cell activation to skin sensitizing chemicals. Since the main limiting step is unsecure epitope formation, we overview current knowledge in the following section.

1.1. Chemical-Induced T Cell Epitopes

Much progress has been made in the understanding of metal-induced T cell epitopes [38–41], which has been reviewed elsewhere [42,43]. Mechanisms of non-metallic chemical-induced T cell epitopes, including those of drug hypersensitivity reactions (DHRs), are illustrated in Figure 1.

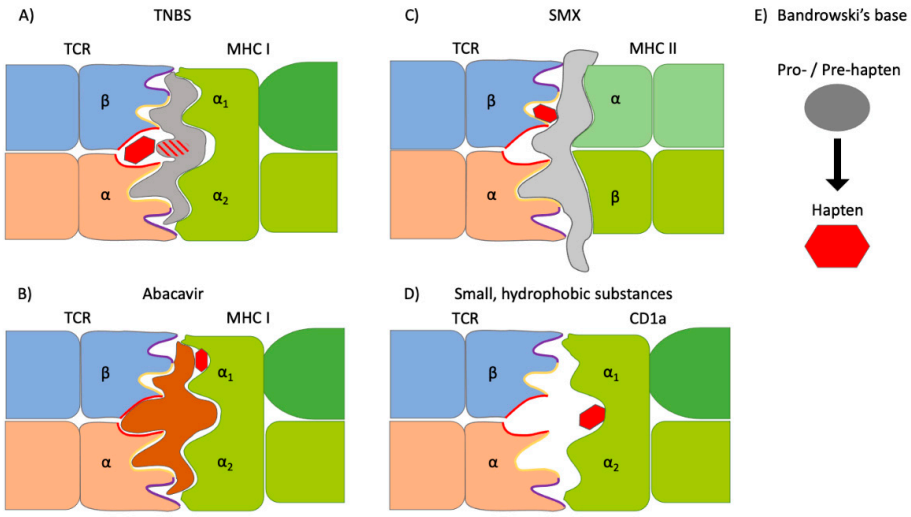

Figure 1. Mechanisms of T cell receptor (TCR) activation by non-metallic chemical allergens. (**A**) Chemical haptens (red trapeze) may bind covalently to major histocompatibility complex (MHC)-presented peptides (hapten concept). This has been shown for MHC I-restricted CD8+ T cells specific for the model chemical 2,4,6-trinitrobenzenesulphonic acid (TNBS) or the β-lactam antibiotic flucloxacillin. Murine responses seem to focus on a lysine modification at peptide position 4 (red-grey striped) [44,45]. (**B**) Some drugs associated with hypersensitivity reactions bind non-covalently, which is called pharmacological interaction (p-i) [46,47]. Binding via p-i has often been described in association with certain MHC alleles, termed human leukocyte antigens (HLAs) in humans (green). Abacavir, for example, binds to the F-pocket of HLA-B*57:01 resulting in the presentation of altered peptides (brown) [48,49]. (**C**) Some chemicals and metal ions form complexes at the TCR-pMHC interface. For sulfamethoxazole (SMX), binding to the complementarity-determining region 2 (CDR2) of TRVB-20-expressing TCR (blue) has been modeled [50]. (**D**) Haptens may displace endogenous lipid ligands on the MHC-like molecule cluster of differentiation (CD) 1a resulting in polyclonal αβ TCR activation to the CD1a surface [51]. (**E**) Pro- or pre-haptens require auto-oxidation or processing by metabolizing enzymes to become protein-binding.

TCRs recognize cognate peptides (p) presented by proteins of the major histocompatibility complex (MHC), also called human leukocyte antigens (HLAs) in humans [52]. Self TCR-pMHC complexes are usually ignored by the immune system due to negative selection in the thymus. In the case of chemical allergens, modified self-structures exceed the threshold for functional T cell binding and induce unintended adaptive immune responses. These mechanisms are grounded in the extensive poly-specificity (also called cross-reactivity) of TCR [43,53,54].

Chemical sensitizers may bind covalently to proteins, a process termed haptenization. Recognition of a covalently bound chemical on MHC-presented peptides by T cells was first shown using the model chemical 2,4,6-trinitrobenzenesulphonic acid (TNBS, Figure 1A) [55]. TNBS generates antigenic trinitrophenyl (TNP) determinants. TNP-modified peptides may replace unmodified peptides on MHC proteins on the surface of APC [55]. Another option is a short-term TNBS modification of APC, which leads to the binding of chemicals to surface pMHC [56–58].

However, most often, haptens are thought to modify extracellular proteins, which afterwards are incorporated and processed by APC leading to the presentation of haptenated peptides on MHC proteins. If the hapten enters the cell, intracellular proteins may get modified. In addition, haptens may influence antigen processing, leading to the presentation of cryptic epitopes by MHC proteins that do not contain the chemical [59].

In mice, TNBS-specific H-2K^b-(MHC I)-restricted CD8+ T cells have unusually high frequencies [60–62]. The underlying mechanism seems to be a carrier peptide-independent

recognition of TNP-modified free ε-amino groups of lysine residues at peptide position (p) 4 by many different TCR [44]. In addition, lysine at p7 may get TNP-modified, but T cells recognize this structure only in the context of a unique peptide and less frequently. Thus, the role of the MHC-presented peptide can vary in chemical-specific T cell recognition and this supposedly has to be individually assessed for each epitope. So far, a common gene segment use among TNBS-specific T cells has been suggested but not confirmed [62,63].

Among relevant human sensitizers, β-lactam antibiotics have been shown to act via covalent binding. The classic example for covalent binding drugs is penicillin G [64]. Another interesting example is flucloxacillin, for which hypersensitivity is strongly associated with HLA-B*57:01. Patient-derived T cells mainly recognize a covalently modified peptide [65,66]. In mice, hypersensitivity could be induced with a peptide modified at a p4 lysine residue [45].

However, flucloxacillin may also bind non-covalently, which is the major recognition mechanism for in vitro T cell activation in non-allergic HLA-B*57:01-expressing individuals [67]. The direct and reversible interaction of drugs with the HLA or the peptide in a non-covalent manner is termed pharmacological interaction (p-i) with immune receptors (Figure 1B) [46,47]. Flucloxacillin activity dependent on high drug concentrations was independent of proteasomal processing and immediate, indicating direct binding to the TCR-pMHC interface [67]. A third mechanism for flucloxacillin T cell epitope formation was recently shown, which involves the binding in the peptide-anchoring pockets of HLA-B*57:01 and the presentation of an altered peptide repertoire (Figure 1B) [45]. In summary, the flucloxacillin case demonstrates the importance of patient analysis to determine the in vivo relevance of different epitope formation mechanisms.

Binding via p-i has often been reported in the context of HLA allele-associated drug hypersensitivities [68–74]. Arguably, the most prominent example is abacavir binding to the F-pocket of HLA-B*57:01, which conceals a carboxy-terminal tryptophan important for peptide anchoring. The shape of the antigen-binding cleft changes upon abacavir binding, resulting in the presentation of an altered peptide repertoire [48,49]. This activates neo-antigen-specific CD8+ T cells in patients [75]. In all mentioned cases of HLA allele-associated binding, the TCR has no direct chemical contact.

Non-covalent interactions with direct chemical TCR contact may involve binding to the MHC outside of the peptide-anchoring pockets, to the presented peptide or to the TCR. TCR binding was modeled using molecular dynamic simulation for a TRBV-20-expressing sulfamethoxazole (SMX)-specific TCR (Figure 1C). Here, the TCR binds to SMX with high affinity through the conserved β-chain complementarity-determining region (CDR) 2 domain. SMX binds via TYRβ57, ASPβ64 and LYSβ65, which in the unbound TCR are responsible for hydrogen bonds to adjacent CDR loops. Therefore, the overall TCR conformation is changed, although a functional link to the allergic reaction remains missing [50].

Recently, a new mechanism of CD1a-restricted chemical-specific T cell activation has been described (Figure 1D) [51]. Several skin cells express CD1a proteins that accommodate endogenous lipid ligands which interfere with the activation of autoreactive CD1a-specific T cells [76,77]. Autoreactive T cells constitute ~1% of the skin T cell pool. Chemical sensitizers such as farnesol displace the endogenous ligands, then the TCR has direct contact with the unliganded surface which provokes autoreactive T cell responses. Alternatively, some chemicals may induce de novo lipid presentation on CD1a in certain APC, which may also activate T cells [78].

PPD, one of the most frequent skin sensitizers, binds non-covalently via a p-i mechanism, but as a pro-hapten, requires prior autoxidation (Figure 1E) [79,80]. Bandrowski's base (BB), a trimeric autoxidation product of PPD, is a pre-hapten requiring cellular metabolism to form T cell epitopes [80].

The variety in chemical reactivity mechanisms and the many different possible target proteins make it difficult to predict T cell epitopes [81,82]. In addition, rare epitopes can be important since T cells can be activated by single ligands [83,84]. While experimental

research on the haptenome of sensitizing chemicals is ongoing [85,86], new insights into possible T cell epitopes are obtained that need to be experimentally validated, e.g., as outlined in the studies reviewed here.

1.2. Review Objectives

In the present review, we systematically review the available literature on in vitro T cell activation achieved with non-metallic chemical allergens in the last 20 years. We focus on skin-sensitizing substances, since these represent one of the most relevant groups of sensitizers on a general population scale. The results and general principles for in vitro T cell activation can be transferred to any sensitizing chemical. Our results aim to provide directions for further attempts on the establishing of in vitro T cell assays for sensitizing chemicals, which are crucial for the further development of predictive and diagnostic tests.

2. Methods

2.1. Search Strategy

The present review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020 statement) [87]. Three screeners (MAS, CC and KS) designed a search strategy including articles indexed and published in the last 20 years (2001–2021) in PubMed and Scopus. We included the following criteria of interest as keywords (see also Supplementary Materials, Table S1).

2.1.1. PubMed

"t-lymphocytes"[MeSH Terms] AND (("2001/01/01 00:00":"3000/01/01 05:00"[Date—Publication] AND "journal article"[Publication Type]) NOT "review"[Publication Type]) AND (("dermatitis, allergic contact"[MeSH Terms] OR "chemical allergen"[Title/Abstract] OR "chemical allergens"[Title/Abstract] OR ("hypersensitivity"[Title/Abstract] AND "dermatitis"[Title/Abstract])) AND (("2001/01/01 00:00":"3000/01/01 05:00"[Date—Publication] AND "journal article"[Publication Type]) NOT "review"[Publication Type])) AND "English"[Language] AND ("human s"[All Fields] OR "humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]).

2.1.2. Scopus

((TITLE-ABS-KEY(T cell) OR TITLE-ABS-KEY(T cells) OR TITLE-ABS-KEY(T-cell) OR TITLE-ABS-KEY(T-cells) OR TITLE-ABS-KEY(T lymphocyte) OR TITLE-ABS-KEY(T lymphocytes) OR TITLE-ABS-KEY(T-lymphocyte) OR TITLE-ABS-KEY(T-lymphocytes)) AND (TITLE-ABS-KEY(allergic contact dermatitis) OR TITLE-ABS-KEY(contact allergy) OR TITLE-ABS-KEY(contact dermatitis) OR (TITLE-ABS-KEY(hypersensitivity) AND TITLE-ABS-KEY(dermatitis)) AND TITLE-ABS-KEY(human)) AND TITLE-ABS-KEY (in vitro)) AND (LIMIT-TO (PUBYEAR,2021) OR LIMIT-TO (PUBYEAR,2020) OR LIMIT-TO (PUBYEAR,2019) OR LIMIT-TO (PUBYEAR,2018) OR LIMIT-TO (PUBYEAR,2017) OR LIMIT-TO (PUBYEAR,2016) OR LIMIT-TO (PUBYEAR,2015) OR LIMIT-TO (PUBYEAR,2014) OR LIMIT-TO (PUBYEAR,2013) OR LIMIT-TO (PUBYEAR,2012) OR LIMIT-TO (PUBYEAR,2011) OR LIMIT-TO (PUBYEAR,2010) OR LIMIT-TO (PUBYEAR,2009) OR LIMIT-TO (PUBYEAR,2008) OR LIMIT-TO (PUBYEAR,2007) OR LIMIT-TO (PUBYEAR,2006) OR LIMIT-TO (PUBYEAR,2005) OR LIMIT-TO (PUBYEAR,2004) OR LIMIT-TO (PUBYEAR,2003) OR LIMIT-TO (PUBYEAR,2002)) AND (LIMIT-TO (LANGUAGE,"English")) AND (LIMIT-TO (DOCTYPE,"ar")).

2.2. Inclusion and Exclusion Criteria

We included only original articles written in English language available in a full-text form from 2001 to 2021 (date of the search: 27 September 2021). The following inclusion criteria were used: (i) in vitro studies using chemicals involved in ACD, (ii) studies investigating in vitro human T cell activation to non-metallic chemical allergens.

We did not consider: (i) reviews, (ii) book chapters, (iii) protocols, (iv) editorials/comments/opinions, (v) publications in languages other than English, (vi) duplicates (articles found in more than one database), (vii) conferences papers, (viii) letters/communications, (ix) articles that did not analyze in vitro human T cell activation upon contact with non-metal chemical allergens and (x) immune-histochemical studies of skin biopsies without further analysis of in vitro T cell activation.

2.3. Data Extraction and Collection

MAS and CC independently revised the articles identified by the search and evaluated whether they met the eligibility criteria to be included in this review. Potential disagreements were resolved through critical discussion with KS. All potentially relevant publications were retrieved in full. In addition, other relevant or up-to-date publications in the field have been included in the introduction and discussion sections.

2.4. Scoring System for Antigen-Specific T Cell Activation

We employed a scoring system to account for the varying degree of experimental evidence obtained for T cell activation to individual chemical allergens. MAS, CC and KS independently assigned a score (+++, ++, +) and the final score was decided on by common agreement. The highest score (+++) was given to chemicals for which multiple independent studies showed antigen-specific T cell activation. A medium degree of experimental evidence was labeled ++ and comprised chemicals that were investigated in at least two independent studies or that were associated with additional confirmation, e.g., by re-stimulation of T cell clones. The remaining chemicals from studies reporting antigen-specific T cell activation were graded +.

3. Results

3.1. Selection of Articles Following PRISMA Guidelines

We conducted searches in the PubMed and Scopus databases, following the strategies described in the methods (Sections 2.1–2.3). All original research articles published between 2001 and 2021 describing the in vitro activation of human T cells by non-metallic chemical allergens in the context of ACD were identified (Figure 2). We identified 238 and 234 publications, respectively. After the screening of the selected articles in PubMed, 208 articles were not included due to a lack of eligibility (see Section 2.2), 11 articles were duplicated in the Scopus database and 19 full-text articles were included in the review. Among the 234 articles obtained in the Scopus database, 216 were excluded because of a lack of criteria (see Section 2.2), leaving 18 records for screening. In total, we reviewed 37 articles and referred to them here with first author and publication year in addition to the bibliography numbering system. Among these, 32 publications described antigen-specific T cell activation (17 from PubMed, 15 from Scopus) and the others non-TCR-mediated T cell activation (2 from PubMed, 3 from Scopus) [88–92].

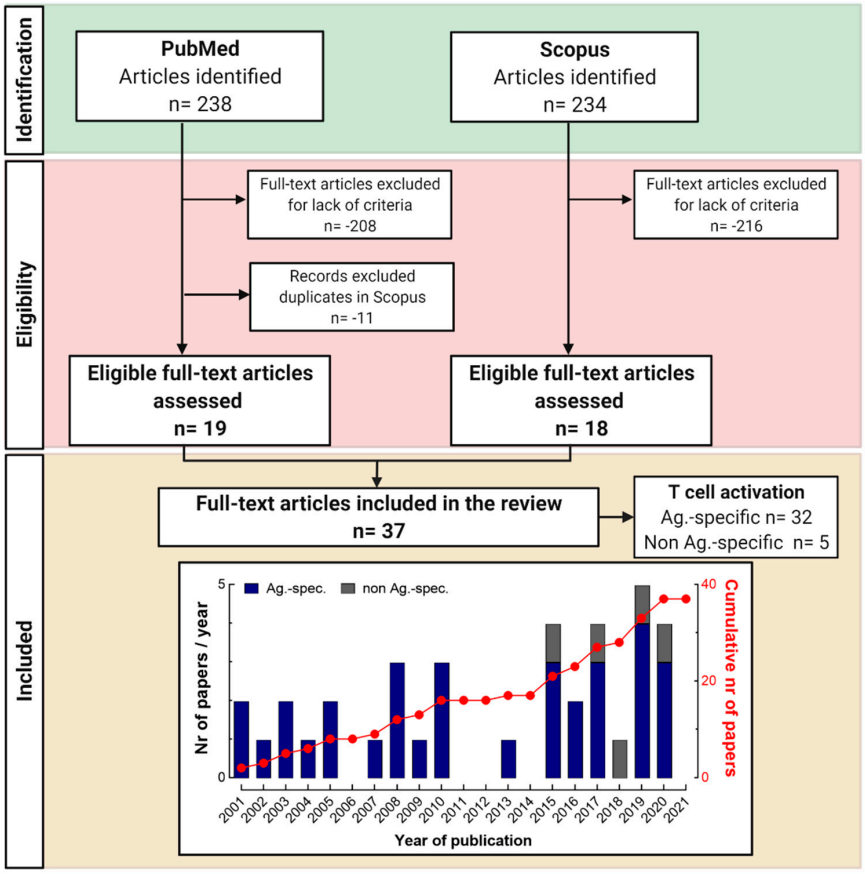

Figure 2. Flowchart of the search strategy applied in this systematic review according to the PRISMA statement 2020 guidelines [87]. The stacked bar histogram represents the time distribution of the articles included by year of publication (antigen (Ag.)-specific, blue, vs. non Ag.-specific, grey) and the red line the cumulative number of papers over the last 20 years (2001–2021).

3.2. Monitoring Chemical-Specific T Cell Responses In Vitro

3.2.1. Investigated Chemical Allergens

From the 32 papers on antigen-specific T cell activation, we identified T cell responses to 31 chemical skin sensitizers (Table 1). Among them, 28 chemicals were of human relevance, including fragrances (12), drugs (8), hair dyes and dye derivatives (2) and 6 other compounds, e.g., plant derivatives, preservatives and pollutants. Additionally, studies described T cell activation to model chemicals ((2,4-Dinitrobenzenesulfoniacid (DNBS), 2,4-Dinitrochlorobenzene (DNCB) and 1-Fluoro-2,4-dinitrobenzene (DNFB)).

To reflect the different experimental evidence obtained for the various chemicals on antigen-specific T cell activation, we applied a score (see Section 2.4). Besides the number of studies that independently assessed T cell activation, we also considered additional experiments, e.g., re-stimulation of T cell clones.

Table 1. Chemical allergens showing in vitro antigen-specific T cell activation in the different reviewed studies.

N°	*Chemical*	*Main Use*	*Score **	*References*
1	Bandrowski's Base (BB)	**	+++	Coulter, 2010 [93]; Gibson, 2015 [94]; Moed, 2005 [95]; Sieben, 2002 [80]
2	*p*-Phenylenediamine (PPD)	hair dye and dye	+++	Bordignon, 2015 [96]; Coulter, 2007 [97]; Coulter, 2010 [93]; Gibson, 2015 [94]; Jenkinson, 2009 [98]; Jenkinson, 2010 [79]; Kneilling, 2009 [99]; Moed, 2005 [95]; Oakes, 2017 [100]; Sieben 2002 [80]; Skazik, 2008 [101]; Wicks, 2019 [102]
3	2,4-Dinitrochlorobenzene (DNCB)	model chemical	++	Betts, 2017 [78]; Newell, 2013 [103]
4	Balsam of Peru	fragrance	++	Nicolai, 2020 [51]
5	Benzyl benzoate	fragrance	++	Nicolai, 2020 [51]
6	Benzyl cinnamate	fragrance	++	Nicolai, 2020 [51]; Schutte, 2019 [104]
7	Coenzyme Q2	fragrance	++	Nicolai, 2020 [51]
8	Eugenol	fragrance	++	Sieben, 2001 [105]
9	Farnesol	fragrance	++	Nicolai, 2020 [51]
10	Fragrance mix	fragrance	++	Cortial, 2015 [106]; Moed, 2005 [95]
11	Methylchloroisothiazolinone (MCI)	preservative	++	Moed, 2005 [95]
	Methylchloroisothiazolinone/ Methylisothiazolinone (MCI/MI)	preservative	++	Masjedi, 2003 [107]
12	Sulfamethoxazole/Trimethoprim (SMX/TMP)	drugs	++	Kim, 2020 [108]
13	1-Fluoro-2,4-dinitrobenzene (DNFB)	model chemical	+	Banerjee, 2003 [109]
14	2,4-Dinitrobenzenesulfoniacid (DNBS)	model chemical	+	Gildea, 2004 [110]
15	Azidamphenicol	drug	+	Sachs, 2001 [111]
16	Benzyl salicylate	fragrance	+	Schutte, 2019 [104]
17	Chloramphenicol	drug	+	Sachs, 2001 [111]
18	Clindamycin	drug	+	Vilchez-Sánchez, 2020 [112]
19	Diltiazem	drug	+	Girardi, 2005 [113]
20	Diphenylcyclopropenone (DPCP)	drug	+	Friedmann, 2017 [114]
21	Geraniol	fragrance	+	Sieben, 2001 [105]
22	Hydroxycitronellal	fragrance	+	Sieben, 2001 [105]
24	Isoeugenol	fragrance	+	Banerjee, 2003 [109]; Sieben, 2001 [105]
23	*Machaerium scleroxylon*	plant	+	Hansel, 2019 [115]

Table 1. *Cont.*

N°	Chemical	Main Use	Score *	References
24	Methylisothiazolinone (MI)	preservative	+	Popple, 2016 [116]
25	Metronidazole	drug	+	Girardi, 2005 [113]
26	Oak moss	fragrance	+	Sieben, 2001 [105]
27	Parthenolide	***	+	Wahlkvist, 2008 [117]
28	Squaric acid dibutylester (SADBE)	drug $	+	Camouse, 2008 [118]
29	Trichloroethylene (TCE)	pollutant	+	Li, 2019 [119]
31	Urushiol	***	+	Kim, 2016 [120]

* Experimental evidence for T cell activation for individual chemicals was graded according to Section 2.4 from best (+++) to little (+). ** PPD-derivative, *** plant component, $ photographic revealer.

The most researched allergen was PPD, which was investigated in 12 independent studies (38%). BB, a trimeric product of PPD, was investigated together with PPD in four studies (13%). Both chemicals were assigned a +++ score regarding their ability to detect TCR-mediated T cell activation. A few sensitizers were investigated in at least two independent studies or T cell activation was additionally confirmed, e.g., by re-stimulation of T cell clones. These chemicals were assigned a ++ score (e.g., benzyl cinnamate, eugenol, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI)). For the remaining chemicals, results were retrieved from only one study or one experimental evidence and a + score was assigned. The following paragraphs will provide more details on the experimental details in the individual studies (summarized in Supplementary Material, Table S2).

3.2.2. Approaches for Chemical-Induced T Cell Epitope Formation

Different APC and epitope generation strategies were used by the different studies to observe antigen-specific T cell activation in vitro. Table 2 summarizes the choice of APC and the method of chemical administration.

The majority of the experimental attempts used PBMC-derived cells (24/32 studies, 75%), which contain all cell types, i.e., APC such as monocytes and B cells and all circulating T cell subsets. Alternatively, monocyte-derived dendritic cells (MoDCs, 6/32 studies, 19%) or Epstein–Barr Virus (EBV)-transformed B cells were used (3/32 studies, 9.4%). The possibility to observe antigen-specific T cell activation is critically dependent on the use of autologous APC except for antigens presented by the conserved MHC I-related molecule CD1a, which may be investigated using monocyte-like cell lines, e.g., K562 as APC (3/32 studies, 9.4%).

Most studies relied on a direct administration of the chemical of interest to the cell culture media to generate allergen-induced T cell epitopes in vitro (23/32 studies, 72%). In nine studies (29%), APC were pulsed with the chemical allergen from 10 min up to 24 h, then washed and co-cultured with T cells. We encountered four publications (13%) where the chemicals (i.e., MI and PPD) were presented as a protein conjugate, i.e., coupled to human serum albumin (HSA).

A practice to determine a non-toxic chemical concentration (e.g., by testing cell viability) before measuring T cell activity upon chemical exposure was used by five studies (16%). All chemical concentrations are listed in Table S2 (Supplementary Material). Protein-conjugated chemicals (i.e., PPD- and MI-HSA) induced a comparable or even better proliferative response compared to the soluble correspondent chemical (Supplementary Material, Table S2) (Jenkinson, 2010; Oakes, 2017; Popple, 2016; Wicks, 2019) [79,100,102,116]. Jenkinson and colleagues (2010) [79] calculated the equivalent molar scale of soluble and HSA-associated PPD to compare the strength of induced activation/proliferation. They revealed that HSA-bound PPD possesses a stronger antigenic capacity. In the case of the protein-bound forms of the chemicals, the induced proliferative responses follow the classi-

cal dose-dependent trend and better correlate to patients' patch test results (Popple, 2016; Wicks, 2019) [102,116]. Soluble chemicals generally become toxic at higher concentrations. Notably, a baseline proliferation response to HSA may be taken into account. Soluble MI and HSA alone induce T cell proliferation in 7 and 9 patients out of 31, respectively, while 17/31 patients responded to MI-HSA (Popple, 2016) [116].

Table 2. APC choices and approaches for the in vitro generation of T cell epitopes used by the reviewed studies.

APC	*Epitope Formation*	*Chemicals*	*References*
PBMC	Direct administration in culture	Azidamphenicol, BB, Benzyl cinnamate, Benzyl salicylate, Chloramphenicol, Clindamycin, Diltiazem, DNCB, DNFB, Eugenol, Fragrance mix, Geraniol, Hydroxycitronellal, Isoeugenol, Metronidazole, *Machaerium scleroxylon*, MCI/MI, MI, Oak mos, Parthenolide, PPD, SMX/TMP, TCE	Banerjee, 2003 [109]; Bordignon, 2015 [96]; Cortial, 2015 [106]; Coulter, 2010 [93]; Friedmann, 2017 [114]; Girardi, 2005 [113]; Hansel, 2019 [115]; Jenkinson, 2009 [98]; Kim, 2020 [108]; Knelling, 2010 [99]; Li, 2019 [119]; Masjedi, 2003 [107]; Moed, 2005 [95]; Newell, 2013 [103]; Popple, 2016 [116]; Sachs, 2001 [111]; Schutte, 2019 [104]; Sieben, 2001 [105]; Sieben, 2002 [80]; Skazik, 2008 [101]; Vilchez-Sánchez, 2020 [112]; Wahlkvist, 2008 [117]; Wicks, 2019 [102]
	Modification (e.g., pulsed APC)	BB, PPD	Sieben, 2002 [80]; Wicks, 2019 [102]
	Protein conjugation (e.g., to HSA)	MI, PPD	Oakes, 2017 [100]; Popple, 2016 [116]; Wicks, 2019 [102]
Dendritic cells	Direct administration in culture	BB, PPD	Coulter, 2010 [93]; Gibson, 2015 [94]
	Modification (e.g., pulsed APC)	BB, DNBS, Fragrance mix, MCI, PPD, SADBE	Camouse, 2008 [118]; Coulter, 2007 [97]; Gildea, 2004 [110]; Moed, 2005 [95]
EBV-transformed B cells	Direct administration in culture	Eugenol, Geraniol, Hydroxycitronellal, Isoeugenol, Oak moss, PPD	Jenkinson, 2010 [79]; Gibson, 2015 [94]; Sieben, 2001 [105]
	Protein conjugation (e.g., to HSA)	PPD	Jenkinson, 2010 [79]
Cell lines (CD1a-expressing)	Direct administration in culture	Balsam of Peru, Benzyl benzoate, Benzyl cinnamate, Coenzyme Q2, Farnesol	Nicolai, 2020 [51]
	Modification (e.g., pulsed APC)	DNCB, Urushiol	Betts, 2017 [78]; Kim, 2016 [120]; Nicolai, 2020 [51]

EBV, Epstein Herpes Virus; HSA, Human Serum Albumin; further abbreviations are listed in Table 1.

3.2.3. Blood as Major T Cell Source

Almost all screened publications (29/32 studies, 91%) relied on PBMC as the source for T cells. Three publications (9.4%) presented results obtained in T cell-like cell lines that sometimes expressed a single TCR. One study studied T cell clones derived from patch test skin lesions in parallel to PBMC (Newell, 2013) [105].

Eight studies (25%) investigated the contribution of the two main CD4+ and CD8+ T lymphocyte subsets (except for one study focusing on CD4+ memory T cells (Kim, 2016) [120]). Three publications explored the involvement of naïve and/or memory T cell subsets (Gibson, 2015; Kim, 2016; Li, 2019) [94,119,120]. Two publications studied cell frequencies of CD4+ and CD8+ naïve and memory T cells (Oakes, 2017; Wicks, 2019) [100,102].

3.2.4. Detection of Chemical-Specific T Cell Activation (Read-Outs)

The read-outs used in the reviewed articles to observe antigen-specific T cell activation in vitro are listed in Table 3. Cellular proliferation was the most frequent read-out (27/32 studies, 91%), measured by thymidine incorporation (21/32 studies, 66%), carboxy fluorescein diacetate succinimidyl ester (CFSE) dilution (2/32 studies, 6.2%) or other methods (4/32 studies, 13%). One study directly assessed the frequencies of antigen-specific T cells by ex vivo enzyme-linked immune-spot (ELISpot) assay (Newell, 2013) [103].

Table 3. T cell activation read-outs.

Read-outs	*Method/Assay*	*Chemicals*	*References*
Proliferation	Thymidine	Azidamphenicol, ** BB, Chloramphenicol, Clindamycin, Diltiazem, DNBS, DPCP, Eugenol, ** Fragrance mix, ** Geraniol, ** Hydroxycitronellal, ** Isoeugenol, ** MCI, ** MCI/MI, ** MI, Metronidazole, ** Oak moss, ** PPD, SADBE	Camouse, 2008 [118]; ** Cortial, 2015 [106]; Coulter, 2007 [97]; ** Coulter, 2010 [93]; Friedmann, 2017 [114]; ** Gibson, 2015 [94]; Gildea, 2004 [110]; Girardi, 2005 [113]; Jenkinson, 2009 [98]; ** Jenkinson, 2010 [79]; Kneilling, 2010 [99]; ** Masjedi, 2003 [107]; ** Moed, 2005 [95]; Oakes, 2017 [100]; Popple, 2016 [116]; Sachs, 2001 [111]; ** Sieben, 2001 [105]; ** Sieben, 2002 [80]; Skazik, 2008 [101]; Vilchez-Sánchez, 2020 [112]; ** Wicks, 2016 [102]
	CFSE	*Machaerium scleroxylon*, SMX/TMP	Kim, 2020 [108]; Hansel, 2019 [115]
	Other	Benzyl cinnamate, ** Benzyl salicylate, ** DNFB, DPCP, ** Isoeugenol, ** TCE	** Banerjee, 2003 [109]; Friedmann, 2017 [114]; ** Li, 2019 [119]; ** Schutte, 2019 [104]
Cytokine production	ELISA	Balsam of Peru, ** BB, Benzyl benzoate, Benzyl cinnamate, Coenzyme Q2, DNCB, ** DNFB, Eugenol, Farnesol, ** Fragrance mix, ** Geraniol, ** Hydroxycitronellal, ** Isoeugenol, ** MCI, ** MI, ** Oak moss, ** PPD, ** TCE	Banerjee, 2003 [109]; Betts, 2017 [78]; ** Cortial, 2015 [106]; ** Coulter, 2010 [93]; ** Jenkinson, 2010 [79]; ** Li, 2019 [119]; ** Masjedi, 2003 [107]; ** Moed, 2005 [95]; Nicolai, 2020 [51]; ** Sieben, 2001 [105]; ** Sieben, 2002 [80]
	ELISpot	** Benzyl salicylate DNCB, PPD, Parthenolide	Bordignon, 2015 [96]; Gibson, 2015 [94]; Newell, 2013 [103]; ** Schutte, 2019 [104]; Wahlkvist, 2008 [117]
	Other	DNCB, Urushiol	Betts, 2017 [78]; Newell, 2013 [103]; Kim 2016 [120]
Gene expression	RT-PCR	BB, PPD, Urushiol	Coulter, 2010 [93]; Kim, 2016 [120]
	Microarray/RNA seq	DNBS, SMX/TMP	Gildea, 2004 [110]; Kim, 2020 [108]
T cell phenotype (e.g., activation markers, cytotoxicity)		BB, DNCB, Eugenol, Geraniol, Hydroxycitronellal, Isoeugenol, *Machaerium scleroxylon*, Oak moss, PPD, SMX/TMP, TCE	Hansel, 2019 [115]; Kim, 2020 [108]; Li, 2019 [119]; Sieben, 2001 [105]; Sieben, 2002 [80]; Wicks, 2019 [102]
T cell clone Proliferation	w/o HLA blocking	PPD	Gibson, 2015 [94]; Jenkinson, 2010 [79]; Skazik, 2008 [101]
	with HLA blocking	BB, PPD	Sieben, 2002 [80]
T cell receptor repertoire	NGS	PPD	Oakes, 2017 [100]
	other	PPD	Skazik, 2008 [101]

** Chemical ability to induce both proliferation and cytokine secretion was measured. CFSE, carboxy fluorescein diacetate succinimidyl ester; HLA, human leukocyte antigen; ELISA, enzyme-linked immuno-sorbent assay; ELISpot, enzyme-linked immuno-spot; NGS, next generation sequencing; RT-PCR, real-time quantitative polymerase chain reaction; further abbreviations are listed in Table 1.

Seventeen studies (53%) measured secretion of inflammatory and/or T_H-subset-specific cytokines (e.g., IL-4, IL-5, IL-17A, IFN-γ) by enzyme-linked immuno-sorbent assay (ELISA) (11/32 studies, 34%), ELISpot (5/32 studies, 16%) or other methods (e.g., intracellular staining, 3/32 studies, 9.4%) following a few days of cellular expansion. In 9 out of these 17 studies, proliferation was measured in parallel (marked with ** in Table 3). We observed a trend for a preferential differentiation towards the T_H2 lineage in the cytokine production (5/17 studies, 29%) for PPD (3/17 studies, 18%) (Coulter, 2010; Jenkinson, 2010; Sieben, 2002) [79,80,93] and MCI/MI (1/17 studies, 6%) (Masjedi, 2003) [107]. Two studies defined a T_H1 cytokine profile of chemical-specific T cells, i.e., for DNCB (note: T_H2 shift in atopic patients) (Newell, 2013) [103] and fragrances (Sieben, 2001) [105]. In three studies (18%), chemical-stimulated cells secreted a mix of T_H1 (e.g., IFN-γ) and T_H2 (e.g., IL-4, IL-5 and/or IL-13) cytokines. Chemicals utilized in these three studies partially overlapped with the ones mentioned above as inducing a T_H2 profile, i.e., PPD, BB, MCI, fragrance mix and parthenolide (Gibson, 2015; Moed, 2005; Wahlkvist, 2008) [94,95,117]. The remaining eight studies (47%) did not measure a conclusive, in this regard, panel of cytokines (e.g., IL-1α/IL-1β or IFN-γ/TNF-α/IL-2 or IFN-γ alone).

Gene expression by real-time quantitative polymerase chain reaction (RT-PCR), microarray or RNA sequencing (4/32 studies, 13%) and cellular phenotype/activation changes (e.g., CD69 expression by flow cytometry, 5/32 studies, 16%) were frequent additional read-outs, especially among more recent publications (Table 3, Supplementary Material Table S2).

None of the studies made conclusive observations on major differences in the activation or role of CD4+ and CD8+ T cell subsets in chemical-associated allergies. Sieben and colleagues (2001) [105] observed that 83% of established eugenol-specific T cell clones were CD4+HLA-DR+, and the remaining 17% were CD8+. Wicks, 2019 [102] and Oakes, 2017 [100] both observed a shift from the central memory (CM) to the effector memory (EM) compartment in PPD and PPD-HSA stimulated CD4+ and CD8+ T cells of allergic patients. Additionally, in the former study, an expansion of naïve T cells was detected in the blood compartment. A simultaneous contraction of the memory T cell population (probably due to recruitment to the site of patch test application) was also observed [102].

Four studies (13%) nailed antigen-specific T cell involvement by generating T cell clones confirming their proliferative ability upon re-stimulation with the original antigens, PPD and BB (Gibson, 2015; Jenkinson, 2010; Sieben, 2002; Skazik, 2008) [79,80,94,101]. Two studies performed HLA-blocking during T cell clone re-stimulation to confirm MHC-restricted T cell activation (Kim, 2020; Sieben, 2002) [80,108].

TCR features were addressed in two PPD-related studies (Oakes, 2017; Skazik, 2008) [100,101]. Oakes, 2017 [100] performed an unbiased high-throughput sequencing of the TCR α- and β-chains of PBMC derived from one PPD-allergic patient in ex vivo conditions after 6 days of culture with PPD-HSA. Approximately 800 TCR α- and β- chain sequences (0.8% of all detected TCR) were considered PPD-specific due to their increased frequencies compared to controls. A skewed V- and J-gene segment usage was observed while a mechanistic association with PPD recognition remains to be defined. The study by Skazik, 2008 [101] showed by flow cytometry that 8 out of 21 PPD-specific T cell clones expressed TRBV14 (Vβ16 in Arden nomenclature), a segment not highlighted in the study of Oakes, 2017 [100].

3.2.5. Features of Chemical-Specific T Cell Responses in Patients

Studies varied in terms of patients' cohort composition and experimental setups. Four case reports (13%) included only one to two patients with drug allergies (Girardi, 2015; Kim, 2020; Sachs, 2001; Vilchez-Sánchez, 2020) [108,111–113]. The remaining articles included cohorts with approximately 10 and up to 200 patch tested allergic patients. The proliferative response of allergic patients' T cells to chemicals showed great variability. Generally, cells derived from patients with a very strong (+++) result in patch tests reacted more often and possessed a higher proliferative response than cells from patients with strong (++) or weak (+) patch test results. A general observation on the existence of a concordance between the patient patch test result and the patient T cell proliferative or cytokine response in vitro has been made by 4 out of 32 studies (13%) in the case of PPD (Bordignon, 2015; Wicks, 2019) [96,102], MCI/MI (Masjedi, 2003) [107] and parthenolide (Wahlkvist, 2008) [117]. Of note, three studies did not confirm this concordance for PPD (Moed, 2005) [95], MI (Popple, 2016) [116] and various fragrances (Sieben, 2001) [105]. Three studies (9.4%) tested the detection of chemical-specific T cells after administration of a cytokine cocktail (e.g., IL-7 + IL-12 or IL-4) to the culture media (Kneilling, 2009; Moed, 2005; Schutte, 2019) [95,99,104]. The addition of cytokines may support the proliferative capacity of chemical-specific T cells.

PPD and its derivative BB were investigated for potential T cell cross-reactivity (2/32 studies, 6.3%). For this purpose, Gibson, 2015 [94] and Sieben, 2002 [80] tested PPD- and BB-specific T cell clones from allergic and healthy donors. Gibson et al. found that 75% of PPD-specific T cell clones reacted exclusively to the original antigen, while Sieben et al. found that most of the 25 PPD-specific T cell clones were BB cross-reactive. Of note, BB-specific T cell responses are observed in all individuals, but PPD-specific T cells have been described only in allergic patients (Coulter, 2010; Gibson, 2015; Sieben, 2002) [80,93,94].

3.3. Monitoring Non-Antigen-Specific T Cell Activation

Five studies assessed T cell responses to chemical sensitizers (42 substances) in a non-antigen-specific manner (Supplementary Material, Table S3). Most chemicals were fragrance agents (13), drugs (11), dyes (5) and model chemicals (3), apart from preservatives, disinfectants and some industrial agents.

Frombach, 2018 [88] assessed immunotoxic influences of chemicals on cytokine secretion as well as IL-23R/CD119, CD124 and CD44 surface expression on expanded T cells derived from mixed lymphocyte reactions containing MoDC, T cells and allogenic keratinocytes. Similarly, Clouet, 2019 [92] monitored T cell proliferation in a mixed-lymphocyte reaction with THP-1 as a DC model. The increase in co-stimulatory capacity by sensitizer-treated DC reflects their potential to support antigen-specific T cell proliferation.

Hou, 2020 [89] used the Jurkat T cell line to measure increased CD69 expression upon exposure to 24 non-metallic sensitizing chemicals compared to control substances (Supplementary Material, Table S3). This approach is reminiscent of systems that assess activation of keratinocytes or DC by sensitizing chemicals [121,122]. While the authors hypothesize that Jurkat T cells may present chemical-induced epitopes, the recognition of this diverse chemical set by the only TCR that Jurkat cells express has not been backed-up by additional experiments.

Baló-Banga, 2015 [90] measured increased IL-6 levels in PBMC cultures from individuals with suspected immediate or delayed drug hypersensitivities 20 min after drug exposure [90]. The cellular IL-6 source, as well as the mechanism of its release, remain to be determined. Mai, 2017 [91] identified increased levels of T_H17- and T_H22-producing T cell subsets in polyclonal stimulated PBMC from formaldehyde-exposed workers with ACD history, indicating the outgrowth of the respective T cell subsets [91].

4. Discussion

In recent years, TCR-mediated in vitro T cell activation has been detected to a number of chemical allergens. Here, we present possible experimental solutions to the unique challenge of chemical-induced epitope formations. We link chemical identities and methodological details with the possibility to detect chemical-specific T cells.

4.1. APC Choice

A multitude of cells have been used as APC for in vitro T cell assays. The reviewed studies mainly employed PBMC and PBMC-derived DC such as MoDC or EBV-transformed B cells (Table 2). In the literature, the use of skin-derived APC such as Langerhans cells (LCs) or fibroblasts has also been described but this APC source is hardly available since it requires scarce autologous skin tissue [123].

EBV-transformed B cells are an intriguing source of APC since they can be propagated limitlessly, e.g., for clone re-stimulation. However, it takes a few weeks to generate EBV-transformed cells and requires a biosafety level 2 lab [94]. HLA-deficient cell-lines transfected with the HLA molecule of interest constitute a further APC option restricted to chemicals for which an HLA association has been identified. Once T cell clones have been established, they usually express MHC II and some can be stimulated without further APC, likely depending on the presented antigen peptide [39]. A few chemical-specific T cell clones tolerant to the HLA haplotype or acting MHC independently have been described [124]. This observation certainly does not warrant a general use of allogenic APC since mixed-lymphocyte reactions usually superimpose any antigen-specific signals. However, CD1a-reactive T cells can be studied using CD1a-transfected cell lines.

Some T cell populations require the presence of specialized APC. For instance, naïve T cells only proliferate upon contact with professional APC such as MoDC [56]. In addition, some chemical-specific T cell clones depend on tissue-restricted epitopes that are not presented by other APC, e.g., PBMC [78,123]. Thus, PBMC-based assays may not capture the complete chemical-reactive T cell pool but probably detect enough representative T cells to allow sound scientific conclusions. In case of pre-haptens, the choice of APC may

influence metabolisms and thus epitope formation. None of the reviewed studies compared T cell responses using different APC. Of note, a high-enough APC density is mandatory to ensure efficient in vitro T cell contact and successful T cell activation [39].

4.2. T Cell Epitope Formation

The most critical step of in vitro T cell assays that investigate chemical allergens is the adequate formation of chemical-induced T cell epitopes. Protein antigen-specific T cells have been detected with frequencies as low as 1 in 10^7 using enrichment methods and a sufficient number of input cells [125]. Thus, techniques are available to interrogate virus-specific cross-reactive T cell memory or the antigen-specific naïve T cell pool [126]. However, if chemical-induced epitopes are formed inefficiently and if this is combined with the rarity of antigen-specific T cells, the detection of T cell activation may become virtually impossible. In addition, epitopes may form in an HLA allele-restricted manner, which is less well investigated for sensitizing chemicals that are not used as drugs [68–72].

The knowledge on T cell epitope identity and the conditions needed for an efficient generation remains very limited and it has to be experimentally determined. Incubation time and chemical concentration are important determinants, as well as temperature and pH value, in order to mimic physiological conditions. In general, three major methods for epitope generation can be distinguished: (i) direct administration of chemicals into the APC–T cell co-culture, (ii) a separate chemical modification of APC and posterior addition to the T cell culture and (iii) allergen-modification of model proteins or peptides as an antigen source.

For haptens that form epitopes directly via covalent binding, APC modification with a high chemical concentration for a short time (e.g., 10–15 min at 37 °C) in PBS seems the most efficient epitope generation method as shown for the model allergens TNBS, DNBS or fluorescein isothiocyanate (FITC) [56,110,127]. This short-term modification method is not suitable for pre- or pro-haptens. Thus, a loss in epitope formation efficiency is expected if the active hapten is only formed during longer culture periods. Variations in experimental conditions, e.g., the addition of a cytochrome P450 cocktail or the antioxidant glutathione may help to evaluate whether a chemical acts as pre- or pro-hapten [80]. In addition, APC fixation or measurements on the timing of T cell responses (Ca^{2+} influx) can inform on the necessity for antigen processing and HLA block on the MHC restriction in experiments using bulk T cell cultures or T cell clones.

For chemicals that bind via a p-i mechanism, the binding affinity decides whether pre-incubated, washed APC, i.e., close to zero concentrations of the free chemical, can be used to detect T cell activation. Abacavir has a high affinity to HLA-B*57:01, so washed APC have been employed [48].

Most commonly, chemicals are directly added to the APC–T cell co-culture (Table 2). Here, toxic effects restrict the use of high chemical concentrations while frequencies of reactive T cells often correlate with the amount of the chemical present in the culture [39,67,128]. The use of rather high (albeit non-toxic) chemical concentrations likely enables the detection of the complete reactive T cell pool. However, in the case of flucloxacillin, in vitro T cell responses to high chemical concentrations observed in non-allergic individuals (processing-independent p-i mechanism) were not relevant in allergic patients (processing-dependent hapten mechanism) [65–67]. This illustrates the need to confirm the in vivo relevance of the obtained epitope–T cell interaction, which may be shaped by low chemical concentrations in vivo, e.g., in the draining lymph nodes.

Chemical-induced epitopes may also be provided by feeding hapten-modified (self-) proteins to APC. As model carrier proteins, most studies use HSA. Within PBMC, monocytes and B cells can capture the antigen proteins and present processed peptides via MHC II to CD4+ T cells. For CD8+ T cell activation, cross-presentation and thus the use of professional APC such as MoDC is necessary [79].

4.3. T Cell Source

Usually, blood-derived T cells are assessed for their chemical reactivity. Only a few studies use skin-derived T cells from ACD lesions or analyze blister fluid [105,108,123,129,130]. The isolation of T cells from the skin may not be efficient and can introduce bias if antigen-specific T cells are restrained by tight immunological synapses [131,132]. Nevertheless, the frequencies of chemical-specific T cells seem increased in situ at sites of the allergic reaction [123,129,130]. Apart from the whole T cell pool, T cell subpopulations may be interrogated, e.g., CD4+ or CD8+ T cells. Magnetic enrichment or untouched depletion techniques may yield purities of ~90% or better. The required number of input T cells determines the limit of detection. Highly frequent antigen-specific T cells, e.g., nickel-specific T cells (200 μM $NiSO_4$) can be detected in one well of a 96-well plate using only 0.8×10^6 PBMC [39]. However, the rarer the antigen-specific T cell population is, the more T cells need to be interrogated, requiring inputs of e.g., 50–100 $\times 10^6$ PBMC or more. The physiological limit is the number of PBMC that can be obtained from a blood donation. Amplified T cell libraries have not yet been used in the field [133].

Conditions for T cell activation may be optimized. Besides, depletion of regulatory T cells (e.g., CD25+ T cells), addition of cytokines such as IL-12 or IL-4 or autologous serum may support the proliferation of chemical-specific T cell subsets [95,127,134,135]. In addition, the presence of co-stimulatory antibodies (e.g., α-CD28, α-CD49a) or checkpoint inhibitor antibodies (e.g., α-programmed death ligand 1/2 (PD-L1/2), α-PD-1, α-cytotoxic T-lymphocyte-associated protein (CTLA)-4) may optimize conditions for T cell activation [136].

4.4. Read-Outs

Proliferation-based methods such as the lymphocyte transformation test (LTT) constitute the most used read-outs for the detection of chemical-specific T cells (Table 3). Staining with pMHC multimers is not an option since chemical-induced T cell epitopes remain unknown. Besides the incorporation of radioactive nucleotides or dye dilution, proliferated T cells may also be detected by determining cytokine levels or metabolite production. Direct quantification of chemical-specific memory T cells can be accomplished ex vivo with ELISpot analysis (DNCB) (Newell, 2013) [103] or with the help of limiting dilution cultures [137]. Using LTT, the reactive T cell pool is usually not comprehensively captured since naïve T cells, for instance, proliferate only in the presence of professional APC. Original frequencies of memory T cell subpopulations will likely be lost in LTT, given the different division speeds [138,139]. Besides, ELISA results do not inform about the number of antigen-specific T cells since individual cytokine amounts secreted per cell differ. For all cytokine-based methods, a parallel analysis of several cytokines will be useful to capture different cytokine-producing subpopulations. This is of particular importance, because polarization patterns differ or have remained unclear for chemical allergens [39,140].

Activation-induced surface marker assays constitute a rather new option for a fast, comprehensive and quantitative analysis of chemical antigen-specific T cells [125,141,142]. Recently, our group adopted this technique to detect nickel-specific CD154+CD4+ naïve and memory T cells [39].

A promising emerging read-out is the analysis of chemical-specific TCR repertoires which may inform on antigen recognition mechanisms [39,143]. Bulk high-throughput sequencing may reveal peculiar gene segment use and inform on clonal expansions while single T cell clone analysis provides information on TCR α- and β-chain pairing. Flow cytometry analysis of TCR V-regions is limited by antibody availability and only informs on TRBV gene segment use. Oakes, 2017 [100] found limited V-gene segment use among ~800 PPD-specific TCR α- and β-chains, e.g., a dominant TRAV29/DV5 use, from one patient, indicating outgrowth of antigen-specific T cell clonotypes. Skazik, 2008 [101] used a panel of 24 Vβ antibodies to identify TRBV14 (Vβ16 in Arden nomenclature) expression by 5/8 PPD-specific T cell clones. Further experiments are needed to investigate the characteristics of PPD-specific TCR. For HLA-B*15:02-associated carbamazepine hypersensitivity, Ko,

2011 [144] identified an overrepresentation of TRBV25-1 (Vβ11) and TRAV9-2 (Vα22) gene segments in antigen-specific T cell lines from eight patients. Interestingly, the TRAV9-2 segment has been mechanistically linked to nickel recognition [38,39], but a connection to carbamazepine recognition remains to be shown.

4.5. Immune Monitoring of Allergic and Non-Allergic Individuals

For diagnostic purposes, differences in the immune responses of allergic and non-allergic individuals have to be identified. Among all chemicals investigated in the studies systematically reviewed here, the ability to detect PPD-specific T cells seems the most promising diagnostic in vitro option [79,80,93,96,99]. Mostly, studies monitor frequency differences, e.g., increased LTT stimulation indexes for allergic individuals. In general, two challenges emerge. Firstly, T cell responses may be detected only for some allergic individuals, i.e., detection levels are not sufficient to identify all allergic individuals as observed for MCI, MI and fragrance mix [95,106,116]. Secondly, frequencies of blood-derived chemical-specific T cells may be similar in allergic and non-allergic individuals, which also impedes allergy detection. BB-specific T cells are frequent in all individuals [80,93], similar to TNBS- or nickel-specific T cells. This likely occurs due to a particular interaction with a larger fraction of the TCR repertoire [39,62,137]. In such cases, allergy-associated T cell subpopulations need to be defined, which has not been accomplished yet.

Another interesting option is a TCR-based diagnosis, which has been recently accomplished for cytomegalovirus or severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infections [145,146]. Pan, 2019 [147] observed one public carbamazepine-specific HLA-B15:02-restricted TCR (TRBV12-4/TRBJ2-2, TCRβ CDR3 "ASSLAGELF"), which had an increased frequency in seven allergic individuals compared to 44 healthy control individuals. A pairing TCR α-chain CDR3 "VFDNTDKLI" was expressed by 83% of carbamazepine-specific TCR. However, without a known HLA association, TCR sequencing data from several hundred to thousands of individuals with defined allergy status have to be available to evaluate a TCR-based diagnostic option, an endeavor for the future when more sequences become available.

4.6. Possible Uses of Assays Investigating Non-Antigen-Specific T Cell Activation

Among the systematically reviewed literature, only a few studies investigated the general effects of sensitizing chemicals on T cells (Section 3.3). One reason is to investigate the T cell activation in a non-antigen-specific manner, similar to the effect that chemicals have on DC maturation or keratinocytes activation [89]. Another purpose is to study immunotoxic chemical effects, e.g., a reduction in cytokine-producing activities. In addition, mixed lymphocyte reactions serve to indicate functional chemical-induced DC maturation [88,92]. With regard to patient analysis, global changes in T cell subsets or function may be associated with the allergic state [90,91].

4.7. Limitations of Our Study

Our selection of original research articles focuses on a relatively small proportion of sensitizing chemicals, i.e., skin sensitizers that have been investigated by T cell assays in vitro. We focus on more recent studies published within the last 20 years. A complete assessment of all chemical allergens, including systemically acting drugs, respiratory sensitizers and additional model chemicals, would be beyond the scope of this review. However, the general findings of the present review are transferable to other sensitizing chemicals and valid in general since the in vitro setup is similar.

5. Conclusions and Outlook

T cell activation mechanistically underlies chemical hypersensitivity reactions. Thus, the in vitro monitoring of human T cell immune response offers a great potential.

Over the past two decades, tremendous progress has been made in the understanding of T cell epitope formation by sensitizing chemicals. Epitopes may form by various methods

that are hard to predict by in silico or in chemico experiments and thus are still defined experimentally. Detected T cell responses are informative, especially if the analysis of patients illustrates in vivo relevance, while a negative result cannot be interpreted [148].

Besides pharmacologically relevant allergens, e.g., drugs, a number of skin sensitizing substances from our daily environment and some model chemicals have been successfully tested for T cell activation. The outlined experimental approaches reviewed here provide a path for the testing of additional chemicals. A broader application of new methods such as activation-induced marker assays, multi-parameter flow cytometry and high-throughput sequencing could advance the characterization of chemical-specific T cells, their phenotypes, functions and TCR characteristics [39].

A unique advantage of T cell assays is their capacity to assess cross-reactivity of individual T cell clonotypes. This can hardly be accomplished in vivo since patch testing relies on skin penetration, which differs for individual allergens and thus confounds results. In addition, prior exposure and co-sensitization cannot be ruled out in humans.

In vitro T cell assays have the potential to improve allergy diagnoses on an individual patient level, enable longitudinal tracking of immune responses, elucidate disease mechanisms and, potentially, may enable public biomonitoring in the future. T cell assays are also well-suited to complement predictive testing strategies for sensitizing chemicals in regulatory toxicology. Current in vivo tests are limited by species differences, ethical considerations and low throughput. In vitro, OECD-validated cell-based methods focus on steps prior to T cell activation, e.g., keratinocytes and DC responses, which represent interactions with the innate immune system. In the beginning era of the new approach methodologies (NAM) and next generation risk assessment (NGRA), the OECD Guideline 497 on "Defined Approaches for Skin Sensitization" has recently been published. The defined approaches currently listed combine several methods to allow hazard assessment and, in some cases, potency prediction, but lack T cell-based read-outs [149].

In summary, the specific influence of T cell activation on the sensitizing capacity of a chemical, TCR cross-reactivity and in vitro diagnostic options remain unclear until reliable T cell assays become available.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells11010083/s1, Table S1: Pubmed search results, Table S2: Chemicals allergens showing in vitro antigen-specific T cell activation in the different reviewed studies (extension of Table 1), Table S3: Studies describing general effects of non-metallic chemical allergens on T cell function.

Author Contributions: K.S., M.A.-S. and C.C. conceived the study; C.C. designed the searching strategy; C.C., M.A.-S. and K.S. carried out the searching strategy; K.S., M.A.-S., C.C. and F.R. wrote the manuscript; visualization was done by C.C. and F.R.; M.A.-S., C.C. and K.S. interpreted data; H.-J.T. and A.L. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by BfR internal grants 1322-718 (to K.S., M.A.-S. and C.C.), 1322-719 (to K.S. and F.R.) and 1322-754 (to H.-J.T.).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACD: allergic contact dermatitis; APC, antigen-presenting cell; CDR, complementarity-determining region; DC, dendritic cell; HSA, human serum albumin; HLA, human leukocyte antigen; LTT, lymphocyte transformation test; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; p-i, pharmacological interaction; TCR, T cell receptor.

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1.7 Objective

The aim of this project was the detection of human chemical allergen-specific CD4+ and CD8+ T cells. The methods used included a CD154- and CD137-based T cell assay as well as high-throughput sequencing (HTS) of the TCR involved. The methods would enable the analysis of the frequencies and TCR characteristics of allergen-specific T cells. In addition, cross-reactivity was investigated as a possible cause of co-sensitisation. Different metal allergens (solutions of $NiSO_4$, $CoCl_2$, $PdCl_2$) and the organic chemical model allergen TNBS were chosen based on their high allergy prevalence and prior experimental work regarding these allergens.

As a first step, control experiments should be performed for each chemical allergen. These should provide information on suitable test conditions, e.g. solubility, toxic effects, and possible interferences of the allergens of choice with flow cytometry. In addition, for different activation-induced markers, expression time course and biologic background expression levels on the total T cell pool were to be assessed to determine optimal incubation times and detection limits for rare antigen-specific T cells.

For the identified chemical-specific T cells, TCR-mediated T cell activation and MHC restriction were to be confirmed, e.g. by co-expression analysis of additional surface markers, the restimulation of single cell clones and the inhibition of MHC interactions by using blocking antibodies. For all assays, established antigens like cytomegalovirus (CMV), tetanus toxoid (TT), or the superantigen staphylococcal enterotoxin B (SEB) served as controls. In addition, relatively high concentrations were to be tested for the metal ions under investigation to capture TCR with low functional avidity. Once the assay conditions were established, the primary objective was identifying possible differences between T cell responses from allergic and non-allergic individuals, facilitated by clinical cooperation.

This thesis's second primary objective was analysing chemical-specific TCR repertoires. A unique-molecular identifier (UMI) and RNA-based approach was to be applied to support error correction and the generation of long sequence reads for reliable V-gene segment identification. For the data analysis, established programs needed to be combined into an automatic workflow to facilitate handling large data sets. In addition, new analysis tools had to be developed to enable, e.g., the analysis of the CDR3 amino acid composition. Programs needed to be developed for the congruence analysis of antigen-specific TCR isolated from independent samples with the same or a potential cross-reactive antigen. Analysis strategies to account for sampling effects had to be established.

Overall, the objective of this doctoral thesis work was to contribute to a better understanding of the immunological mechanisms of contact allergen-specific T cell responses. In addition, this work contributes to the development of novel predictive in vitro tests, potentially to be applied in regulatory and diagnostic settings.

2. Results

2.1 Research paper: TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells

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Published online on 16 April 2020 in:
Allergy. 2020; 75: 2574– 2586
DOI: 10.1111/all.14322
Link: https://doi.org/10.1111/all.14322

Involvement of the author within this publication: Project execution, data analysis, literature research, writing and visualisation of the manuscript.

Author contributions as published: MAS, FR, ML, and KS performed experiments and analysed data. GH, MW and SM provided samples and patient data. BT and HK performed library quantification and assisted in sequencing reactions and sequencing data analysis. DMC assisted in TCR sequencing experiments and TCR data analysis. KS, PB, AS, HJT, GH, and AL conceived the project, and KS directed the project. KS, MAS, and FR prepared the figures and wrote the manuscript. AL, BT, MW, GH, DMC, HJT, and AS corrected the manuscript. All authors approved the submitted version.

Online Supplementary Material is presented in Annex I.

Received: 4 November 2019 | Revised: 24 March 2020 | Accepted: 31 March 2020

DOI: 10.1111/all.14322

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells

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Funding information
Bundesinstitut für Risikobewertung, Grant/Award Number: SFP 1322-595, SFP 1322-663, SFP 1322-718 and SFP 1322-719; Ministry of Science and Higher Education of the Russian Federation, Grant/Award Number: 075-15-2019-1789

Abstract

Background: Nickel is the most frequent cause of T cell–mediated allergic contact dermatitis worldwide. In vitro, CD4+ T cells from all donors respond to nickel but the involved αβ T cell receptor (TCR) repertoire has not been comprehensively analyzed.

Methods: We introduce CD154 (CD40L) upregulation as a fast, unbiased, and quantitative method to detect nickel-specific CD4+ T cells ex vivo in blood of clinically characterized allergic and non allergic donors. Naïve (CCR7+ CD45RA+) and memory (not naïve) CD154+ CD4+ T cells were analyzed by flow cytometry after 5 hours of stimulation with 200 µmol/L $NiSO_4$., TCR α- and β-chains of sorted nickel-specific and control cells were studied by high-throughput sequencing.

Results: Stimulation of PBMCs with $NiSO_4$ induced CD154 expression on ~0.1% (mean) of naïve and memory CD4+ T cells. In allergic donors with recent positive patch test, memory frequencies further increased ~13-fold and were associated with markers of in vivo activation. CD154 expression was TCR-mediated since single clones could be

Abbreviations: APC, antigen presenting cell; CDR, complementarity determining region; CLA, cutaneous lymphocyte-associated antigen; CMV, cytomegalovirus; MHC, major histocompatibility complex; Ni, nickel; PBMCs, peripheral blood mononuclear cells; PMA-I, PMA-ionomycin; PT, patch test; SEB, staphylococcus enterotoxin B; TCR, T cell receptor; TRAV9-2, TCR α-chain V segment.

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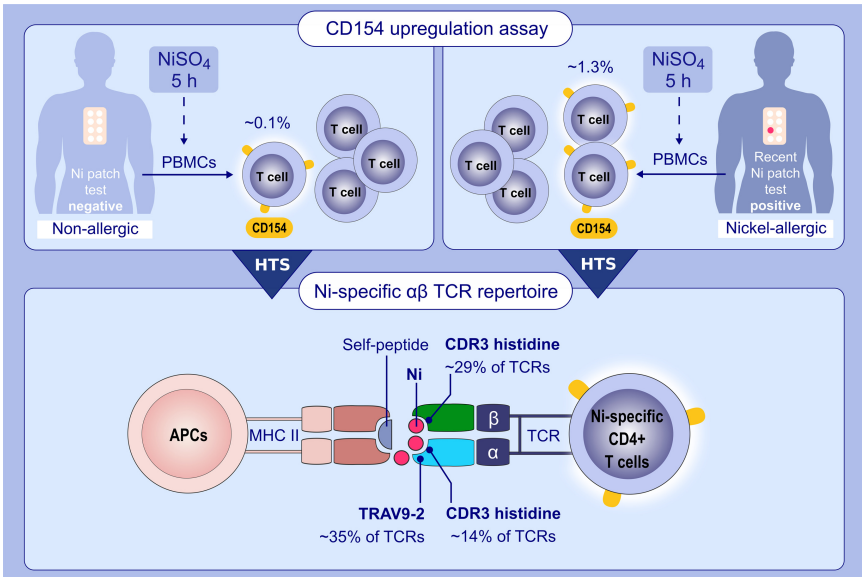
specifically restimulated. Among nickel-specific CD4+ T cells of allergic and non allergic donors, TCRs expressing the α-chain segment TRAV9-2 or a histidine in their α- or β-chain complementarity determining region 3 (CDR3) were highly overrepresented.

Conclusions: Induced CD154 expression represents a reliable method to study nickel-specific CD4+ T cells. TCRs with particular features respond in all donors, while strongly increased blood frequencies indicate nickel allergy for some donors. Our approach may be extended to other contact allergens for the further development of diagnostic and predictive in vitro tests.

KEYWORDS

CD154 upregulation assay, CDR3 histidine, human allergic contact dermatitis, nickel-specific CD4+ T cells, TCR α-chain segment TRAV9-2



GRAPHICAL ABSTRACT

We find increased frequencies of Nickel-specific memory CD4+ T cells in PBMCs of some nickel allergic individuals, applying a new approach based on CD154 upregulation. High-throughput sequencing of αβ T cell receptors reveals an overrepresentation of α-chain segment TRAV9-2 and CDR3 histidines.

Abbreviations: CDR, complementarity determining region; HTS, high throughput sequencing; MHC, major histocompatibility complex; Ni, nickel; $NiSO_4$, nickel sulfate; PBMCs, peripheral blood mononuclear cells; TCR, T cell receptor; TRAV9-2, TCR α-chain V-segment.

1 | INTRODUCTION

Nickel (Ni) contact allergy affects approximately 11% of the general population.[1] It is a T cell–mediated type 4 hypersensitivity reaction directly linked to Ni exposure[2,3] with little genetic influences.[4]

In allergic individuals, skin contact with Ni triggers allergic contact dermatitis mediated by allergen-specific CD4+ and CD8+ T cells.[5-8] Patch testing reproduces this reaction as current diagnostic standard because no reliable in vitro test exists.

Ni-specific T cells have been analyzed in vitro by proliferation or cytokine secretion assays (eg, lymphocyte transformation test, ELISpot assay). These studies found similar blood frequencies for many allergic and non allergic donors, especially in the CD4+ T cell compartment,[9-12] a limited correlation with patch test results,[11-13] and mixed T_H1/T_H2 cytokine secretion.[14-16] A

possible reason for the frequent in vitro activation of CD4+ T cells by Ni could be bystander activation due to unspecific mitogenic effects of Ni.[11,17] However, Ni-reactive clones were often generated[9,14] arguing for T cell receptor (TCR)-mediated activation.

Several mechanisms for the interaction of Ni with the TCR-peptide-major histocompatibility complex (MHC) II surface area have been proposed while the exact epitopes remain unknown.[19] Ni can bind to histidines on MHC II-presented peptides as has been shown for a single peptide.[20] Also, peptide mimotopes have been identified that activate a Ni-specific TCR and likely replace an unknown Ni-loaded peptide.[21] Alternatively, an interaction with TCR β-chain segment TRBV19 (Vβ17 in Arden nomenclature) has been discussed[17,22,23] but mutation analysis of three TRBV19 clones rejected an involvement in Ni binding.[24] Still, the idea that conserved TCR segments are associated with the recognition of Ni seems intriguing since it could explain the activation of a larger fraction of TCRs.

So far, a comprehensive analysis of the Ni-reactive TCR repertoire has been missing due to technological limitations. Only β-chains were analyzed because many V segment antibodies are available and the limited number of J segments facilitates multiplex PCR approaches.[22,25,26]

To analyze the Ni-reactive αβ TCR repertoire more comprehensively, we here adopted induced CD154 (CD40L) expression[27-29] and combined it with high-throughput sequencing of both TCR α- and β-chains[30]. Our approach revealed that Ni mainly activates TCRs with certain characteristics providing an explanation for the high numbers of Ni-specific CD154+ CD4+ T cells in non allergic individuals.

2 | METHODS

2.1 | Blood samples

Blood samples (~50 mL) were obtained from individuals with defined status regarding Ni sensitization and allergic contact dermatitis with written informed consent according to the current version of the declaration of Helsinki at the Department of Dermatology and Allergology (Charité, Berlin) and at the German Federal Institute for Risk Assessment (BfR, Berlin; ethic votes EA4/071/13, EA2/228/17). Details on donors and methods are given in Table S1 and Appendix S1 "Supplemental Methods."

2.2 | Antigen stimulation assays

Peripheral blood mononuclear cells (PBMCs) were stimulated with 200 µmol/L $NiSO_4$ in the presence of CD40 blocking antibody (1 µg/mL, HB14, Miltenyi Biotec).[27,28,29] After 5 hours, cells were stained for CD154 and other markers and analyzed by flow cytometry. CD154+ CD4+ T cells were sorted for TCR sequencing or expanded as clones for restimulation assays. Details on antigen stimulation assays and further methods are provided in Appendix S1 "Supplemental Methods."

2.3 | TCR sequencing

RNA- and UMI-based high-throughput sequencing of TCR α- and β-chains was performed on the Illumina MiSeq platform basically as described.[30] Appendix S1 "Supplemental Methods" lists full methodological details, Table S2 lists sorted cells and TCR sequence numbers. For primers and PCR conditions, see Table S3 and for sequences of single clones, see Table S4. Raw reads from αβ TCR sequencing are available on the European Nucleotide Archive (study accession no. PRJEB37836, https://www.ebi.ac.uk/ena/data/view/PRJEB37836).

3 | RESULTS

3.1 | Detection of Ni-specific CD4+ T cells by induced CD154 expression

To assess whether Ni-specific CD4+ T cells are detectable by CD154 (CD40L) upregulation similar to protein-specific cells,[27,28,30] blood was collected from non allergic donors (n = 12, Table S1) and from allergic donors with recent positive Ni patch test (n = 11). As a third group, donors with former positive Ni patch test were included (n = 8, ≥3 weeks between patch test and blood draw).

PBMC cultures were left without antigen or stimulated with 200 µmol/L $NiSO_4$. After 5 hours, we analyzed CD154 expression on naïve (CCR7+ CD45RA+, gating strategy Figure S1A) and memory (non-naïve) CD4+ T cells by flow cytometry. Without antigen stimulation, CD154 expression was low (Figure 1A shows two representative donors). After stimulation with 200 µmol/L $NiSO_4$, increased CD154 expression was detectable at various frequencies for 53 out of 62 naïve and memory CD4+ T-cell populations from all 31 donors on a 96-well plate (Figure 1B, Table S1).

We then compared Ni-induced CD154 expression among the different groups. Similar increased frequencies were observed for non allergic donors and for donors with former positive Ni patch test (~0.1%, Figure 1B). For donors with recent positive Ni patch test, memory frequencies increased significantly to 1.3%. Receiver operating characteristics curve analysis yielded a cutoff value of 0.53% that discriminates donors with recent positive patch test from nonallergic donors with 100% specificity and 90% sensitivity (Figure 1C). Therefore, many Ni-allergic individuals with recent positive patch test but not those with former positive patch test could be discriminated from non allergic controls.

Stimulation of PBMC cultures with the superantigen staphylococcus enterotoxin B (SEB) confirmed that T cells from the different groups did not differ in their general capacity to express CD154 (Figure S1B). Only CD4+ T cells expressing the SEB-interacting TCR

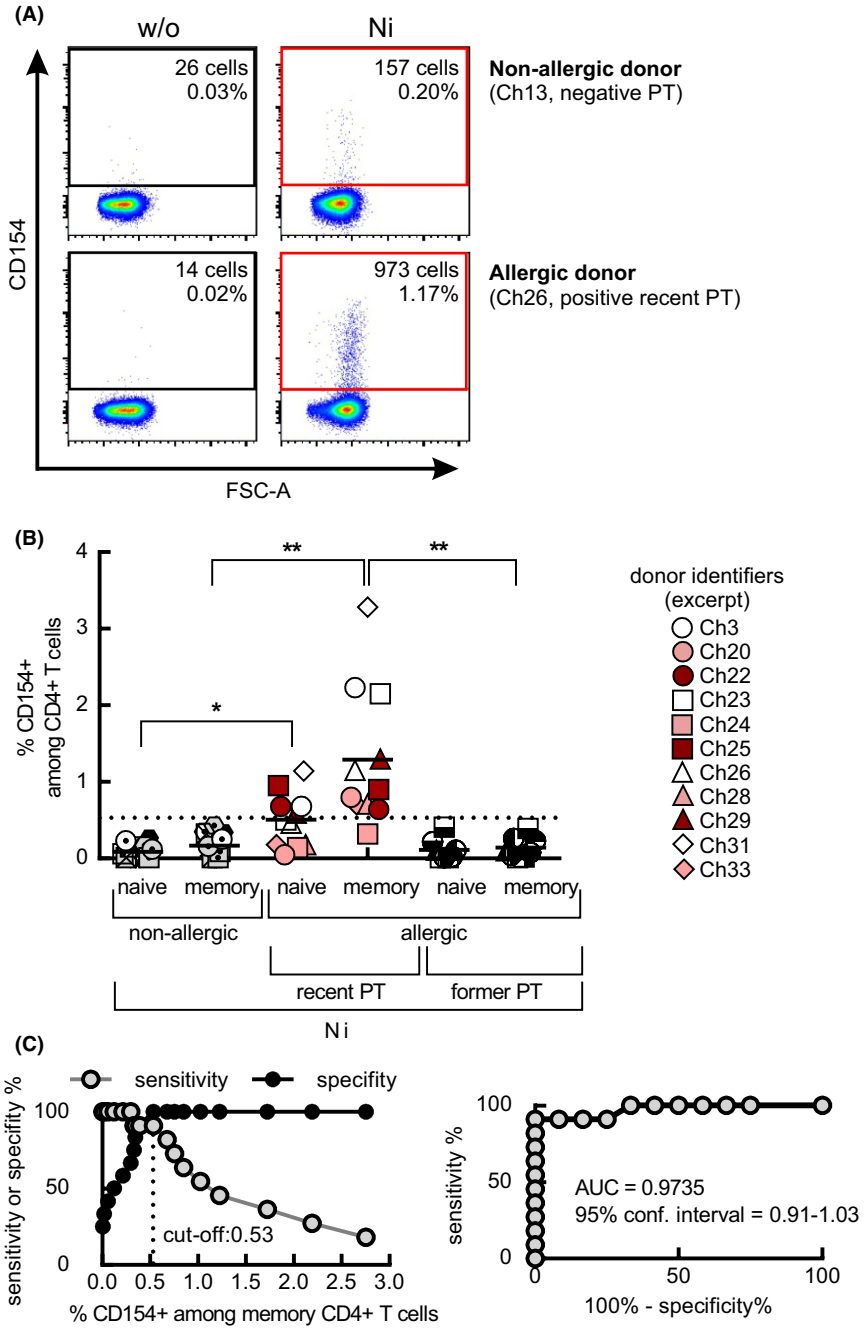


FIGURE 1 Detecting Ni-specific CD4+ T cells by CD154 upregulation assay. (A) Dot plots showing frequencies and cell numbers of CD154+ memory CD4+ T cells without antigen stimulation (w/o, left panels) and after 5 h of stimulation with 200 µM $NiSO_4$ (Ni, right panels) on a 96-well plate. Data are from one representative non allergic (Ch13) and from one Ni-allergic donor with recent positive patch test (PT; Ch26, gating scheme Figure S1A). (B) Frequencies of Ni-specific naïve and memory CD154+ CD4+ T cells in blood from all donors. Values from samples without antigen stimulation were subtracted. Donors were separated into three groups (non allergic, n = 12; allergic with recent positive PT, n = 11; and allergic with former positive PT, n = 8). For all donor identifiers and frequency values, see Table S1. Lines indicate the means. The dashed line indicates a cutoff value obtained by receiver operator characteristic (ROC) curve. One-way nonparametric ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between groups (*P < .05, **P < .01). (C) ROC curve analysis for frequencies of Ni-specific memory CD4+ T cells from allergic donors with recent positive PT and from non allergic donors discriminates between both groups: area under the curve (AUC) = 0.9735 (95% CI: 0.91-1.03), cutoff: 0.53% for 90% sensitivity and 100% specificity

β-chain TRVB19 (Vβ17) but not the noninteracting β-chain TRBV6-5 (Vβ13) stained CD154+ (Figure S1C). This confirms TCR-mediated and not bystander-mediated CD154 expression even if large fractions of T cells become activated, analogue to previous findings.[27] We did not find interferences with unrelated immune responses. For instance, donor Ch32 (non allergic) had many cytomegalovirus (CMV) pp65-specific but hardly Ni-specific cells (Figure S1D). Vice versa, donor Ch23 (allergic but CMV IgG negative) had increased frequencies of Ni-specific but not CMV pp65-specific CD4+ T cells.

In additional experiments, we further characterized Ni-induced CD154 expression. Upregulation peaked 2-4 hours after SEB and Ni stimulation and then stayed constant for more than 12 hours (Figure S1E). CD154 expression was dependent on PBMC density, that is, the presence of antigen presenting cells (APCs), arguing for TCR-mediated activation, whereas PMA-ionomycin (PMA-I) stimulation was APC-independent (Figure S1F). $NiSO_4$ titration showed increasing frequencies up to ~1 mmol/L (Figure S1G) while toxic effects, for example, decreasing monocyte numbers, started at ~400 µmol/L. We then chose 200 µmol/L $NiSO_4$ as standard concentration.

Regarding Ni-specific CD154+ CD8+ T cells, we observed a trend toward higher frequencies for allergic donors with outliers

among non allergic donors (Figure S1H). Since only ~20% of memory CD8+ T cells express CD154[32] and because Ni-specific CD8+ T cells seem less frequent in general,[9] they cannot be reliably detected on a 96-well plate format.

3.2 | Effector immune responses in donors with recent positive Ni patch test

To further estimate the in vivo relevance of Ni-specific CD4+ T cells, we analyzed co-expression of activation markers for donors with recent positive patch test. This was possible due to the high numbers of responding cells in this group (mean 1049 cells/well, 96-well plate).

Cutaneous lymphocyte antigen (CLA) has been associated with skin homing and Ni allergy.[33,34] Among Ni-activated CD154+ memory CD4+ T cells, 26% (mean) expressed CLA compared to 22% of total T cells (Figure 2A, Table S1). Increased CLA expression was not observed for non-skin–associated CMV pp65- or SEB-specific cells (12% and 14%, respectively). Since Ni is also contained in some foods, we determined the expression of the gut-homing chemokine receptor CCR9 to dissect food-associated from skin-associated Ni-specific T cells.[35] CCR9 was expressed by few total and CD154+ memory CD4+ T cells (<5%, Figure S2A), arguing against gut involvement in most cases.

Ni-specific cells of some donors expressed Ki-67, a marker for an active cell cycle,[36] to a higher percentage compared to the total cell pool and SEB-stimulated cells (Figure 2B; means 5%, 2%, 2%). Ki-67

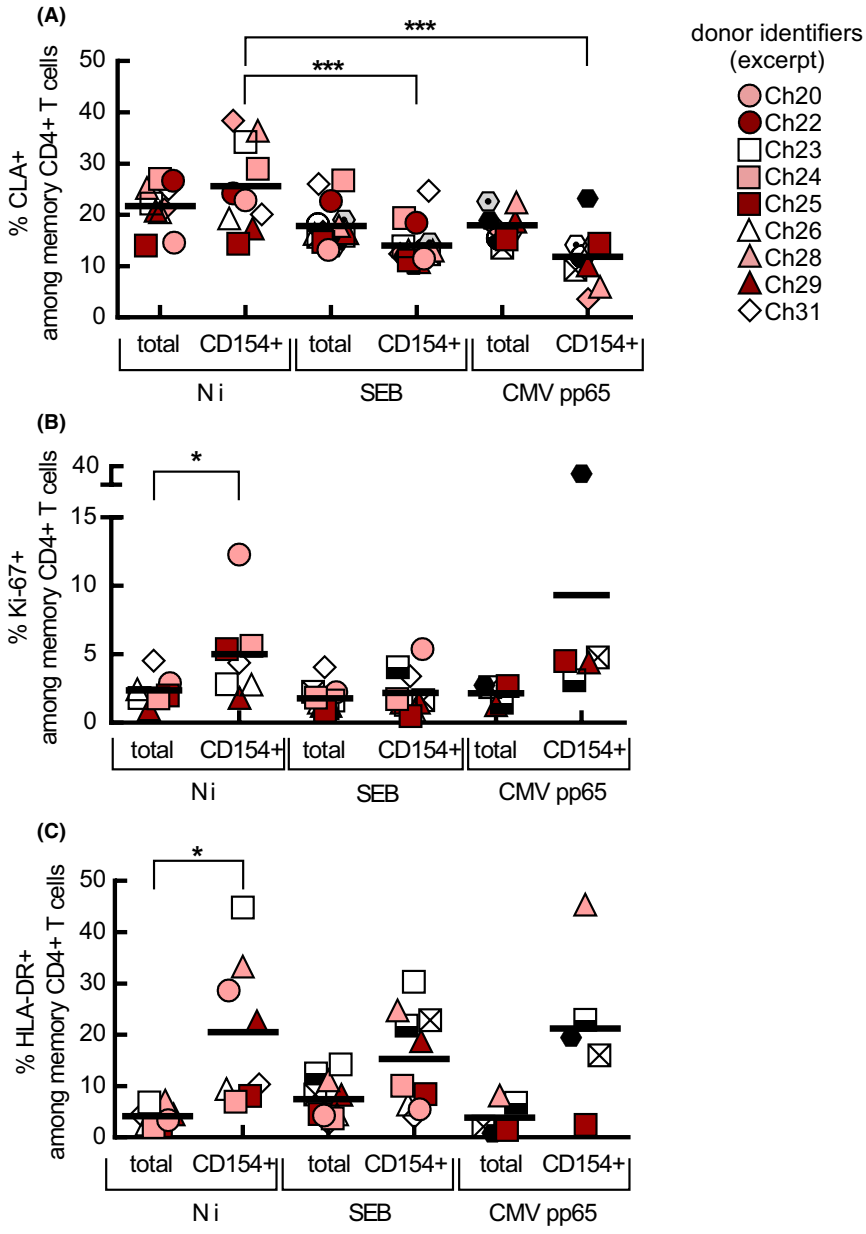


FIGURE 2 In vivo activation of Ni-specific T cells in allergic donors. Expression of (A) CLA, (B) Ki-67, and (C) HLA-DR by total or antigen-specific CD154+ CD4+ memory T cells in donors with recent positive patch test after 5 h of in vitro stimulation . Only populations with ≥20 cells are shown. Lines indicate the means. For all donor identifiers and frequency values, see Table S1. One-way ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between populations stimulated with different antigens. Wilcoxon signed-rank test was used to observe paired differences between total and CD154+ CD4+ T cells after stimulation with the same antigen (*P < .05, ***P < .001)

expression was also increased among CMV pp65-specific cells in some donors with positive CMV IgG titer (9%), likely due to ongoing chronic immune responses.

HLA-DR, a late activation marker,[37] was also more frequently expressed by Ni-specific T cells (Figure 2C). Nur77 and CD69, markers for TCR-mediated activation,[38,39] were induced on nearly all CD154+ CD4+ T cells after ~5 hours of stimulation (Figure S2B,C) arguing against bystander activation.

Co-expression of CLA, Ki-67, and HLA-DR differed among donors. For some donors, increased expression by Ni-specific cells

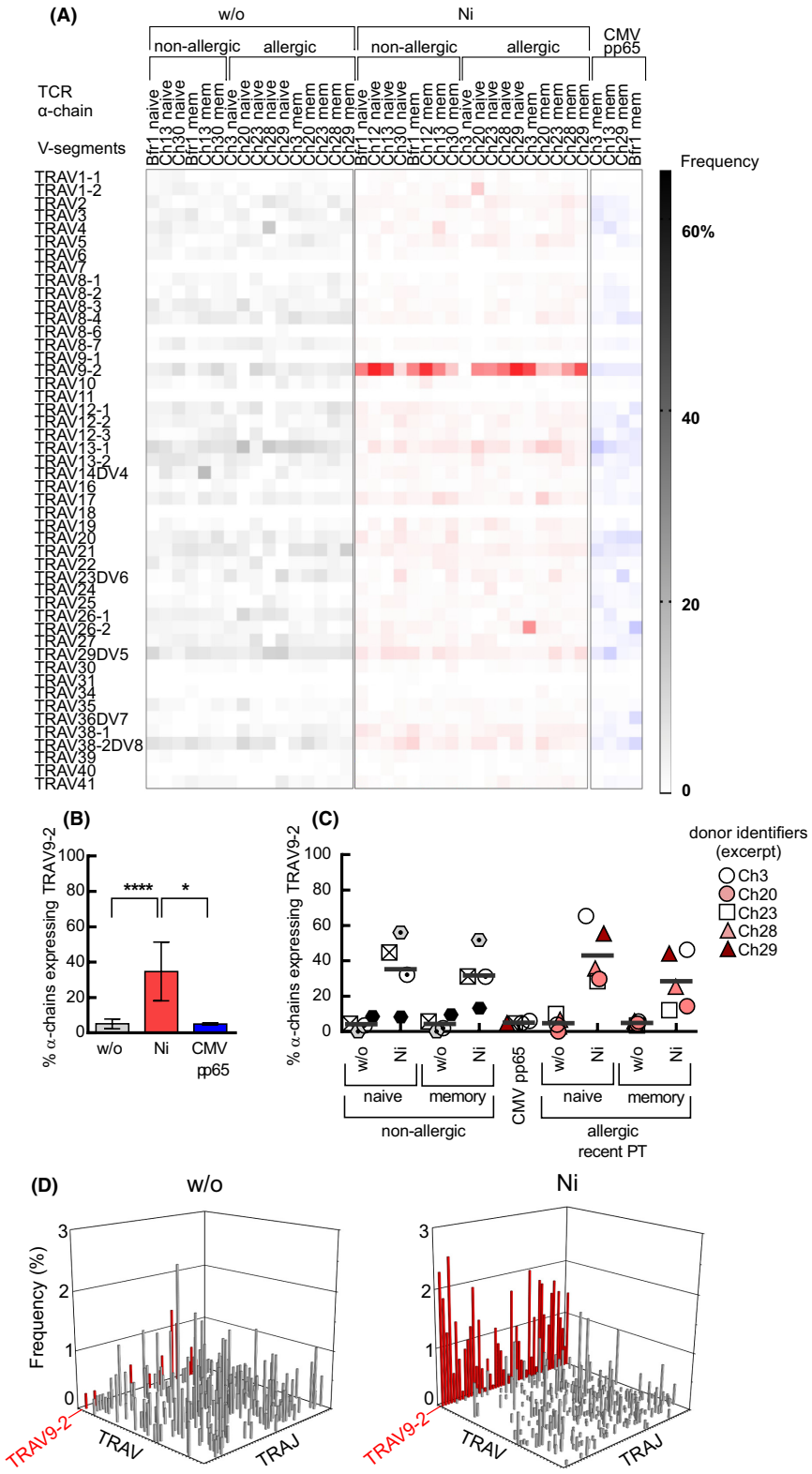


FIGURE 3 TCR α-chain segment TRAV9-2 is overrepresented among Ni-specific TCRs. (A) Heat plot depicting TCR α-chain V segment use among randomly sorted (without antigen stimulation, w/o, grey), Ni-specific (red), and CMV pp65-specific (blue) CD4+ T cells (frequencies). Cells were sorted from CD154 upregulation assays (~1000-3000 cells per sample, Table S2), and their TCRs were analyzed by high-throughput sequencing. (B) Mean frequencies of TRAV9-2+ TCRs among Ni- and CMV pp65-specific or randomly sorted (w/o) CD4+ T cells (means ± SD). (C) Frequencies of TRAV9-2+ TCRs among the indicated T-cell populations from individual donors (same data as in (A)). For all donor identifiers, see Table S1. One-way ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between groups (B and C, $^{*}P < .05$; $^{****}P < .0001$). (D) Representative 3D bar plots depicting TCR α-chain V- and J segment use for randomly sampled CD4+ T cells (left, w/o—without antigen stimulation) and for Ni-specific CD154+ CD4+memory T cells (right; frequencies, donor Ch29)

was observed (eg, Ch20) arguing for ongoing Ni-related immune responses. On the contrary, donor Ch29 had a strongly positive patch test (+++) and a high frequency of Ni-specific cells (1.3%) but showed no signs of recent immune activation, that is, no increased percentages of CLA+ or Ki-67+ T cells among Ni-specific cells. This donor also lacked recent dermatitis (personal report) and may therefore have a long-term increased memory response.

Ni-specific CD154+ CD4+ T cells expressed different cytokines (Figure S3), in line with previous reports.[14-16] Many expressed INFγ (T_H1), with similar or lower percentages compared to SEB-activated CD154+ cells. For some donors, IL-17A or IL-4 was expressed by a relatively high percentage of Ni-activated cells compared to SEB-activated cells indicating outgrowth of T_H17 or T_H2 cells during Ni allergy. For non allergic donors and allergic donors with former positive patch test, cytokine-producing cells were below detection limits (≤20 cells) but most cytokines tended to be elevated in Ni-stimulated cell culture supernatants. We observed no correlation for the type of Ni-specific cytokine response with the atopic status of the donor. As expected, CMV pp65-specific CD4+ T cells mainly expressed IFNγ.

3.3 | Ni-specific CD4+ T cells are linked to TCRs expressing α-chain segment TRAV9-2

Given the relatively high frequencies of Ni-specific CD4+ T cells in nonallergic donors, we wondered whether certain αβ TCR repertoire features are involved and whether repertoires differ between allergic and nonallergic individuals. To address this, we sorted Ni-specific CD154+ naïve and memory CD4+ T cells from 5 allergic donors and from 4 nonallergic donors (Table S2). Typically, 1000-3000 cells were collected from ~10 million stimulated PBMCs from a 12-well plate. As controls, random naïve and memory CD4+ T cells from samples without antigen stimulation, CMV pp65-specific T cells, and Jurkat T cells were sorted. In total, we analyzed 102 TCR α- and β-chain libraries, yielding 42 713 functional sequence reads (counts) of 20 397 distinct clonal lineages (TCR diversity; Table S2). On average, we obtained 0.2 ± 0.2 (mean ± SD) TCR cDNA counts per sorted cell for TCR α- and β-chains, similar to literature data.[40] Slightly higher cDNA counts were observed for activated CD154+ CD4+ T cells (0.3 ± 0.2) compared to randomly sorted cells (0.1 ± 0.1 counts/cell) for both naïve and memory CD4+ T cells. This is probably due to a slightly higher TCR mRNA amounts in the former.

In samples from Jurkat T cells, we only found the expected αβ TCRs (except for one erroneous α-chain with one sequence count among 1380 correct sequence counts) indicating that TCR sequences were correctly identified and that little spillover occurred between samples.

TCR β-chain segment TRBV19, which has been hypothesized to be involved in Ni allergy,[17,22-24] was equally expressed by randomly sorted CD4+ T cells (~5% of sequence counts) and by Ni-specific CD154+ CD4+ T cells (~3% of sequence counts, Figure S4).

Instead, we found a Ni-related overrepresentation of TCR α-chain segment TRAV9-2 (Figure 3A,B). TRAV9-2 is one out of 47 functional α-chain gene segments with ~5% background expression among randomly sorted CD4+ T cells. Among Ni-specific naïve and memory cells, TRAV9-2 was expressed by 43% ± 15% (mean ± SD) and 28% ± 14% of T cells, respectively, in allergic individuals and by 35% ± 18% and 32% ± 14% of T cells in nonallergic individuals (Figure 3C). Ni-specific TRAV9-2+ TCRs combined with different J segments (Figure 3D).

Because ~5% of randomly sampled T cells express TRAV9-2 and just ~0.1% of T cells respond to Ni in nonallergic donors, not all TRAV9-2+ T cells react to Ni. As a rough estimate, less than ~1 out of 100 TRAV9-2+ cells recognizes Ni.

A prior mutation study with a single TRAV9-2+ Ni-reactive T cell clone showed Ni recognition via $tyrosine_{36}$ in the CDR1 of TRAV9-2.[41,42] Although TRAV40 has a similar CDR1 compared to TRAV9-2 (TRAV40: STGYPT, TRAV9-2: ATGYPS), the TRAV40 segment was rarely expressed among randomly sorted T cells and not commonly enriched among Ni-reactive CD4+ T cells. Summarizing, apart from TRAV9-2, no other V- or J segments were commonly enriched and/or had relevant sequence counts among Ni-reactive CD4+ T cells (Figure S4).

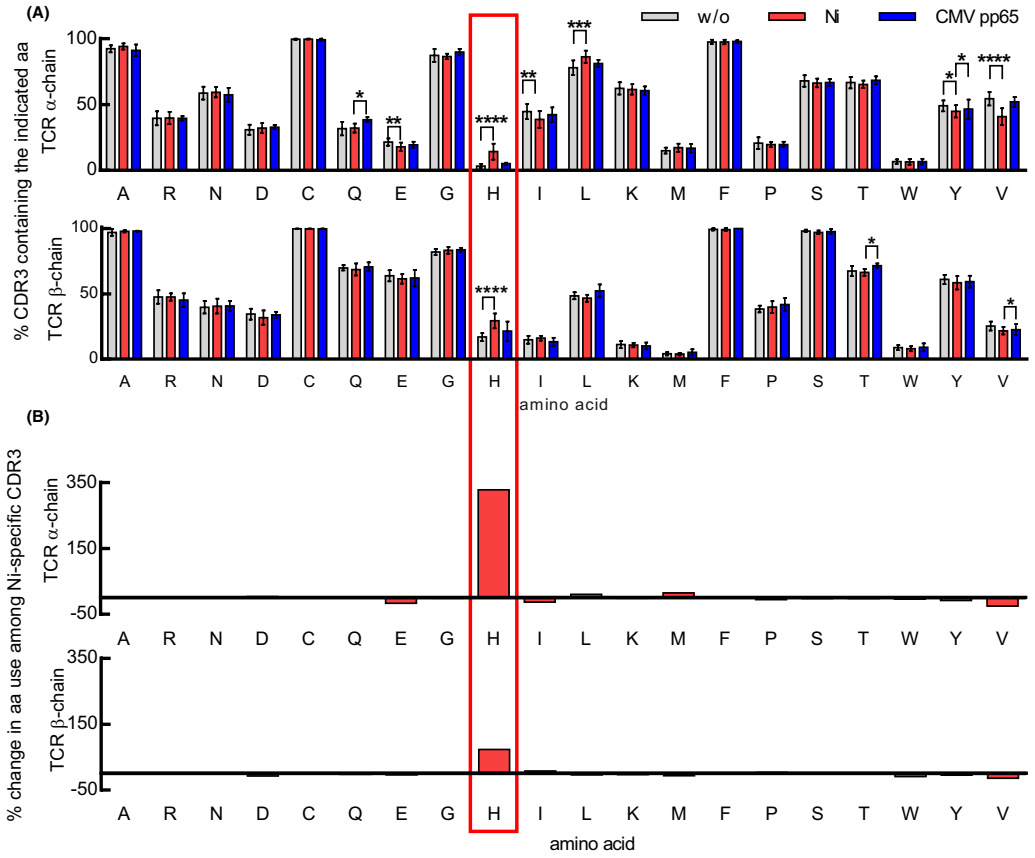
3.4 | Increased abundance of a histidine residue among Ni-specific CDR3

Given that only ~35% of Ni-specific TCRs express TRAV9-2, we further analyzed the CDR3 which is mainly responsible for peptide antigen recognition. We observed an increased use of the Ni-binding amino acid histidine among Ni-specific CDR3 (Figure 4A). Among randomly sorted, Ni-specific, and CMV pp65-specific α-chain CDR3, 3%, 14%, and 5% contained a histidine, respectively. Among β-chain CDR3, 17%, 29%, and 21% contained a histidine. No other amino acid was similarly increased (Figure 4B).

We mainly found one histidine per CDR3. Two or more histidines were present in 4% and 13% of Ni-specific TCR α- and β-chains, with similar results for random, Ni, or CMV pp65-specific TCRs (Figure 4C). Only few Ni-specific TRAV9-2+ TCR contained a CDR3 histidine arguing for largely independent mechanisms (Figure 4D).

As for TRAV9-2 overrepresentation, we observed a trend for an increased occurrence of a CDR3 histidine among Ni-specific naïve and memory CD4+ T cells of allergic and nonallergic donors (Figure 4E). The proportion of Ni-specific TCRs expressing a CDR3 histidine seemed less frequent among memory CD4+ T cells of allergic donors. This effect was not significant but could indicate less efficient in vivo selection.

Every donor had different Ni-specific TCRs. Not one overlapping clone was observed on CDR3 amino acid or nucleotide sequence level for β-chains (Figure S5A). To assess differences in TCR repertoire diversity, we calculated the Shannon index, which considers TCR diversity and the abundance of each clone in a sample. As expected, repertoire diversity was higher for naïve compared to memory T cells (Figure S5B). Ni-specific and CMV pp65-specific


(A)
w/o
Ni
CMV pp65
% CDR3 containing the indicated aa
TCR α-chain
TCR β-chain
A R N D C Q E G H I L K M F P S T W Y V
amino acid
(B)
% change in aa use among Ni-specific CDR3
TCR α-chain
TCR β-chain
amino acid

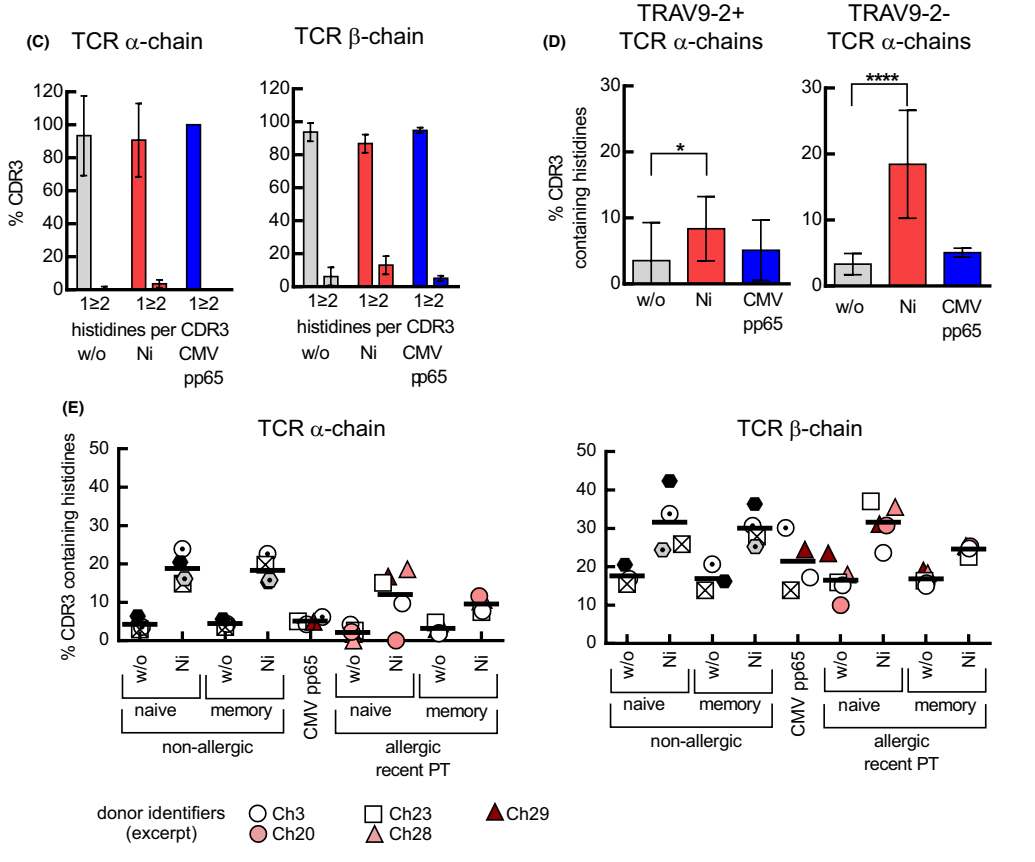

(C) TCR α-chain
TCR β-chain
% CDR3
1≥2 1≥2 1≥2
histidines per CDR3
w/o Ni CMV pp65
(D)
TRAV9-2+ TCR α-chains
TRAV9-2- TCR α-chains
% CDR3 containing histidines
w/o Ni CMV pp65
(E)
TCR α-chain
TCR β-chain
% CDR3 containing histidines
naive memory
non-allergic
CMV pp65
allergic recent PT
donor identifiers (excerpt)
Ch3 Ch20 Ch23 Ch28 Ch29

FIGURE 4 Ni-specific TCR α- and β-chains frequently contain a histidine in their CDR3. (A) Percentage of α- and β-chain CDR3 that contain the indicated amino acids among randomly sorted (without antigen stimulation, w/o, grey), Ni-specific (red), and CMV pp65-specific (blue) CD4+ T cells (diversity). Cells were sorted from CD154 upregulation assays (~1000-3000 cells per sample, Table S2), and their TCRs were analyzed by high-throughput sequencing. The first CDR3 cysteine (C) and last phenylalanine (F) were included in the analysis. Data were pooled according to antigen specificity (w/o n = 16, Ni n = 18, CMV pp65 n = 4; means ± SD, diversity values). (B) Percent change in amino acid use for α- and β-chain CDR3 of Ni-specific TCRs compared to randomly sampled TCRs. The same data as in (A) were used. (C) Analysis of the number of histidines in α- or β-chain CDR3 that are specific for the indicated antigens (means ± SD). (D) Percentage of CDR3 containing a histidine among TRAV9-2+ and TRAV9-2- TCRs for the indicated antigens (means ± SD). (E) Percentage of CDR3 with histidine for the indicated CD4+ T cell populations from individual donors. For all donor identifiers, see Table S1. One-way ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between groups (*P < .05, **P < .01, ***P < .001, ****P < .0001)

memory T cells had a lower diversity compared to randomly sorted cells indicating similar selection of antigen-specific TCRs. In general, there was no difference in repertoire diversity between allergic and nonallergic donors, that is, in terms of clonal expansions. Frequency and TCR diversity percentages were similar for the different repertoire features (Figure 5SC). However, donor Ch3 (allergic) had two expanded Ni-reactive clones occupying 26% (TRAV26-2) and 17% (TRAV9-2) of α-chain sequences (Figure 4A) causing a low Shannon index (Figure S5B). Since we observed β-chains with similar frequencies (33% TRBV12-4 and 13% TRBV27), matching TCR αβ chains may be assigned from bulk sequencing data in this case.[40]

3.5 | Ni-specific CD154+ CD4+ T cell clones can be specifically restimulated

Single Ni- or CMV pp65-specific CD154+ memory CD4+ T cells were sorted, expanded, and restimulated with the original and control antigens (Figure 5A). Restimulation frequencies were similar for Ni- and CMV pp65-specific CD4+ T cells with 34/47 (72%) of Ni-specific clones and 16/21 (76%) of CMV pp65-specific clones reacting to their respective original antigen (Figure 5B). This shows that sorted cells were indeed antigen-specific and that background expression of CD154 hardly interferes. We did not observe cross-reactive clones; for example, 0/17 CMV pp65-specific clones were Ni-reactive and *vice versa* (0/20 clones). Among Ni-specific clones, 27% (6/22) expressed the TRAV9-2 gene segment and 41% (9/22) a CDR3 histidine (8 clones β-chain, 1 clone α-chain), reproducing data from bulk sequencing (Table S4). Some clones from nonallergic and allergic donors reacted to 2 μmol/L $NiSO_4$ (3/16) and some did not require additional APCs (2/6 TRAV9-2+, 3/9 TRAV9-2-). Ni- and CMV pp65-induced CD154 expression was blocked to a similar extent by the addition of anti-MHC blocking antibodies (clones tu39 and ac122) in both bulk PMBC cultures and clone restimulation assays (Figure 5D) indicating TCR-mediated CD154 expression.

4 | DISCUSSION

The present study characterizes the frequencies and αβ TCR repertoires of Ni-specific CD154+ CD4+ T cells in blood of allergic and nonallergic donors.

The CD154 upregulation assay has several advantages.[27,28,30] It is fast, quantitative, and unbiased because it does not depend on proliferative or cytokine-producing capacity and it includes even naïve cells. Prior epitope knowledge is not required, and natural background expression of CD154 is low. Ni-induced CD154 expression was mainly TCR- but not bystander-mediated as demonstrated by the specific restimulation of clones and inhibited activation after the addition of MHC blocking antibodies, in agreement with previous work.[9,25]

We observed relatively high frequencies of Ni-specific CD4+ T cells for nonallergic individuals, similar to proliferation-based studies (ie, ~0.1% with 200 μmol/L $NiSO_4$ here vs 0.02% with ~40 μmol/L $NiSO_4$[9]). We used a high nontoxic concentration of $NiSO_4$ to also capture low-affinity clones. The importance of low-affinity clones becomes increasingly recognized even in chronic T cell responses,[43] in contrast to antibodie responses that undergo affinity maturation. The identified Ni-specific cells seem relevant in vivo, given elevated frequencies and expression of in vivo activation markers like CLA, Ki-67, and HLA-DR in some allergic donors. Lower Ni concentrations do not appear to improve the distinction of allergic and nonallergic individuals.[11]

Analyzing thousands of Ni-specific αβ TCRs, we identified two commonly shared characteristics: increased expression of the α-chain segment TRAV9-2 (~35% of TCRs) or a CDR3 histidine (~14% of α-chains, 29% of β-chains). Apart from these features, TCRs differed for each donor as expected since individual repertoires usually comprise ≥ 100 million, mainly unique, clonotypes.[44]

The use of the TRAV9-2 segment has been previously described for only one Ni-specific clone termed "SE9".[41,42] Mutation studies of "SE9" showed that it recognizes Ni via $tyrosine_{36}$ in the CDR1 of TRAV9-2 and via $histidine_{81}$ in the β-chain of human MHC II proteins, which is in close proximity and forms a major contact site in the canonical docking mode of TCRs.[45] Given that MHC II $histidine_{81}$ is expressed by most HLA-DRB1 alleles and no polymorphisms are known for the CDR1 of TRAV9-2 (IMGT database, February 24, 2020), TRAV9-2-mediated Ni recognition could occur in most donors and is compatible with a lack of HLA association in Ni allergy.[4,46] Contrary, Beryllium disease, another metal allergy, has a HLA allele-associated mechanism where Be^{2+} has no direct TCR contact.[47] Additionally, drugs may cause type IV hypersensitivity by noncovalent binding to certain HLA alleles via "pharmacological interactions".[48,49] This is, to our knowledge, the first report of an antigen-specific immune response linked to one conserved segment of the adaptive immune receptor repertoire.

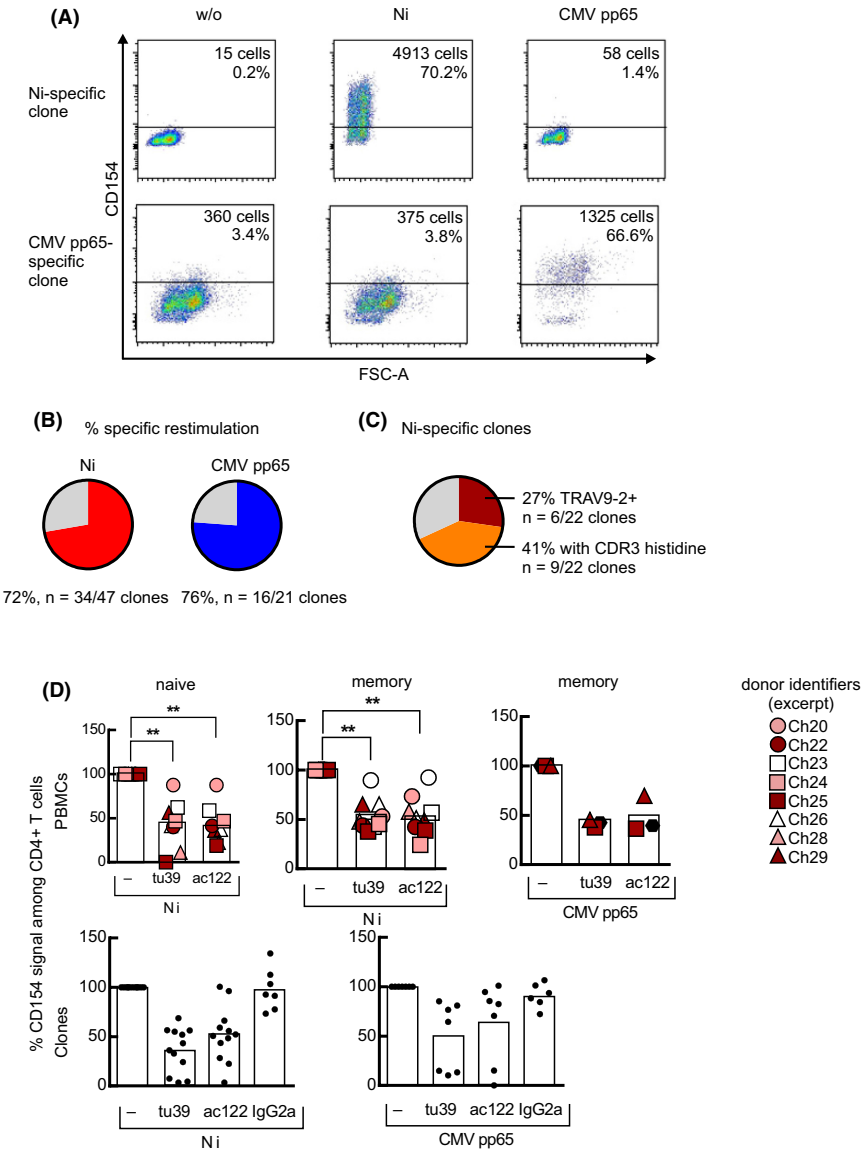


FIGURE 5 Ni-induced CD154 expression is TCR-mediated. (A) Representative dot plots depicting CD154 expression after 5 h of restimulation of a Ni- and a CMV pp65-specific T cell clone with the different antigens. Clones were derived from single CD154+ CD4+ memory T cells that were sorted from a CD154 upregulation assay. Cells were expanded in vitro for 2-3 wk and restimulated with autologous APCs and the indicated antigens. Gated on live, CD3+, single, CD4+ T cells. W/o—without antigen stimulation. (B) Restimulation efficiencies for Ni- (red) and CMV pp65-specific clones (blue, clones from n = 9 donors). (C) Frequency of Ni-specific clones expressing TRAV9-2 or a CDR3 histidine (n = 22 clones from 3 donors, TCR sequences are listed in Table S4). (D) Inhibited CD154 expression after the addition of MHC blocking antibodies to PMBCs bulk cultures (upper panels; n = 3-9 donors; for donor identifiers see Table S1) and single antigen-specific CD4+ T cell clones in restimulation assays (lower panel; n = 7-12 clones). MHC blocking antibodies (clones tu39, ac122) were added to the culture medium 30 min prior to stimulation with Ni or CMV pp65. After 5 h, expression of CD154 was monitored on CD4+ T cells. Graphs illustrate the percentage in signal reduction due to MHC block. Cultures without MHC blocking antibodies (upper panels) or with IgG2a isotype control antibody (lower panels) served as controls. White bars represent the means. One-way nonparametric ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between groups (**$P < .01$)

Only a fraction of TRAV9-2+ T cells reacted to Ni, indicating that it is not a classical superantigen-like interaction. The presented self-peptide may interfere with Ni binding or may not provide enough interactions to overcome a signaling threshold. Clone "SE9" lost its reactivity when tyrosine$_{108}$ in the α-chain CDR3 was mutated to histidine, suggesting a role in peptide but not Ni binding.[41] Accordingly, some Ni-reactive clones are dependent on peptides presented by certain APCs, as reported here and elsewhere.[50,51]

As second feature, TCRs with a CDR3 histidine were commonly enriched among Ni-specific TCRs (mainly TRAV9-2

negative), suggesting Ni binding via a CDR3 histidine is one mechanism of Ni-mediated T cell activation. This has not been hypothesized before. Future mutation or crystallization studies could solve structural details of the recognition mechanism. Together, TRAV9-2–mediated and CDR3 histidine-mediated Ni recognition cover a large proportion of Ni-specific TCRs (~78%) in both nonallergic and allergic donors with increased frequencies indicating that the identified cells are recruited in vivo. This renders repertoire features or clonal expansions unsuitable for the detection of Ni allergy.

Usually, specific memory CD4+ T cells comprise less than ~0.05% of all cells in the absence of effector responses, as shown for tetanus toxoid, measles virus,[52] vaccinia virus (IFNγ-read-out),[53] or food allergens.[30] Ni activates already ~0.1% of cells in nonallergic individuals. Therefore, only strongly increased blood frequencies can be linked to Ni allergy. For these cases, CD154 upregulation could represent an alternative in vitro test if patch tests cannot be applied or are less reliable, for example, for implant-related allergies. We do not expect strong interferences with unrelated immune responses since only ~0.1% of TCRs are cross-reactive but this requires further confirmation.

Given the relatively high frequencies of Ni-specific CD4+ T cells in blood of nonallergic donors, we hypothesize that the local density of Ni-specific CD4+ and CD8+ T cells in the skin is decisive for the Ni-allergic state and patch test results.[5,6] However, proof for the existence of global skin-resident nickel-specific CD4+ or CD8+ T-cell memory is missing.

For allergic donors without increased blood frequencies of Ni-specific CD4+ T cells, in vitro tests remain challenging. Possibly, rarer subpopulations within the Ni-specific pool are linked to Ni allergy.[15,16] Outgrowth of cytokine-producing clones may be detected by the CD154 upregulation assay if compared to unspecifically stimulated cells or to cells from non allergic donors (but not by simple cytokine analysis of cell culture supernatants). This requires large input cell numbers and magnetic enrichment of Ni-reactive T cells[28] and was incompatible with TCR sequencing here.

Summarizing, this study shows for the first time that TCRs with α-chain segment TRAV9-2 and a histidine in their α- or β-chain CDR3 are linked to Ni-specific CD4+ T cell activation. The CD154 upregulation assay may help to identify active allergy and may be extended to other contact allergens for the future development of diagnostic and predictive tests.[54-56]

ACKNOWLEDGMENTS

We thank PD Dr Klaus Abraham and Prof Cornelia Weikert for collecting blood samples at the BfR, PD Dr Burkhard Malorny and Maria Borowiak for their excellent assistance in Illumina MiSeq sequencing, and Daniel Siewert and Dr Julia Katharina Schlichting for help in analyses involving R. Open Access funding enabled and organized by Projekt DEAL.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

MAS, FR, ML, and KS performed experiments and analyzed data. GH, MW and SM provided samples and patient data. BT and HK performed library quantification and assisted in sequencing reactions and sequencing data analysis. DMC assisted in TCR sequencing experiments and TCR data analysis. KS, PB, AS, HJT, GH, and AL conceived the project, and KS directed the project. KS, MAS, and FR prepared the figures and wrote the manuscript. AL, BT, MW, GH, DMC, HJT, and AS corrected the manuscript. All authors approved the submitted version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Aparicio-Soto M, Riedel F, Leddermann M, et al. TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells. *Allergy*. 2020;75:2574–2586. https://doi.org/10.1111/all.14322

2.2 Research paper: Unique and common TCR repertoire features of Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific human CD154+CD4+ T cells

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Published online on 25 August 2022 in:
Allergy. 2022; 00: 1–13
DOI: 10.1111/all.15494
Link: https://doi.org/10.1111/all.15494

Involvement of the author within this publication: Project planning, project execution, data analysis, literature research, writing and visualisation of the manuscript.

Author contributions as published: KS conceived the study, supervised the project, the experimental work and data analysis. FR and KS planned the experiments. WKP selected patients, provided patient samples and clinical data. FR, MAS, LM and KS performed cell culture and flow cytometry experiments. FR, MAS, LM and CAC analysed the data. FR performed TCR sequencing experiments and analysed the data. FR and AA programmed CDR3 amino acid positional exact analysis and analysis of shared TCR clonotypes. FR, MAS, CAC and KS interpreted the data, prepared tables and figures and wrote the manuscript. WKP, AL, HJT, LM and AA corrected the manuscript. All authors provided discussion and agreed to the submitted version of the manuscript.
The authors declare that they have no conflicts of interest.

Online Supplementary Material is presented in Annex II.

Received: 28 March 2022 | Revised: 21 July 2022 | Accepted: 8 August 2022

DOI: 10.1111/all.15494

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

ORIGINAL ARTICLE

Unique and common TCR repertoire features of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific human CD154 + CD4+ T cells

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Funding information
Bundesinstitut für Risikobewertung; Deutsche Forschungsgemeinschaft

Abstract

Background: Apart from Ni^{2+}, Co^{2+}, and Pd^{2+} ions commonly trigger T cell-mediated allergic contact dermatitis. However, in vitro frequencies of metal-specific T cells and the mechanisms of antigen recognition remain unclear.

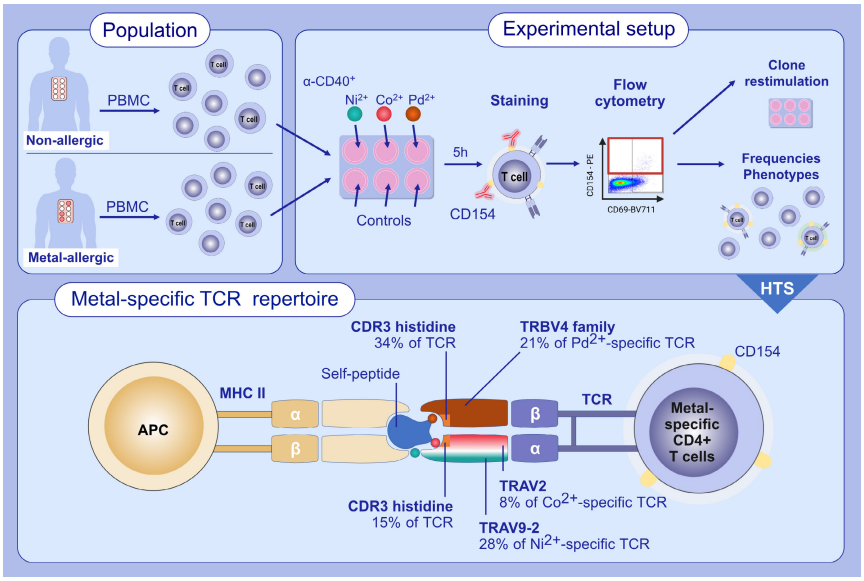
Methods: Here, we utilized a CD154 upregulation assay to quantify Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD4+ T cells in peripheral blood mononuclear cells (PBMC). Involved αβ T cell receptor (TCR) repertoires were analyzed by high-throughput sequencing.

Results: Peripheral blood mononuclear cells incubation with $NiSO_4$, $CoCl_2$, and $PdCl_2$ increased frequencies of CD154+CD4+ memory T cells that peaked at ~400 μM. Activation was TCR-mediated as shown by the metal-specific restimulation of T cell clones. Most abundant were Pd^{2+}-specific T cells (mean 3.5%, $n = 19$), followed by Co^{2+}- and Ni^{2+}-specific cells (0.6%, $n = 18$ and 0.3%, $n = 20$) in both allergic and non-allergic individuals. A strong overrepresentation of the gene segment TRAV9-2 was unique for Ni^{2+}-specific TCR (28% of TCR) while Co^{2+} and Pd^{2+}-specific TCR favorably expressed TRAV2 (8%) and the TRBV4 gene segment family (21%), respectively. As a second, independent mechanism of metal ion recognition, all analyzed metal-specific TCR showed a common overrepresentation of a histidine in the complementarity determining region 3 (CDR3; 15% of α-chains, 34% of β-chains). The positions of the CDR3 histidine among metal-specific TCR mirrored those in random repertoires and were conserved among cross-reactive clonotypes.

Conclusions: Induced CD154 expression allows a fast and comprehensive detection of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD4+ T cells. Distinct TCR repertoire features underlie the frequent activation and cross-reactivity of human metal-specific T cells.

KEYWORDS
CD154 upregulation assay, complementarity determining region 3 (CDR3) histidine, metal allergens Ni^{2+}, Co^{2+}, Pd^{2+}, T cell receptor (TCR) repertoire, TCR α-chain segment TRAV9-2

Abbreviations: AIM, activation-induced marker assay; CDR, complementarity determining region; CLA, cutaneous lymphocyte-associated antigen; CMV, cytomegalovirus; HLA, human leukocyte antigen; HTS, high-throughput sequencing; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; SEB, staphylococcus enterotoxin B; TCR, T cell receptor; TRAV9-2, TCR α-chain V segment 9–2; TT, tetanus toxoid.



GRAPHICAL ABSTRACT

Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD4+ T cells can be detected by a short-term CD154 upregulation assay. Overrepresentation of certain individual gene segments or a CDR3 histidine represent distinct TCR repertoire characteristics that underlie metal ion binding and cross-reactivity. Frequent metal-specific T cell activation in allergic and non-allergic individuals challenges blood-based allergy detection in vitro.

1 | INTRODUCTION

Metal allergies are the most common cause of allergic contact dermatitis and may underlie implant failure.[1-3] This public health concern is dominated by nickel allergy, affecting ~11% of the general population in Europe and North America.[4] Co-sensitization to cobalt and palladium, adjacent elements in the periodic table of the elements, is frequent but the mechanisms of antigen recognition and the extent of immunological cross-reactivity remain unclear.[5-9]

Metal allergies are mainly T cell-mediated diseases.[10-12] CD4+ T cell activation by metal ions has been shown to be dependent on the T cell receptor (TCR) and involves a self-peptide and protein of the major histocompatibility complex (MHC, in humans termed human leukocyte antigen [HLA]).[13-16] During an initial sensitization phase, chemicals do also activate the innate immune system. For example, Ni^{2+}, Co^{2+}, and Pd^{2+} bind to the human toll-like receptor 4, with a possible contribution of heterologous stimulatory signals.[17-22] Activated dendritic cells migrate to draining lymph nodes and present allergen-induced epitopes triggering antigen-specific T cell proliferation, differentiation, and memory formation.[12,21,23]

To date, there is a lack of validated in vitro T cell-based diagnostic and regulatory tests. One major challenge is the frequently observed metal-induced T cell activation in peripheral blood mononuclear cells (PBMC) from non-allergic individuals, as mainly shown for Ni^{2+} and CD4+ T cells.[16,24-27] Thus, metal-specific T cells are generally more abundant than conventional protein antigen-specific T cells in the absence of immune responses.[23,28] Some authors suspected unspecific mitogenic effects of metal ions, but the specific restimulation of T cell clones argues for antigen-specific T cell receptor (TCR)-mediated interactions.[16,29,30]

Every human individual expresses more than 100 million different, mainly unique αβ TCR generated through somatic V(D)J gene segment recombination with a hypervariable complementarity-determining region 3 (CDR3) mainly responsible for antigen recognition.[31] Usually, a tiny fraction of all TCR recognizes a given epitope consisting of a peptide presented by a protein of the MHC. Some epitope changes are tolerated, which underlies TCR cross-reactivity.[23,32]

In the case of metal allergens, hypotheses and preliminary data describe different possibilities for the non-covalent interactions of metal ions with the TCR-self-peptide–MHC interface.[14,15,23,33-35] The unusually frequent T cell activation led to speculations on a possible metal ion binding to conserved TCR gene segments.[13,36,37] Recently, our group comprehensively analyzed the TCR of Ni^{2+}-specific CD4+ T cells by high-throughput sequencing (HTS) identifying an overrepresentation of the TCR gene segment TRAV9-2 and a metal-binding CDR3 histidine among Ni^{2+}-specific CD4+ T cells.[16]

The present study analyzed the metal-mediated T cell activation with a focus on Co^{2+} and Pd^{2+} ions alongside Ni^{2+} ions. We quantified Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific human CD4+ T cells in allergic and non-allergic individuals by a CD154-based activation-induced marker (AIM) assay. We revealed unique TCR gene segments and a common CDR3 histidine overrepresentation among metal-specific TCR that also underlies their cross-reactivity.

2 | METHODS

2.1 | Blood samples and PBMC isolation

Fresh blood samples (~50 ml) were obtained from individuals with clinically defined allergy status with written informed consent according to the current version of the Declaration of Helsinki at the Department of Dermatology and Phlebology of the Vivantes Klinikum im Friedrichshain, Berlin, Germany (ethic vote EA4/045/19). Details on blood donors are listed in Table S1. PBMC was isolated by standard gradient density centrifugation as previously described.[16]

2.2 | T cell antigen stimulation assay

Peripheral blood mononuclear cells we seeded at a density of $2.5\times10^6/cm^2$ and incubated with 400 μM $NiSO_4$, $CoCl_2$, or $PdCl_2$ or control antigens in the presence of CD40 blocking antibody (1 μg/mL, HB14, Miltenyi Biotec). After 5 h, cells were stained for CD154 and additional surface marker expression and analyzed by multiparameter flow cytometry. Frequencies of activated T cells were calculated as CD154+ cell numbers within the total CD4+ memory T cell pool. CD154+CD4+ memory T cells were sorted for clone restimulation or TCR HTS.[16]

2.3 | TCR sequencing

RNA- and unique molecular identifier (UMI)-based HTS of TCR α- and β-chains was performed using Illumina's MiSeq platform as described.[16,38] TCR sequences are available on the European Nucleotide Archive (study accession no. PRJEB53204, https://www.ebi.ac.uk/ena/data/view/PRJEB53204).

Further information on methods can be found in Appendix S1 "Supporting methods".

3 | RESULTS

3.1 | Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD4+ T cells can be detected by induced CD154 expression

In a first step, we tested whether Co^{2+}- and Pd^{2+}-specific human CD4+ T cells can be detected by induced CD154 expression similar to Ni^{2+}-specific cells.[16] PBMC were stimulated with $NiSO_4$, $CoCl_2$, or $PdCl_2$ for 5 h, stained, and analyzed by flow cytometry. In samples without antigen stimulation, hardly any CD154 background expression was detected while metal allergens induced CD154+CD4+ memory T cells (Figure 1A; gating strategy depicted in Figure S1). Cytomegalovirus (CMV) lysate was chosen as a control protein antigen because infected individuals show high percentages of CMV-specific memory T cells ("memory inflation").[39,40] The ubiquitous vaccination antigen tetanus toxoid (TT) served as a second control antigen since only a fraction of people are CMV infected.[41]

Percentages of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD154+CD4+ memory T cells depended on the metal ion concentration and peaked between 400 and 800 μM (Figure 1B). Pd^{2+}-specific T cells were the most abundant. Activation was similar for CD4+ naive T cells (data not shown). We here focus on the memory compartment because these cells are the main mediators of allergic reactions and their lower TCR diversity enables cross-reactivity analysis.

At higher metal salt concentrations, live cell numbers of CD14+ monocytes and CD19+ B cells declined and the percentages of apoptotic Annexin V+ cells increased[42] (Figure S2A; B cell data not shown). The most sensitive read-out for analyzing the effect of metal salts was a reduced CD154 upregulation on CD4+ T cells, monitored by stimulation with the superantigen staphylococcus enterotoxin B (SEB; Figure S2B). SEB links certain TCR β-chains with MHC proteins independent from the presented antigen peptide activating a large proportion of T cells.[43] SEB-induced T cell activation covered metal-specific signals except for stimulation with 200–400 μM $PdCl_2$ that activated additional T cells (Figure S2B). In summary, metal-specific T cell activation and toxic effects intersect at rising metal salt concentrations until the latter become dominant.

3.2 | Increased percentages of CLA+ metal-specific T cells in some allergic individuals

Percentages of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD154+CD4+ memory T cells were investigated in blood samples from clinically characterized allergic and non-allergic individuals (Figure 1C; donor list Table S1). A standard metal salt concentration of 400 μM was chosen that likely captures the complete reactive T cell pool (Figures 1B). Percentages of activated T cells varied considerably among donors with similar mean values among non-allergic and allergic individuals (Ni^{2+}: 0.3 ± 0.2% vs. 0.3 ± 0.2%; Co^{2+}: 0.5 ± 0.8% vs. 0.6 ± 0.7%; means ± standard deviation [SD]; Figure 1C; Table S2).

An increased percentage of cutaneous lymphocyte antigen (CLA) expressing CD154+CD4+ memory T cells compared with the total CD4+ memory T cell pool (≥5% increase) was detected for 3/7 nickel allergic (VF4, VF9, VF14) and 2/7 cobalt allergic individuals (VF7, VF19; Figure S3; Table S2). CLA expression is linked to skin-directed immune responses and nickel-induced allergic contact dermatitis.[16,44-48] For one nickel and one cobalt allergic donor (VF10, VF5), CLA co-expression was increased among allergy unrelated Co^{2+}- and Pd^{2+}-specific T cells, respectively. Increased percentages of CLA co-expressing cells were not observed for CMV-, TT-, and SEB-specific T cells, which are not expected to home to the skin (data not shown).[16]

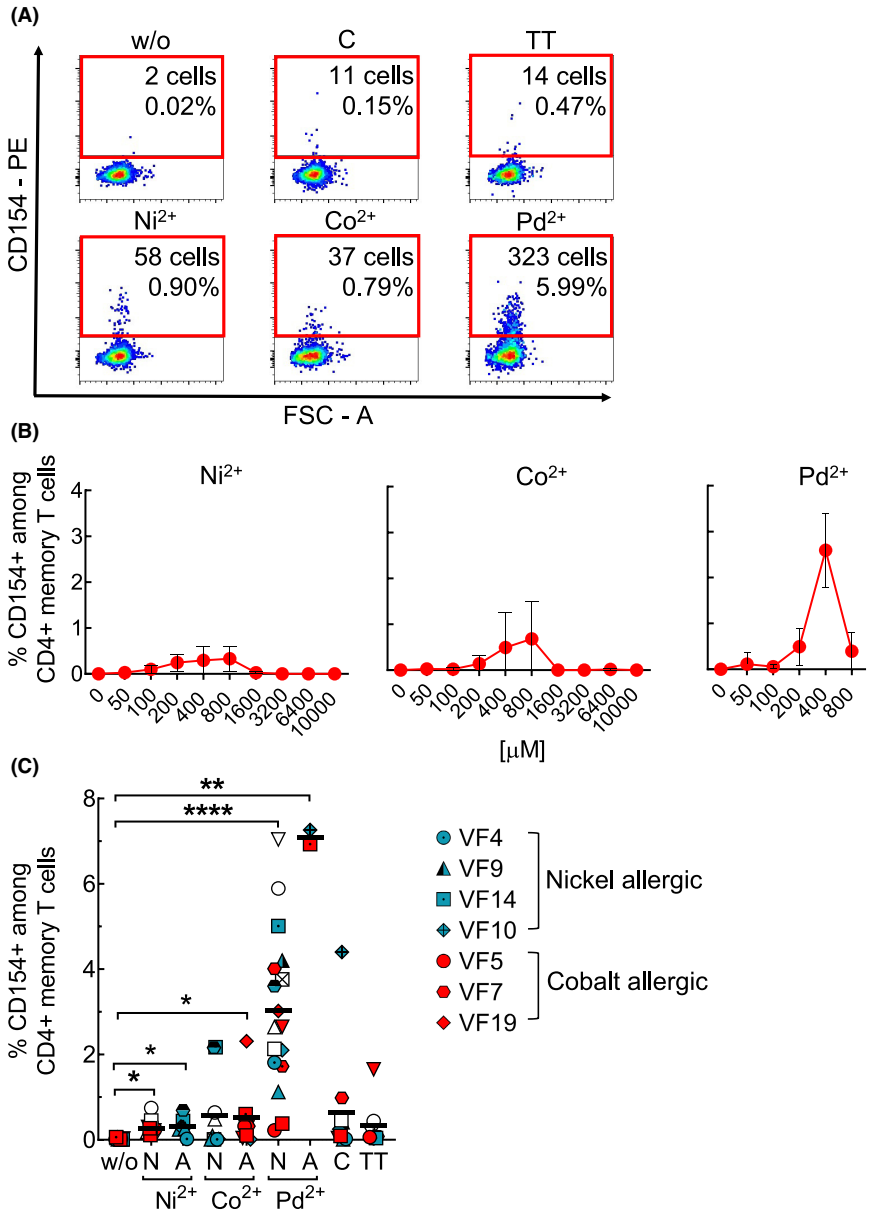


FIGURE 1 Detection of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD154+CD4+ T cells by CD154 upregulation assay. (A) Representative dot plots. PBMC were incubated without antigen (w/o) or with 400 µM $NiSO_4$ (Ni^{2+}), $CoCl_2$ (Co^{2+}), $PdCl_2$ (Pd^{2+}), CMV (C), or TT for 5 hours, stained and analyzed by flow cytometry. Indicated are total numbers and frequencies of CD154+CD4+ memory T cells (for gating strategy, see Figure S1; data from donor VF11, non-allergic, see Table S1; results from 100,000 total events, Table S2 lists values for all acquired events). (B) Metal salt titration. PBMC from buffy coats were incubated with the indicated metal salt concentrations ($n = 4–6$ donors). (C) Frequencies of antigen-specific CD154+CD4+ memory T cells among non-allergic (N) and allergic (A) donors. For CMV stimulation, only results for CMV IgG+ donors are shown. Each symbol represents one donor (Table S1, Ni^{2+}: $n = 13$ (N) and 7 (A); Co^{2+}: $n = 10$ (N) and 8 (A); Pd^{2+}: $n = 17$ (N) and 2 (A); C: $n = 11$; TT: $n = 8$). Horizontal lines indicate the mean values, vertical lines the SD. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal–Wallis) with Dunn's post-hoc test (*$p < .05$; **$p < .01$; ****$p < .0001$ vs. w/o)

3.3 | Metal ion-induced CD154 expression is TCR-mediated

To exclude a non-specific upregulation of CD154 on CD4+ T cells by non-TCR-mediated stimulatory effects of metal salts, we monitored CD69 co-expression, MHC restriction and restimulation of T cell clones.

CD69 is an early marker of T cell activation expressed with similar kinetics as CD154 that also mediates tissue retention and immune responses.[49] Compared with CD154, CD69 background expression and antigen-induced CD69+CD4+ memory T cell frequencies were both higher, suggesting variable levels of bystander activation that render CD69 less useful as sole activation marker[50-52] (Figure S4A; Table S2). However, CD69 co-expression analysis confirmed antigen-specificity, being mostly above 80% for different non-toxic metal salt concentrations[28] (Figure S4B, C). The addition of anti-MHC II blocking antibodies prevented metal ion-induced CD154 expression to a similar extend as in other studies[16,53] (Figure S4D). Out of three antibody clones, anti-HLA-DR clone "L243" showed a tendency for the best MHC II blocking effects. These results illustrate both TCR-mediated antigen recognition and MHC restriction for the majority of metal-specific T cells.

Metal-specificity was further confirmed by restimulation of T cell clones established from single sorted antigen-specific CD154+CD4+ memory T cells (Figure S5; Table S3). Most clones reacted positively to their original antigen (Ni^{2+}: 16/19; Co^{2+}: 9/14; Pd^{2+}15/18). Some clones reacted to lower metal salt concentrations, as suggested by the titration results. Ni^{2+}-specific clones favorably cross-reacted to Pd^{2+} (8/19 clones), while other clones were also cross-reactive (Ni^{2+}: 2/19 to Co^{2+}; Co^{2+}: 3/14 to Ni^{2+}; and 5/14 to

Pd^{2+}; Pd^{2+}: 2/18 to Ni^{2+} and 2/18 to Co^{2+}). A generally lower cross-reactivity of Pd^{2+}-specific clones with Ni^{2+} and Co^{2+} is expected, given the overall higher abundance of Pd^{2+}-specific T cells. After in vitro expansion, T cell clones usually express MHC II (HLA-DR+, data not shown). Several clones could be activated without additional antigen-presenting cells, that is, they presented the metal-induced antigens themselves, as reported earlier for Ni^{2+}-specific clones[54] (Ni^{2+}: 6/12 clones; Co^{2+}: 2/8; Pd^{2+}: 11/15). Some clones showed broader polyspecificity, for example, additional recognition of Pt^{2+}, Au^{3+}, or Cu^{2+}, but not Cr^{3+} and Be^{2+} ions indicating complex and varying cross-reactivity patterns (Figure S5).

3.4 | Gene segments are uniquely overrepresented among metal-specific TCR

The high frequencies of metal-specific CD4+ T cells and the previous discovery of Ni^{2+}-specific TCR repertoire features[16] prompted a comparative analysis of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific TCR. Metal- and control antigen-specific CD154+CD4+ memory T cells (400–15,000 cells/sample) as well as random CD4+ memory T cells (23,000–100,000 cells/sample) were sorted from 12 individual donors (Table S4). An RNA- and unique molecular identifier-based TCR HTS protocol was applied, which counts each transcribed cDNA and allows efficient error correction.[16,38,55] In total, 134 TCR libraries, derived from 1,246,173 sorted T cells yielded 639,951 sequence counts (0.2±0.2 counts per cell, mean±SD) and 311,341 unique TCR clonotypes (Table S4).

A distinctive overrepresentation of the gene segments TRAV9-2, TRAV2, and the TRBV4 family was observed among Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific TCR, respectively (Figure 2). Among Ni^{2+}-specific TCR, 28±16% (mean±SD) expressed TRAV9-2 compared with 6.4±1.0% of random TCR and a background-like expression among Co^{2+}-, Pd^{2+}, CMV- or TT-specific TCR (Figure 2A). The increase of Co^{2+}-specific TRAV2+ TCR compared with random TCR was also statistically significant but less pronounced than changes in TRAV9-2 segment use, that is, 8±4% vs. 3±1% (Figure 2B). A trend for a preferred TRAV2 expression by Pd^{2+}-specific TCR was also observed. The three gene segments of the TRBV4 family, TRBV4-1, TRBV4-2, and TRBV4-3, were exclusively abundant among Pd^{2+}-specific TCR (7±4% vs. 2±1% in random TCR; 7±3% vs. 2±0%; 6±6% vs. 1±1%, respectively; Figure 2C). No other α- and β-chain V- and J-gene segment expression was commonly changed among metal-specific TCR repertoires compared with random repertoires (Figure S6). Allergic individuals showed on average a slightly lower overrepresentation of TRAV9-2, TRAV2, and TRBV4 compared to non-allergic individuals, indicating possible in vitro effects (Figure 2). However, one cobalt allergic donor (VF19) had high frequencies of Co^{2+}-specific CD4+ T cells, high CLA+ co-expression and a prominent TRAV2 segment use, arguing for the in vivo relevance of Co^{2+}-specific TRAV2+ TCR. We did not observe clonal expansions among metal-specific compared with protein antigen-specific TCR repertoires (data not shown).

3.5 | A CDR3 histidine is commonly enriched among metal-specific TCR

TCR gene segment associations underlie only a part of the frequent metal-induced T cell activation and we therefore further analyzed the CDR3. We observed an increased abundance of a metal-binding histidine in CDR3α and CDR3β among metal-specific TCR (Figures 3, S7A). Other amino acids were not commonly modified and the changes were less pronounced, as visualized by a background (random TCR) corrected display (Figure S7B). The large majority of CDR3 contained only one histidine (Figure S7C). TCR with a CDR3 histidine were less abundant among metal-specific TCR that expressed gene segments TRAV9-2, TRAV2, and TRBV4. Thus, both mechanisms of metal ion recognition act independently (Figure S8).

Among Ni^{2+}-specific CDR3α, 19±8% (mean±SD) contained a histidine compared with 4±0% of random CDR3α, an increase of 375%. Similarly, 34±7% of Ni^{2+}-specific CDR3β contained a histidine compared with 17±1% of random CDR3β, an increase of 100% (Figure 3A). Results were similar for Co^{2+}- and Pd^{2+}-specific CDR3α and CDR3β (Co^{2+}: 15±5% and 30±4%; Pd^{2+}: 12±3% and 38±8%). The average abundance of CDR3β with a histidine tended to be higher in allergic than in non-allergic individuals. In addition, CDR3α with a histidine were frequent among Co^{2+}-specific CD4+ T cells with increased CLA co-expression (e.g., cobalt allergic donor VF7), which supports a possible in vivo role of the identified TCR. Additionally, we analyzed the occurrence of amino acids at individual positions in the CDR3 to determine whether a metal-binding histidine occurs at a preferred position. As the length of the CDR3 varies, both the 5′-3′ as well as the 3′-5′ directions were analyzed for patterns of individual amino acid appearance (Figure S9). Histidine expression is mainly restricted to position 4–8 in the CDR3α (5′-3′) but occurs more often along the whole length of the CDR3β starting at position 5 (5′-3′) with a dominant peak at the end (position 2, 3′-5′; Figure 3B). The increase of histidine-containing CDR3 among metal-specific TCR mirrored the histidine positions in the random repertoires, without indications for a preferred metal ion binding site. Apart from histidine, glutamic acid (E) has been discussed as a possible Ni^{2+} ion binding site at the end of the CDR3β.[56] In our data, glutamic acid occurred frequently at position 4 (3′-5′; 47% of random TCR; mean) but was not further increased among Ni^{2+}-specific TCR (~42%; Figure S9).

3.6 | TCR repertoire features are preserved among cross-reactive metal-specific TCR

TCR cross-reactivity has been suspected as the underlying cause of metal allergen co-sensitizations, but it is challenging to study due to the vast TCR diversity. Here, we sequenced Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific TCR from CD4+ memory T cells of the same individuals by HTS, which identified cross-reactive TCR

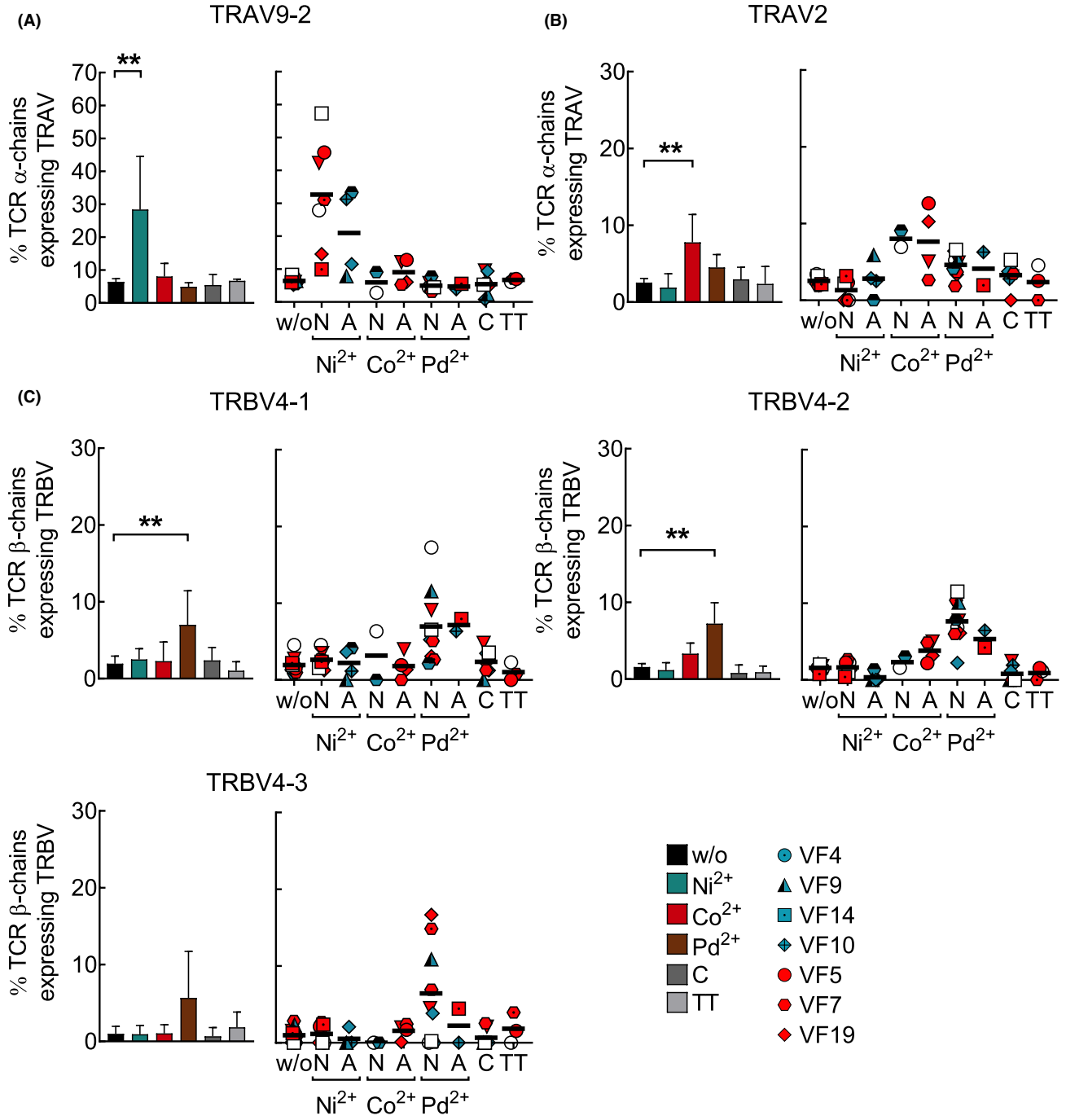


FIGURE 2 Distinct TCR gene segment overrepresentation by metal-specific TCR. Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells were sorted after 5 h of antigen stimulation and their TCR were sequenced. Random TCR were obtained from samples without antigen stimulation (w/o, black) and antigen-specific TCR from samples incubated with 400 µM $NiSO_4$ (Ni^{2+}, blue), $CoCl_2$ (Co^{2+}, red) or $PdCl_2$ (Pd^{2+}, brown), CMV (C, dark gray) or TT (gray, Table S4). (A) TRAV9-2 segment use. Depicted are frequencies of TCR α-chains expressing TRAV9-2 (diversity percentages). The left graph shows overall mean values and SD, the right graph individual donor data for non-allergic (N) and allergic (A) individuals with horizontal lines indicating mean values (wo: $n = 12$; Ni^{2+}: $n = 7$ (N) and 4 (A); Co^{2+}: $n = 2$ (N) and 4 (A); Pd^{2+}: $n = 9$ (N) and 2 (A); C: $n = 7$; TT: $n = 3$). (B) TRAV2 segment use. Similar mean and individual donor data graphs are shown as in (A). (C) TRBV4 segment use. Similar mean graphs and individual donor data graphs as in (A) are shown for the three genes of the TRBV4 segment family. Each symbol represents one donor (Table S1). Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal–Wallis) with Dunn's post-hoc test (**$p < .01$ vs. w/o)

(intra-individual analysis; Table S5). We observed a trend for an increased TCR cross-reactivity among metal-specific compared with metal vs. protein antigen-specific TCR (Figure S10). Among metal-specific cross-reactive TCR we observed increased percentages of TRAV9-2+, TRAV2+, and TRBV4+ TCR (Figure 4A). Thus, although gene segment use is uniquely associated with the

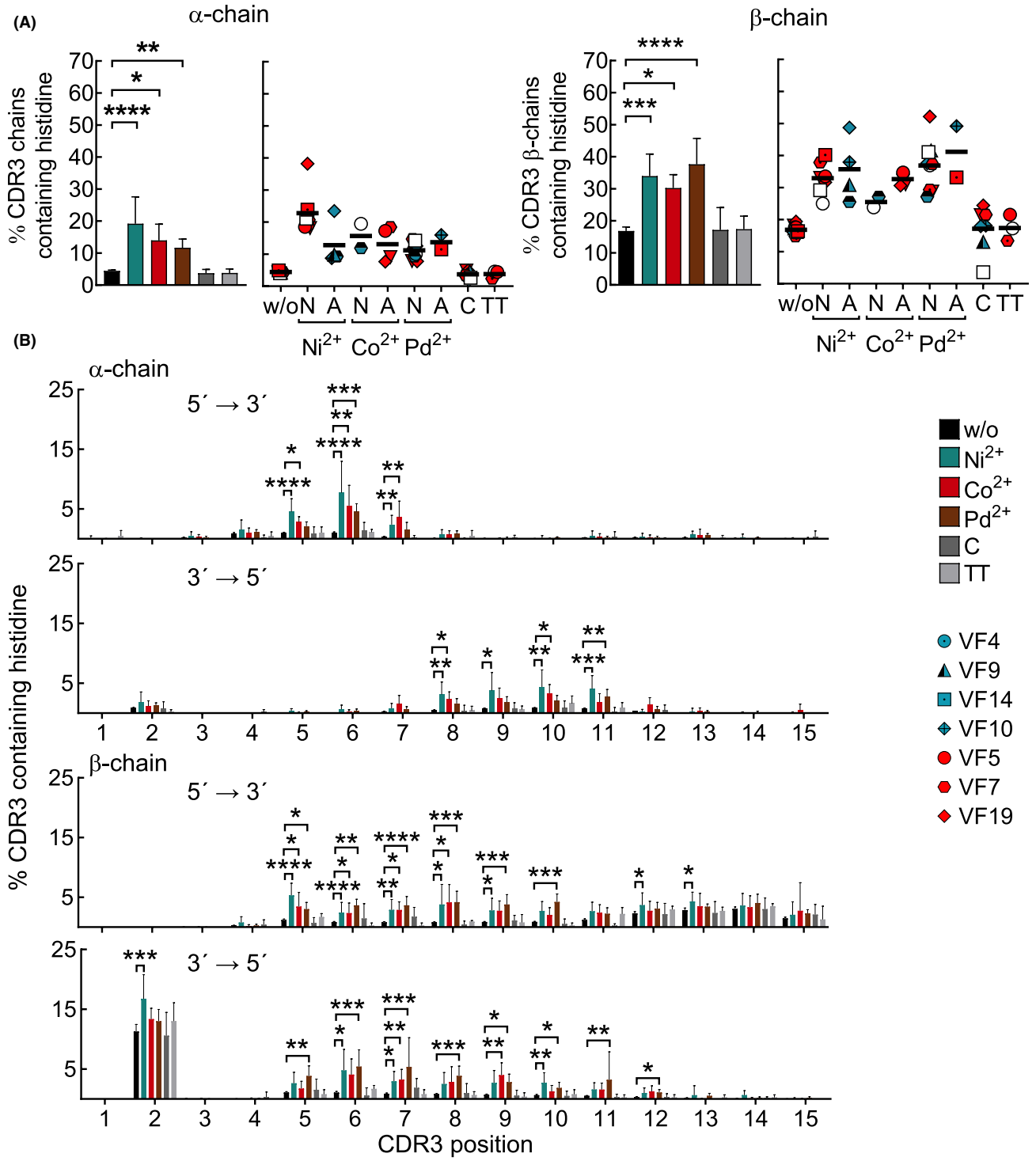


FIGURE 3 Metal-specific TCR commonly overrepresent a CDR3 histidine. Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells were sorted after 5 h of antigen stimulation and their TCR were sequenced. Random TCR were obtained from samples without antigen stimulation (w/o, black) and antigen-specific TCR after stimulation with 400 μM $NiSO_4$ (Ni^{2+}, blue), $CoCl_2$ (Co^{2+}, red) or $PdCl_2$ (Pd^{2+}, brown), CMV (C, dark gray) or TT (gray, Table S4). (A) Frequency of CDR3α and CDR3β with a histidine. The left graph shows mean values and SD of the percentages of TCR from the indicated repertories that express a histidine in their CDR3 α-chain (TCR diversity percentages). The right graph shows individual donor data for non-allergic (N) and allergic (A) individuals with horizontal lines indicating mean values (wo: $n = 12$; Ni^{2+}: $n = 7$ (N) and 4 (A); Co^{2+}: $n = 2$ (N) and 4 (A); Pd^{2+}: $n = 9$ (N) and 2 (A); C: $n = 7$; TT: $n = 3$). (B) CDR3 histidine position analysis. Shown are the frequencies of TCR with a CDR3 histidine at the indicated position. Due to different lengths of the CDR3, analysis was performed in 5′-3′ and 3′-5′ direction. We show positions 1–15, which cover most of the CDR3 (average length 12–15 amino acids). The same donor data as in (A) were used. Each symbol represents one donor (Table S1). Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal–Wallis) with Dunn's post-hoc test (*$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$ vs. w/o)

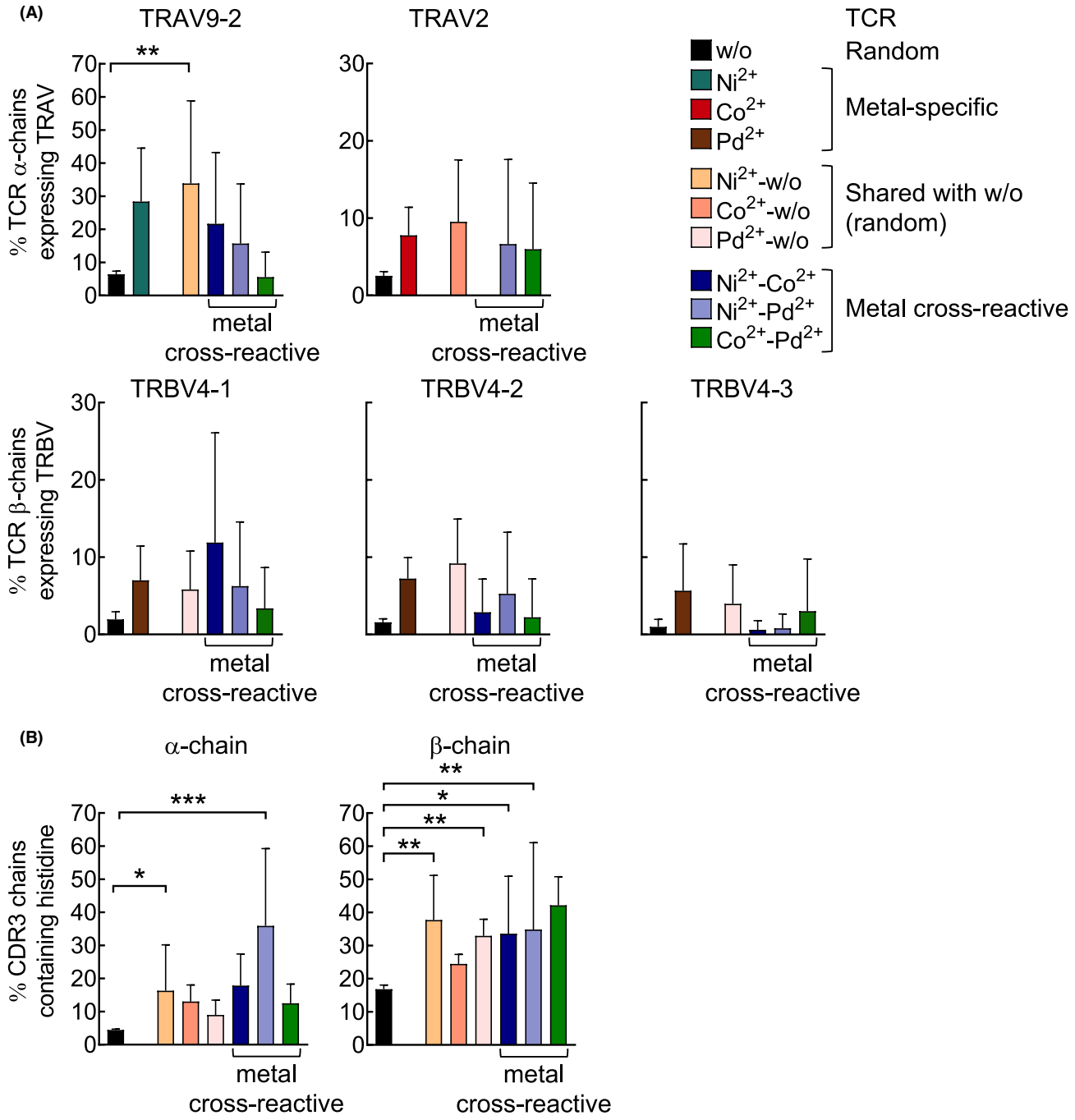


FIGURE 4 Repertoire features are conserved among cross-reactive metal-specific TCR. Cross-reactive TCR from different samples of the same donor were identified by overlap analysis (intraindividual analysis; Table S5). (A) Gene segment use. Depicted are the percentages of TCR expressing TRAV9-2, TRAV2, or one of the three segments of the TRBV4 family in the indicated (cross-reactive) repertoires (diversity percentages; w/o: $n = 12$; w/o - Ni^{2+}: $n = 11$; w/o - Co: $n = 6$; w/o - Pd: $n = 11$; Ni^{2+}-Co^{2}: $n = 4$–5; Ni^{2+}-Pd^{2+}: $n = 7$–8; Co^{2+}-Pd^{2+}: $n = 5$). (B) Frequencies of CDR3 with a histidine. Depicted are frequencies of CDR3 with a histidine in the indicated (cross-reactive) TCR repertoires (mean values and SD). The same donor data as in (A) were used. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal–Wallis) with Dunn's post-hoc test (*$p < .05$; **$p < .01$; ***$p < .001$ vs. w/o)

different analyzed metal-specific TCR, the respective TCR contain a cross-reactive subpopulation. TCR with a CDR3 histidine were similarly overrepresented among cross-reactive compared with total metal-specific TCR, also with respect to the individual CDR3 positions (Figure 4B; CDR3 position data not shown). Taken together, metal-specific TCR that express the identified repertoire features may contain a cross-reactive fraction that likely becomes activated by interchangeable metal ion binding.

4 | DISCUSSION

The present study illustrates that Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD4+ T cells can be detected by a CD154-based AIM assay. AIM assays have been employed in the characterization of protein-specific T cells but are not yet broadly established for chemical allergens.[16,28,57,58,59,60] The advantages of AIM assays include their speed, comprehensiveness, and sensitivity.[28,61-65] AIM assays are quantitative and do not require prior knowledge on the involved HLA allele, the presented antigen or self-peptide, and the chemical modifications at the TCR-self-peptide–MHC interface.

Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific CD154+CD4+ T cells were observed in PBMC from all analyzed individuals. As in other studies, frequencies of activated metal-specific T cells increased with the metal ion concentration until toxic effects became dominant. Short term assays generally enable the use of rather high concentrations compared with proliferation-based assays.[8,16,24,66-68] At all investigated metal salt concentrations, T cell activation was TCR-mediated as shown by co-expression of CD69 and the metal-specific restimulation of T cell clones. MHC II blocking antibodies inhibited CD154 upregulation, illustrating MHC restriction. Pd^{2+}-specific CD4+ T cells were more frequent than Ni^{2+}- and Co^{2+}-specific cells.

To date, optimal metal salt concentrations for the in vitro distinction of allergic and non-allergic individuals have not been established.[16,67,69] Relatively high metal salt concentrations likely capture the complete reactive T cell pool but may activate T cells without in vivo relevance. So far, lower metal ion concentrations have not been associated with improved nickel allergy detection.[8,23,24,67] Data on the in vitro detection of cobalt and palladium allergy are even more limited and inconclusive about optimal concentrations.[8,70-74] In the present study, allergic individuals did not exceed the background levels of metal-specific CD4+ T cell frequencies observed in non-allergic donors, rendering the identification of individual allergy-associated TCR unlikely. The frequencies of metal-specific T cells were considerably higher than those for the control antigen CMV- and TT-specific CD4+ memory T cells. Antigen-specific human memory T cells usually comprise <0.05% of the total T cell pool, as shown for example for food allergens,[75] tetanus toxoid and measles virus.[76] Even effector immune responses involve less than 5% of antigen-specific T cells.[28] We observed average frequencies of 3% Pd^{2+}-specific T cells in non-allergic individuals. Therefore, the inclusion of higher donor numbers in the study would not improve the distinction of allergic and non-allergic donors under the given conditions.

Increased percentages of metal-specific CLA+ T cells nevertheless indicate skin homing in some individuals.[16,44-48,77] The metal ion concentrations in situ remain unknown but may be high in injured skin or within the small volumes of tissue-draining lymphatic vessels. High metal ion concentrations could activate pre-existing memory T cells with potent effector functions, driving sensitization.[78,79] Cross-reactive memory B cells are efficiently recruited into de novo immune responses in humans, as shown by the fast emergence of highly hypermutated antibodies.[80] However, TCR lack somatic hypermutation and thus the involvement of pre-existing cross-reactive memory T cells in metal allergies cannot be shown in a similar manner. The influence of local metal ion concentrations in the skin may be illustrated by the improved detection of palladium allergy using water-soluble 3% Na_2PdCl_4 instead of the standard 1% $PdCl_2$ preparation.[81,82]

The frequent T cell activation by Ni^{2+}, Co^{2+}, and Pd^{2+} ions prompted a search for common TCR repertoire characteristics that provide insights into the mechanisms of antigen recognition. Due to technical challenges, previous studies focused on the TCR β-chain,[37,66,83,84] but the analysis of the α-chain proved essential. On average, about one third of Ni^{2+}-specific CD4+ T cells expressed the gene segment TRAV9-2, confirming previous results.[16] A mutation study on a single Ni^{2+}-specific TRAV9-2+ TCR previously had demonstrated Ni^{2+} complexation via the conserved $tyrosine_{36}$ in the CDR1 and $histidine_{81}$ in the MHC II β-chain.[13] Our study shows that Co^{2+}- and Pd^{2+}-specific T cells overrepresent different segments, that is, TRAV2 and the TRBV4 segment family, respectively, indicating unique metal ion binding characteristics.

A link between T cell activation and TCR V-gene segments has been described for superantigens that act independently from the antigen peptide, a recent example being the SARS-CoV-2 spike protein.[43,85] For metal-specific CD4+ T cells, the role of the MHC-presented peptide varies. Some metal-specific TCR respond to allogeneic or, as shown in this study, autologous T cell clones as untypical antigen-presenting cells, indicating some independence from the MHC-presented peptide.[13,16] However, other metal-specific T cell clones strongly depend on a certain antigen-presenting cell presenting distinct self-peptides, which argues against a conventional superantigen-like recognition.[29,86] Polar amino acids such as histidine, tyrosine, or glutamic acid have been discussed in Ni^{2+} complexation.[13,16,56] Further crystallization and mutation studies are needed to identify the exact binding sites for which representative TCR may be selected considering the features identified in this study.

Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific TCR showed a common overrepresentation of CDR3 containing a histidine which was an independent mechanism of antigen recognition. One histidine seems sufficient for metal ion recognition, as suggested before.[16,34] Since the amino acid composition of the CDR3α and CDR3β varies for individual positions, the analysis of control repertoires is essential to pinpoint changes associated with metal allergen recognition. Our study revealed that a CDR3 histidine among metal-specific TCR occurs at positions that favorably contain this amino acid in the random repertoire. This finding argues against a preferred metal ion binding site. The activation of TCR via their CDR3 had been hypothesized and modelled for organic chemical sensitizers and was recently demonstrated for 2,4,6-trinitrobenzenesulfonic acid (TNBS)-specific T cells.[60,87,88] The abundance of the identified TCR repertoire features varied among individuals but combined data argued for a possible involvement in allergic immune responses. However, the in vivo relevance of the identified metal-specific TCR requires further verification, for example, by showing a local enrichment in tissues affected by allergic reactions.

Complexation of metal ions by certain amino acid residues in the TCR and MHC molecules may exceed the activation threshold of suboptimal TCR-self-peptide–MHC interaction. This is one explanation for the high frequencies of metal-specific T cells in non-allergic individuals. In particular, the high frequencies of Pd^{2+}-specific T cells can be attributed to the fact that Pd^{2+} is bound by the TCR more frequently than Ni^{2+} at the aforementioned binding sites (gene segments and CDR3 histidines). For example, one in seven TRBV4-expressing TCR binds to Pd^{2+}, whereas only one in 80 TRAV9-2+ TCR binds to Ni^{2+}. The spatial conformation of amino acid residues in the TRBV4 gene segments may fit better for Pd^{2+} complexation than those in the TRAV9-2 gene segment for Ni^{2+}.

The common overrepresentation of TCR with histidine-containing CDR3 and the activation of T cell clones by different metal allergens suggest the existence of profound metal-specific TCR cross-reactivity.[29,30,66] Cross-reactivity can hardly be assessed by patch testing due to differences in metal salt skin migration, an unknown history of metal allergen-specific immune responses and varying local density of tissue-resident memory T cells. Thus, metal allergen co-sensitization frequencies vary a lot in the literature, for example, for nickel-cobalt co-sensitization between 2% and 72%.[9,89,90] However, TCR HTS opens new avenues for cross-reactivity analysis. While sampling effects reduce the possibility to detect cross-reactive TCR, a lower TCR diversity and larger clonotype expansion in the memory compared with the naive T cell compartment facilitates their detection. We identified cross-reactive Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific TCR that preserved the repertoire features of the individual metal-specific TCR indicating interchangeable metal ion binding. Cross-reactivity was more pronounced within metal-specific TCR compared with protein antigen-specific TCR. Further studies are required to analyze the cross-reactivity more comprehensively and to cover additional metal allergens at different metal ion concentrations. These studies optimally need to include a representative number of clonotypes from several individuals, given the inter-individual variations observed.

Future AIM assays may include additional metal allergens and T cell populations.[2,62,64,91,92] Since high background frequencies of metal-specific T cells impede in vitro allergy diagnosis, smaller allergy-associated T cell subpopulations need to be identified. One possibility is an immunome-based approach that utilizes a few distinctly expanded metal-specific public TCR clonotypes, as accomplished for CMV and SARS-CoV-2 infection diagnosis.[93,94] In a regulatory setting, T cell assays and the experimental assessment of TCR cross-reactivity are still largely unexplored but hold a great potential for an improved risk assessment.

AUTHOR CONTRIBUTIONS

KS conceived the study, supervised the project, the experimental work, and data analysis. FR and KS planned the experiments. WKP selected patients, provided patient samples and clinical data. FR, MAS, LM, and KS performed cell culture and flow cytometry experiments. FR, MAS, LM, and CAC analyzed the data. FR performed TCR sequencing experiments and analyzed the data. FR and AA programmed CDR3 amino acid positional exact analysis and analysis of shared TCR clonotypes. FR, MAS, CAC, and KS interpreted the data, prepared tables and figures, and wrote the manuscript. WKP, AL, HJT, LM, and AA corrected the manuscript. All authors provided discussion and agreed to the submitted version of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Dr. Sven Knueppel and Narges Ghoreishi for their support in statistical analysis, Melanie Leddermann for the excellent technical assistance, and PD Dr. Burkhard Malorny and Dr. Maria Borowiak for the support with Illumina sequencing. Azize Degirmenci-Tasasiz, Manon Grondkowski, Daniela Schaller, and Sandra Sekuterski (Department of Dermatology and Phlebology, Vivantes Klinikum im Friedrichshain) are acknowledged for support with patient recruitment, patch testing and collection of blood samples. This work was supported by BfR internal grants SFP 1322-718, 1322 -719, and Deutsche Forschungsgemeinschaft (DFG) project number 500312706. The authors declare no competing financial interests.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Riedel F, Aparicio-Soto M, Curato C, et al. Unique and common TCR repertoire features of Ni^{2+}-, Co^{2+}-, and Pd^{2+}-specific human CD154 + CD4+ T cells. *Allergy*. 2022;00:1-13. doi: 10.1111/all.15494

2.3 Research paper: Frequencies and TCR repertoires of human 2,4,6-trinitrobenzenesulfonic acid-specific T cells

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Published online on 22 February 2022 in:

Frontiers in Toxicology. 4:827109

DOI: 10.3389/ftox.2022.827109

Link: https://doi.org/10.3389/ftox.2022.827109

Involvement of the author within this publication: Project execution, data analysis.

Author contributions as published: KS conceived the study and supervised the project, the experimental work and data analysis. KS, MA-S, and CC planned the experiments. CC, MA-S, IW and AB performed cell culture and flow cytometry experiments and analysed flow cytometry data. CC and FR performed TCR sequencing and analysis. AA contributed to data sequencing analysis. MA-S, CC, and KS interpreted data and wrote the main manuscript. HJ-T and AL revised the manuscript. All authors provided discussion and agreed to the final version of the manuscript.

Online Supplementary Material is presented in Annex III.

frontiers in Toxicology

ORIGINAL RESEARCH
published: 22 February 2022
doi: 10.3389/ftox.2022.827109

Frequencies and TCR Repertoires of Human 2,4,6-Trinitrobenzenesulfonic Acid-specific T Cells

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OPEN ACCESS

Edited by:
Stefan F. Martin, University of Freiburg Medical Center, Germany

Reviewed by:
Philipp R. Esser, Medical Center-University of Freiburg, Germany
Govindarajan Thangavelu, University of Minnesota Twin Cities, United States
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Specialty section:
This article was submitted to Immunotoxicology, a section of the journal Frontiers in Toxicology

Received: *01 December 2021*
Accepted: *20 January 2022*
Published: *22 February 2022*

Citation:
Curato C, Aparicio-Soto M, Riedel F, Wehl I, Basaran A, Abbas A, Thierse H-J, Luch A and Siewert K (2022) Frequencies and TCR Repertoires of Human 2,4,6-Trinitrobenzenesulfonic Acid-specific T Cells. Front. Toxicology 4:827109. doi: 10.3389/ftox.2022.827109

Allergic contact dermatitis is a widespread T cell-mediated inflammatory skin disease, but *in vitro* monitoring of chemical-specific T cells remains challenging. We here introduce short-term CD154/CD137 upregulation to monitor human T cell responses to the experimental sensitizer 2,4,6-trinitrobenzenesulfonic acid (TNBS). Peripheral blood mononuclear cells (PBMC) from healthy donor buffy coats were TNBS-modified and incubated with unmodified PBMC. After 5 and 16 h, we detected TNBS-specific activated CD154+CD4+ and CD137+CD8+ T cells by multi-parameter flow cytometry, respectively. Activated cells were sorted for restimulation and bulk T cell receptor (TCR) high-throughput sequencing (HTS). Stimulation with TNBS-modified cells (3 mM) induced CD154 expression on 0.04% of CD4+ and CD137 expression on 0.60% of CD8+ memory T cells, respectively (means, n = 11–17 donors). CD69 co-expression argued for TCR-mediated activation, which was further supported by TNBS-specific restimulation of 10/13 CD154+CD4+ and 11/15 CD137+CD8+ T cell clones and lines. Major histocompatibility complex (MHC) blocking antibodies prevented activation, illustrating MHC restriction. The high frequencies of TNBS-specific T cells were associated with distinct common changes in the TCR β-chain repertoire. We observed an overrepresentation of tryptophan and lysine in the complementarity determining regions 3 (CDR3) (n = 3–5 donors), indicating a preferential interaction of these amino acids with the TNBS-induced epitopes. In summary, the detection of TNBS-specific T cells by CD154/CD137 upregulation is a fast, comprehensive and quantitative method. Combined with TCR HTS, the mechanisms of chemical allergen recognition that underlie unusually frequent T cell activation can be assessed. In the future, this approach may be adapted to detect T cells activated by additional chemical sensitizers.

Keywords: activation-induced marker assay, allergic contact dermatitis, CDR3 amino acids, chemical sensitizer, high-throughput sequencing, t cell, T cell receptor, trinitrobenzene sulfonate

Abbreviations: AIM, activation-induced marker; APC, antigen-presenting cells; CDR, complementarity determining region; CFSE, carboxyfluorescein diacetate succinimidyl ester; HTS, high-throughput sequencing; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PMA/iono, phorbol myristate acetate/ionomycin; TCR, T cell receptors; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl.

INTRODUCTION

Chemical allergens bind to self-proteins to produce immunogenic epitopes recognized by T cell receptors (TCR). In the presence of appropriate stimulatory signals for the innate immune system (Martin and Esser, 2022), chemical-specific T cell activation leads to adaptive immune responses. Once sensitized, clinical symptoms, such as allergic contact dermatitis (ACD) occur, even after otherwise relatively harmless chemical exposures (Peiser et al., 2012; Esser and Martin, 2017; Martin et al., 2018). To date, only a few chemical-induced T cell epitopes, mainly for model proteins or peptides, have been elucidated (Martin et al., 2004; Thierse et al., 2005; Meng et al., 2018; Pichler, 2019; Riedel et al., 2021). Experimental hardships linked to inefficient epitope generation and the rarity of antigen-specific T cells have delayed the development of T cell-based assays (Martin et al., 2010; Ogese et al., 2020; Hammond et al., 2021; Riedel et al., 2021). However, alternative *in vitro* tests that include T cells are urgently needed for improved diagnosis and predictive purposes by worldwide regulatory authorities (Corsini et al., 2018).

One of the best-researched chemical model allergens is 2,4,6,-trinitrobenzenesulfonic acid (TNBS). TNBS and its lipophilic form, trinitrochlorobenzene (TNCB), promote T cell activation *in vitro* and *in vivo*, respectively (Gerberick et al., 1992; Moulon et al., 1993; Martin et al., 2000; Dietz et al., 2010; Richter et al., 2013). TNCB provides sufficient irritant signals by itself to sensitize germ-free mice (Martin et al., 2008) and has been used in murine colitis models (Antoniou et al., 2016). *In vitro*, TNBS binds covalently to free amino groups at a wide range of pH values by nucleophilic aromatic substitution (Habeeb, 1966; Freedman and Radda, 1968; Sarantonis et al., 1986). Thus, mainly antigenic trinitrophenyl (TNP) determinants are generated on accessible lysine residues of proteins or on their free N-terminal amino groups (Gevaert et al., 2003). The TNBS adductome, i.e., *in vivo* protein target sites for TNBS, remains unknown as for most chemical allergens (Ndreu et al., 2020).

Since the 1970ies, TNBS-modified cells, proteins or peptides have been employed to activate murine and human T cells. CD4+ and CD8+ T cells were shown to recognize TNP-modified peptides presented by proteins of the major histocompatibility complex (MHC) I and II, respectively, but not by TNP-modified MHC proteins themselves (von Bonin et al., 1992; Kohler et al., 1995). In H-2k^b C57BL/6 mice, CD8+ T cells mainly interact with TNP moieties at peptide position P4 independent from the amino acid sequence of the carrier peptide. A second set of TCR binds TNP-modified lysine at position P7 in combination with additional unmodified amino acids at positions P3 and P4 (Martin et al., 1993; Martin et al., 2003). TNBS-induced epitopes seem to activate an unusually large fraction of murine T cells (Hamann et al., 1983; Iglesias et al., 1992; Kohler et al., 1995; Martin et al., 2003). Recently, T cell priming assays confirmed the presence of TNBS-specific CD4+ and CD8+ naive T cells in humans, while exact frequencies remain elusive (Dietz et al., 2010; Richter et al., 2013).

High frequencies of murine TNBS-specific T cells and the analysis of TNBS-specific T cell clones led to speculations on a preferential interaction of TNBS-induced T cell epitopes with common TCR elements. The extensive diversity of TCR ($\geq 100 \times 10^6$TCR per individual) (Robins et al., 2009) is generated through V(D)J gene recombination and junctional random nucleotide insertions and deletions that yield to the complementarity determining region (CDR) 3. The CDR3 is mainly responsible for protein antigen binding (Rudolph et al., 2006). Possible interactions with TNBS include binding to certain TCR gene segments or to amino acids in the CDR3 (Hochgeschwender et al., 1986; Hochgeschwender et al., 1987; Kempkes et al., 1991; Martin et al., 1995). So far, bulk T cell analysis did not confirm a bias in TCR β-chain segment use for murine TNBS-specific CD8+ T cells (Martin et al., 2003). Technological advances over the last decades now enable the exploration of TCR repertoires by high-throughput sequencing (HTS) (Britanova et al., 2014; Aparicio-Soto et al., 2020; Barennes et al., 2021).

We here introduce a new short-term activation-induced marker (AIM) assay based on CD154 (CD40L) and CD137 (4-1BB) upregulation to quantify TNBS-specific CD4+ and CD8+ T cells. Both activation markers have been used for the detection of protein-specific T cells (Frentsch et al., 2005; Wolfl et al., 2007; Wehler et al., 2008; Bacher et al., 2013; Reiss et al., 2017; Elias et al., 2020; Saggau et al., 2021). TNBS-specific T cells were isolated for *in vitro* restimulation and TCR HTS. This proof-of-principle study elucidates frequencies and TCR repertoires of human TNBS-specific CD4+ and CD8+ T cells.

MATERIALS AND METHODS

Blood Samples and Isolation of Peripheral Blood Mononuclear Cells

Since TNBS is an experimental allergen, patient samples are not available. We used buffy coats from healthy, most likely non-allergic, donors (German Red Cross, **Supplementary Table S1**). Buffy coats (~80 ml) were diluted with an equal volume of MACS buffer (Miltenyi) and peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient centrifugation with Ficoll Paque Plus (GE Healthcare), as described (Aparicio-Soto et al., 2020). Cells were cultured in complete RPMI 1640-based T cell media (TCM, see **Supplementary Methods**). Cell numbers were determined with a CASY Cell Counter (OMNI Life Science). Experiments were conducted according to the current version of the declaration of Helsinki (Charité's ethics committee, vote EA1_217_19).

Antigen Presenting Cell Preparation

PBMC were labeled with carboxyfluorescein succinimidyl ester (CFSE) (0.5 μM in PBS, CellTrace™ CFSE, Thermofisher) for 15 min. After CFSE labeling, PBMC were incubated with phosphate buffered saline (PBS, control) or TNBS (0.05–25 mM in PBS) for 10 min at 37°C and washed extensively, as described to yield modified PBMC (Shearer et al., 1974; Richter et al., 2013).

T Cell Antigen Stimulation Assay

Modified PBMC and unmodified "responder" PBMC were cultured in a 1:1 ratio at a density of 2.5×10^6 cells/cm^2 in flat bottom tissue culture plates (TPP) at 37°C in a 5% CO^2 >95% humidified atmosphere. To prevent ligand-induced downregulation of CD154, CD40 blocking antibody was added (Yellin et al., 1994; Frentsch et al., 2005). In some experiments, staphylococcal enterotoxin B (SEB) (1 µg/ml) or phorbol myristate acetate-Ionomycin (PMA-I) (10 ng/ml and 1 µg/ml, all Sigma-Aldrich) were used as positive controls for T cell activation. For MHC blocking experiments, antibodies were added 30 min prior to antigen stimulation. T cell assays were performed for 5 and/or 16 h.

Antibody Staining and Flow Cytometry Analysis

Cells were stained for 20 min at room temperature with different combinations of fluorochrome-conjugated antibodies and dead cell stain (see **Supplementary Methods**). Analysis and sorting were performed on a BD FACSAria III with BD Diva7.0 Software (BD). Data were further analyzed in FlowJo (V.10.7.1, BD Biosciences).

Cell Sorting, *in vitro* Expansion and T Cell Restimulation

TNBS-specific CD154+CD4+ and CD137+CD8+ memory T cells (single cells and lines) were sorted in single cell mode into 200 µL TCM supplemented with 20% human AB serum, 300 U/ml interleukin (IL)-2 (IS grade), 30 ng/ml anti-CD3 (both Miltenyi Biotec) and ~200.000 allogeneic mitomycin C-treated PBMC as feeder cells on 96-well flat-bottom plates. After one week, half of the media was replaced with fresh TCM supplemented with 15% human AB serum and 200 U/ml IL-2. Cells were further expanded on 48-well plates pre-coated with anti-CD3 antibody (OKT-3) (1 µg/ml in PBS for 1 h at 37°C) and, if required for CD4 clones, 10.000 CD3/CD28 coated beads per well (Dynabeads Human T-Activator, Thermo Fisher Scientific). Before restimulation, clones rested for ~3 days in TCM supplemented with 10% human AB serum (without IL-2 and/or CD3/CD28 beads). Restimulation was performed with cryopreserved, thawed and overnight rested autologous CD3-depleted PBMC (human CD3 MicroBeads and LD columns, Miltenyi Biotech) modified with TNBS (3 mM) or PBS (control) in 384-well plates. Conditions were the same as used for bulk culture stimulation assays, except for the additional administration of co-stimulatory CD28 antibody (1 µg/ml).

T Cell Receptor High-Throughput Sequencing and Data Analysis

TNBS-specific CD154+CD4+ memory T cells and CD137+CD8+ memory T cells were sorted in purity mode directly into 1 ml buffer RLT (Qiagen) and stored at −80°C. RNA was extracted with RNeasy Micro Kit (Qiagen). Reverse transcription with the introduction of a unique molecular identifier (UMI)-containing SMART adapter, PCR amplification, library preparation and Illumina sequencing for TCR αβ chains were performed as described (Aparicio-Soto et al., 2020). PCR products were purified with QIAquick PCR Purification Kit (Qiagen), pooled, size selected to ~300–600 bp with beads (0.65 ratio, CleanPCRNA kit, GC Biotech), and sequencing adapters were annealed using TruSeq DNA PCR-Free Low Throughput Library Prep Kit (Illumina). Libraries were quantified using a Qubit device (Qubit™ dsDNA HS Assay Kit, Thermo Fisher Scientific). Sequencing was done with MiSeq Reagent Kit v3 (2 × 250 bp paired-end sequencing, Illumina). Raw data were demultiplexed and error corrected with MIGEC (v1.2.9; over sequencing threshold ≥4 reads per UMI) (Shugay et al., 2014). TCR were extracted with MIXCR (v3.0.13; library repseqio. v1.6) (Bolotin et al., 2015) and sequences with identical V-, (D-), and J-gene segments as well as CDR3 nucleotide sequence were considered as one TCR clonotype. Further analysis was performed with VDJtools v1.2.1 (e.g., removal of non-functional clonotypes) (Shugay et al., 2015) and own custom Python software (available upon reasonable request). GraphPad Prism 8 was used for visualization. International ImMunoGeneTics information system nomenclature (IMGT) is used throughout the manuscript.

Quantification and Statistical Analysis

Statistical tests and n values are specified in the respective figure legends. A p value < 0.05 was considered statistically significant. For flow cytometry data, statistical analysis was only performed with populations containing at least 20 cells.

Supplementary Methods and Data Availability

Further experimental details can be found in the Supplementary Material, **Supplementary Methods**. TCR sequencing data are available at European Nucleotide Archive (ENA) under study number PRJEB49381 (https://www.ebi.ac.uk/ena/data/view/PRJEB49381).

RESULTS

Trinitrobenzene Sulfonate Efficiently Modifies Peripheral Blood Mononuclear Cells

TNBS produces antigenic TNP-determinants mainly by binding to free ε-amino groups of lysine in peptides presented by MHC proteins on the surface of antigen presenting cells (APC, **Figure 1A**) (Weltzien et al., 1996). As APC, we used autologous PBMC. We monitored extracellular TNBS surface modification by anti-TNP staining. TNBS modification was reproducible for different TNBS lots (**Supplementary Figure S1A**). Compared to T cells, monocytes and B cells showed an increased shift in the mean fluorescence intensity. This indicates the modification of a larger number of cell surface proteins, which may reflect the generation of more T cell epitopes

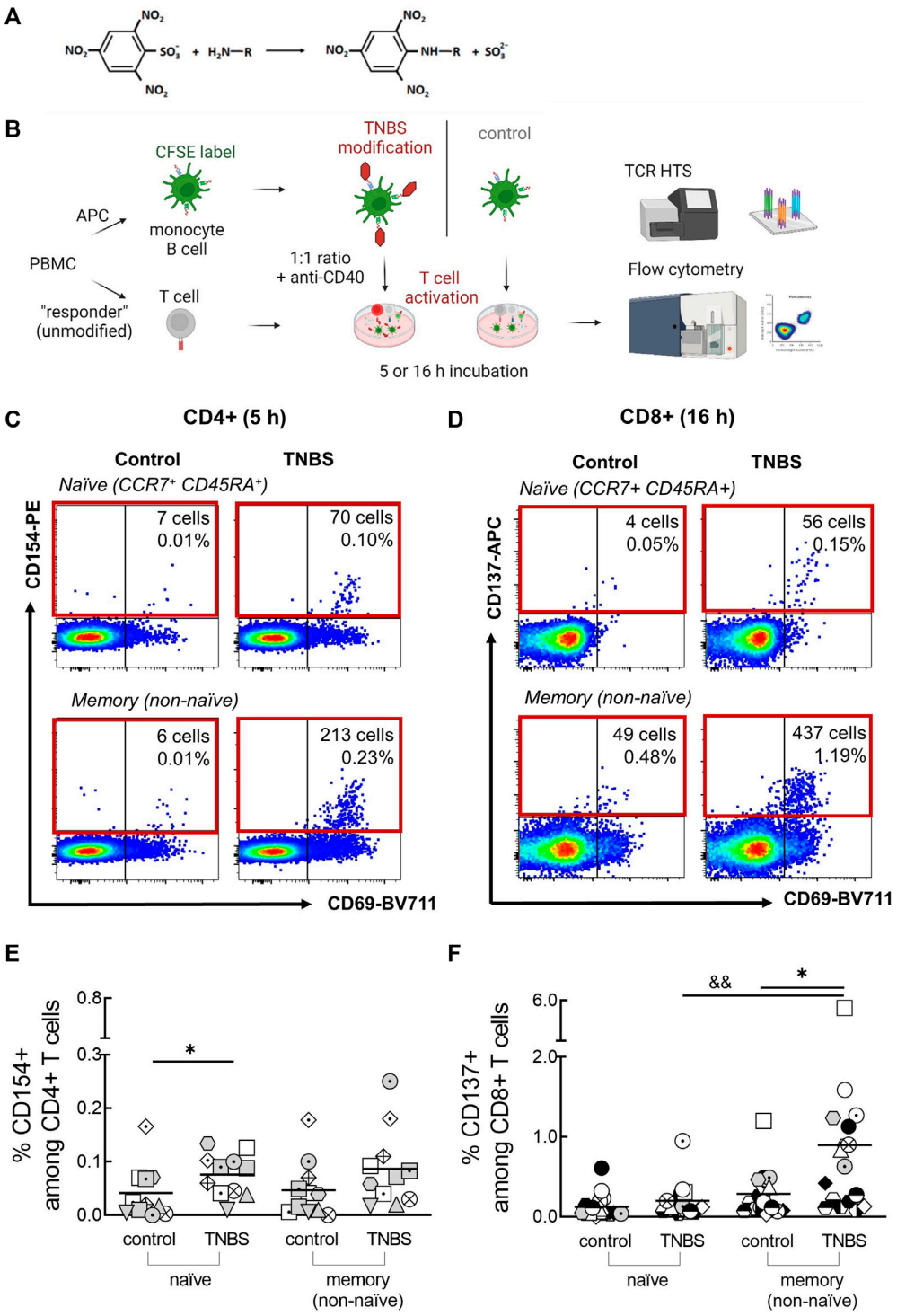

FIGURE 1 | Detection of TNBS-specific T cells by CD154/CD137 upregulation. **(A)** TNBS reaction scheme. TNBS mainly binds to primary amino groups. **(B)** Assay setup. PBMC were labeled with CFSE, modified with PBS or TNBS and mixed as APC with unmodified autologous "responder" PBMC. CD4+ and CD8+ T cell activation was detected by flow cytometry after 5 and 16 h, respectively, and activated cells were sorted for TCR HTS. Created with BioRender.com. **(C,D)** Representative dot plots showing numbers and frequencies of CD154+CD4+ [**(C)**, MLB20] and CD137+CD8+ [**(D)**, MLB31] naive and memory T cells after incubation with PBS- (control) or TNBS-modified APC. **(E,F)** Summarized frequencies of TNBS-specific CD4+ **(E)**, $n = 11$) and CD8+ T cells [**(F)**, $n = 17$]. Each symbol represents one donor (buffy coats, **Supplementary Table S1**). The gating strategy can be found in **Supplementary Figure S4**. Horizontal lines indicate the mean values. Statistical significances were determined by non-parametric Mann-Whitney *t*-test (*$p < 0.05$ vs. control; $^{\&\&}p < 0.01$ vs. naïve TNBS).

(**Supplementary Figure S1B**). TNBS concentrations ranging between 0.8 and 3.1 mM resulted in the brightest anti-TNP stain while input cell numbers were preserved.

Trinitrobenzene Sulfonate-Modified Antigen Presenting Cells are Non-toxic in CD154/CD137 Upregulation Assays

To exclude toxic effects of TNBS on PBMC in CD154/CD137 upregulation assays, we monitored total live cell numbers and T cell activation capacity to the superantigen Staphylococcal enterotoxin B (SEB). SEB activates a large fraction of T cells independent of their antigen-specificity and thus it superimposes any TNBS-specific T cell activation. We tested a two-fold dilution series of TNBS concentrations (25–0.05 mM) for APC modification and analyzed both TNBS-labeled PBMC (APC) and unlabeled "responder" PBMC (**Figure 1B**).

Among TNBS-modified PBMC, toxic effects occurred after modification with 12.5 mM or higher TNBS concentrations. This was exemplified by a decline in total CD4+ and CD8+ memory T cell numbers and reduced CD154/CD137 upregulation capacity upon SEB stimulation after 5 or 16 h of antigen stimulation (**Supplementary Figures S2, S3**). Similar data were obtained for naive T cell, CD14+ monocytes and CD19+ B cells (data not shown). T cells from TNBS-modified PBMC can respond to SEB and remain functional upon modification with up to 6.3 mM TNBS. However, we did not further analyze activated T cells within the APC fraction.

T cells from unmodified "responder" PBMC did not show sign of toxicity upon incubation with TNBS-modified cells (**Supplementary Figures S2, S3**). SEB-induced T cell activation stayed constant even if APC numbers were drastically reduced after modification with 12.5 mM TNBS or 25 mM TNBS at the end of the incubation period. Thus, harsh conditions may ensure efficient *in vitro* T cell epitope formation in this experimental setup.

We chose 3 mM TNBS as standard concentration for APC preparation in CD154/CD137 upregulation assays. This concentration was the highest with bright anti-TNP stain and in the non-toxic range for TNBS-modified APC. In addition, most studies in the literature use 3 mM TNBS (Dietz et al., 2010; Richter et al., 2013).

Trinitrobenzene Sulfonate-specific Human T Cells are Frequent

The main aim of this study was to determine frequencies and TCR repertoires of human TNBS-specific CD4+ and CD8+ T cells using CD154 and CD137 upregulation, respectively. The experimental setup is illustrated in **Figure 1B**. PBMC were CFSE-labeled, TNBS or control (PBS)-modified and mixed as APC in a 1:1 ratio with non-modified "responder" PBMC in the presence of anti-CD40 antibody to prevent ligand-induced down-regulation of CD154 (Yellin et al., 1994; Frentsch et al., 2005). After 5 or 16 h of antigen stimulation, cells were stained and frequencies of CD154+CD4+ and CD137+CD8+ T cells were determined by multi-parameter flow cytometry. The incubation times were chosen according to the required times for maximum induced CD154 (~5 h) and CD137 expression (~16–24 h) (Frentsch et al., 2005; Wolfl et al., 2007; Wehler et al., 2008; Aparicio-Soto et al., 2020). The gating strategy to detect expression of both activation markers is depicted in **Supplementary Figure S4**.

Background expression of CD154 was negligible on control-stimulated CD4+ T cells (0.04% ± 0.05 and 0.05% ± 0.05%, naive and memory cells, respectively; means ± standard deviation, **Supplementary Table S1**). Incubation with TNBS-modified APC substantially increased frequencies to 0.08% ± 0.04% (naive) and 0.09% ± 0.07% (memory) CD154+CD4+ T cells (n = 11 buffy coats, **Figures 1C,E**). Signals were similar after 16 h of antigen stimulation (0.08% ± 0.1 and 0.13% ± 0.18%, **Supplementary Figure S5A**). A constant CD154 signal over this timeframe was expected from earlier studies (Frentsch et al., 2005; Aparicio-Soto et al., 2020). However, some buffy coats showed an increased signal in 16 h experiments compared to 5 h experiments, e.g., LMB1, MLB31. Increased 16 h signals were accompanied by high frequencies of TNBS-specific CD137+CD8+ T cells and may thus be cytokine-induced (**Supplementary Figure S5A**) (Skov et al., 2000). To avoid the analysis of activated bystander CD4+ T cells, we analyzed TCR repertoires and T cell clones from 5 h experiments. In summary, we observed 0.04% TNBS-specific CD154+CD4+ naive and memory T cells in human buffy coats.

Background expression of CD137 on CD8+ T cells was higher and more variable among different buffy coats compared to CD154 (**Figures 1D,F**; **Supplementary Table S1**). After 16 h of stimulation with PBS-modified APC (control), we observed 0.13% ± 0.15 and 0.29% ± 0.28% CD137+CD8+ naive and memory T cells, respectively. Stimulation with TNBS-modified APC resulted in 0.20% ± 0.21 and 0.90% ± 1.2% of CD137+CD8+ T cells (n = 17 buffy coats, **Figure 1E**). A small signal was already visible after 5 h, as expected from the slower expression kinetics of CD137 compared to CD154 (0.1% ± 0.1 and 0.4% ± 0.2%, n = 8 buffy coats, **Supplementary Figure S5B**). TNBS-induced CD137+CD8+ T cells were rarer among the naive compared to the memory compartment. In summary, we detected 0.6% TNBS-specific CD137+CD8+ memory T cells in human buffy coats.

CD69 is another activation marker that is upregulated by TCR engagement (Testi et al., 1989; Yamashita et al., 1993; Beeler et al., 2008). However, background expression of CD69 on control-stimulated T cells was very high (**Supplementary Figures S5C, D**). We only detected signals for TNBS-specific CD69+CD8+ memory T cells (**Supplementary Figures S5C, D**). In summary, CD69 was less suited as a specific activation marker compared to CD154 (for CD4+ T cells) and CD137 (for CD8+ T cells).

Early CD137 expression on CD4+ T cells can be used to identify antigen-specific regulatory T cells (Schoenbrunn et al., 2012; Saggau et al., 2021). However, we observed a rather high background expression of CD137 on CD4+ T cells and did not detect TNBS-specific regulatory T cells (**Supplementary Figure S5E**). Similarly, we did not detect TNBS-specific CD154+CD8+ T cells in most buffy coats (data not shown), since only a minority

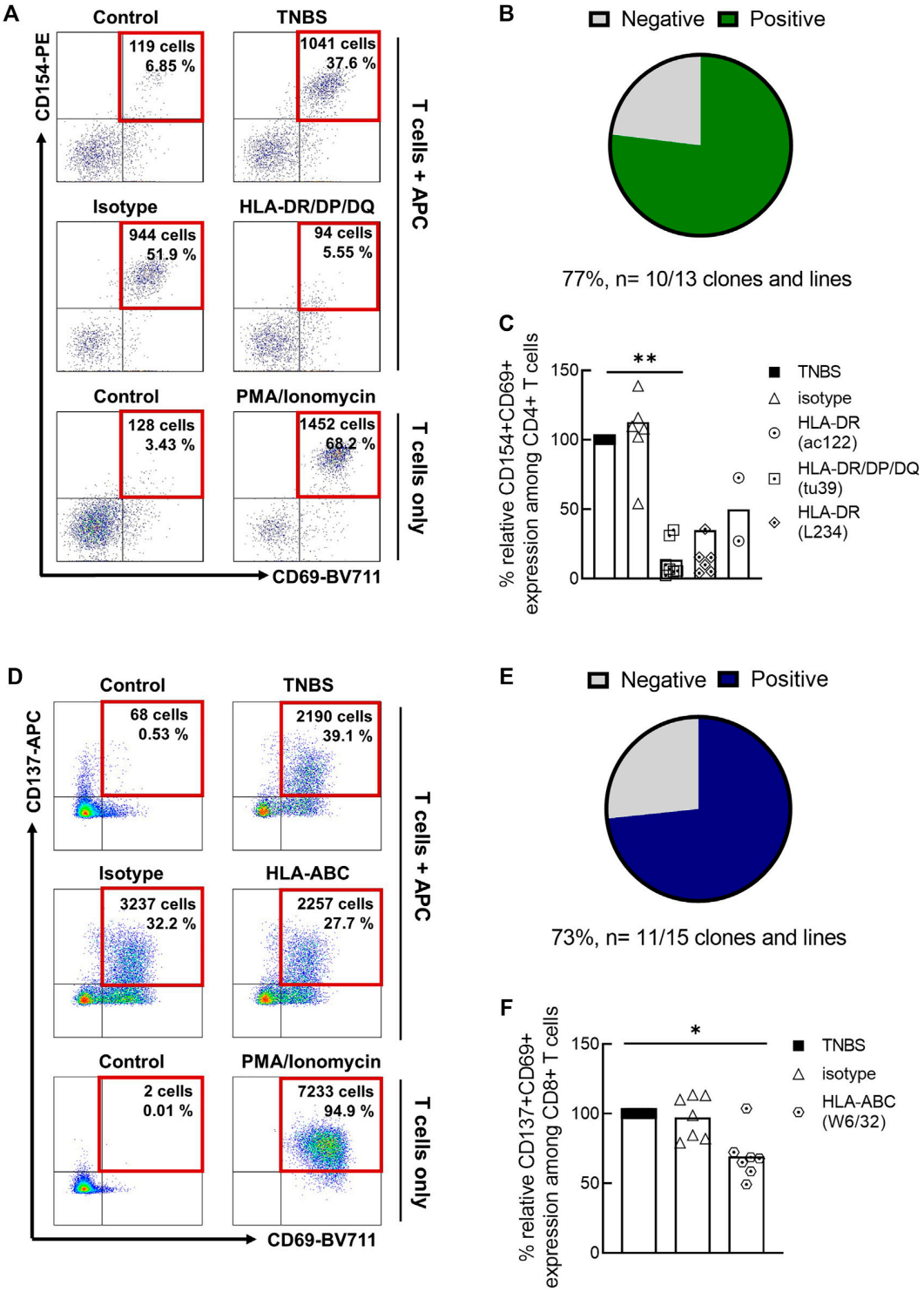

FIGURE 2 | Restimulation of TNBS-specific T cells. TNBS-specific CD154+CD4+ or CD137+CD8+ memory T cells were sorted from 5 to 16 h experiments, respectively. After *in vitro* expansion, cells were restimulated with TNBS-modified CD3-depleted autologous PBMC. **(A,D)** Representative dot plots for one CD4+ T cell clone [CACB3, **(A)**] and one CD8+ T cell clone [MLB42a, **(D)**]. As a negative control, T cells were incubated with PBS-modified APC (control). PMA/iono stimulation was used as a positive control for T cell reactivity. Gated on live, CD3+, single, CD4+ or CD8+ T cells. **(B,E)** Summary of restimulation experiments for TNBS-specific CD4+ **(B)** and CD8+ **(E)** T cell clones and lines (listed in **Supplementary Table S2**). **(C,F)** MHC blocking experiments. MHC II or MHC I blocking antibodies were added to APC 30 min before incubation with T cells. Shown is the relative inhibition of induced CD154 and CD137 expression on TNBS-specific CD4+ and CD8+ T cell clones and lines, respectively. Signals for stimulation with TNBS-modified APC without antibodies were set to 100%. White bars represent the mean values. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal-Wallis) with Dunn's test as post-hoc test (*$p < 0.05$; **$p < 0.01$ vs. TNBS).

of CD8+ T cells expresses this activation marker (Frentsch et al., 2013).

T Cell Receptor-Mediated CD154/CD137 Upregulation by Trinitrobenzene Sulfonate-specific T Cells

We confirmed TCR-mediated CD154/CD137 upregulation on TNBS-specific T cells by analyzing CD69 co-expression, restimulation of activated T cells and effects of MHC blocking antibodies.

High levels of CD69 co-expression were observed among TNBS-specific CD154+CD4+ and CD137+CD8+ naive and memory T cells (**Supplementary Figure S6A, B**; **Supplementary Table S1**). CD69 co-expression is a well-established indicator for TCR-mediated activation for protein and nickel-specific CD4+ T cells (Bacher et al., 2013; Aparicio-Soto et al., 2020). Among TNBS-specific CD8+ T cells, CD69 co-expression was less frequent (74 and 64%, means of naive and memory T cells, respectively, $n = 11$ buffy coats) compared to the co-expression in TNBS-specific CD4+ T cells (71 and 82%, $n = 6$). CD69 co-expression among control activated T cells likely indicates *ex vivo* activation or auto-reactivity and was not further considered. In summary, co-expression of the CD69 activation marker among the majority of CD154+ or CD137+ T cells indicates antigen-specificity and TCR-mediated TNBS recognition.

An independent method to investigate antigen-specificity is the sorting of TNBS-activated T cells, *in vitro* expansion and restimulation (**Figure 2**; **Supplementary Table S2**). As APC, we used autologous TNBS or control-modified CD3-depleted PBMC. Among TNBS-specific CD4+ T cell clones and lines, 10/13 (77%) responded to TNBS restimulation with CD154 upregulation (**Figures 2A,B**). The presence of different MHC II blocking antibody clones prevented T cell activation, further confirming antigen-specificity and conventional MHC II restriction for TNBS-specific CD4+ T cells (**Figure 2C**). Results were similar for TNBS-specific CD137+CD8+ memory T cell clones and lines. 11 out of 15 (73%) TNBS-specific clones or lines were activated by TNBS-modified APC (**Figures 2D,E**) and activation was reduced in the presence of an MHC I blocking but not isotype control antibody clones (**Figure 2F**).

Taken together, CD69 co-expression, restimulation of TNBS-specific T cell clones and lines and MHC block experiments confirm that a large fraction of TNBS-activated CD154+CD4+ and CD137+CD8+ T cells recognize TNBS-induced epitopes *via* their TCR.

Trinitrobenzene Sulfonate-specific CD4+ and CD8+ T Cell Receptor Repertoires Show Distinct Features

Although TNBS has been used extensively to study T cell activation, it remained unclear whether TNBS-induced epitopes preferentially interact with TCR gene segments or with specific amino acids in the CDR3. To address this research objective, we sorted ~450–2500 TNBS-specific CD154+CD4+ and CD137+CD8+ memory T cells from several buffy coats and analyzed their TCR by HTS. For comparison, we also analyzed randomly sorted CD4+ and CD8+ memory T cells. Using an RNA and UMI-based protocol, we obtained transcribed TCR α- and β-chain cDNA (counts, **Supplementary Table S3**) for each sample, distributed among different TCR clonotypes (diversity, **Supplementary Table S3**). Results on TCR repertoire analysis are shown for TCR clonotypes (diversity percentages) because there is no exposure to TNBS and clonotype expansions are not linked to a TNBS allergy. For some samples, relatively small repertories were analyzed and coincidental clonotype (count) frequencies of expanded TNBS-specific cross-reactive memory T cells could influence the results.

Gene segment use by random TCR α- and β-chains varied considerably among buffy coats (**Supplementary Figure S7**). We observed a relatively small common increase of the TRBV20-1 gene segment among TNBS-specific CD4+ T cells (8% of random compared to 13% of TNBS-specific TCR, adj. p-value 0.004; **Supplementary Figures S7B, D**). Among TNBS-specific CD8+ T cells, changes of similar magnitude were observed for several V-gene segments, e.g., TRAV26-1 and TRBV28 (adj. p-value n.s.; **Supplementary Figures S7G, H**). Changes in J-gene segment use were similar but not further considered. Antigen recognition is mainly mediated by the three CDR regions with the CDR1 and CDR2 occurring in the V-gene segments. In general, we found no hints that TNBS-induced T cell activation is based on the interaction with one major TCR gene segment.

Most interactions for conventional antigen recognition by TCR occur in the CDR3 region. To assess the possible involvement of certain amino acids in TNBS-induced epitope recognition, we analyzed the amino acid composition of TNBS-specific and random TCR (**Figure 3**). Inter-individual variations in the CDR3 amino acid composition were lower than in the gene segment use analysis. The amino acid composition differs for TCR α- and β-chains, but these differences were similar for CD4+ and CD8+ T cells (**Figures 3A–D**). For instance, isoleucine (I) and lysine (K) residues are more common among TCR α-chains (CD4: I 44% vs. 18% and K 60% vs. 11%, CD8: I 53% vs. 11%, K 60% vs. 16%, α- vs. β-chain, respectively) while histidine (H) occurs more frequently among TCR β-chains (CD4: 6 vs. 22%, CD8: 3% *vs.* 13%, α- vs. β-chain, respectively) (**Figures 3A,C**).

Comparing TNBS-specific and random TCR, nearly all CDR3 amino acids were similarly represented. Exceptions were an increased frequency of TCR β-chains with lysines (K) in TNBS-specific CD8+ T cells and tryptophans (W) in TNBS-specific CD4+ and CD8+ T cells (**Figures 3B,D–F**). Lysine overrepresentation was focused on central amino acids in the β-chain CDR3 and also occurred in the central amino acids among TNBS-specific CD4+ T cells (**Figure 3G**, **Supplementary Figure S8A**). The most pronounced changes in tryptophan use occurred among the central and N-terminal CDR3 amino acids (**Figure 3G**, **Supplementary Figure S8B**). We further observed a statistically significant increase in the use of asparagine (N), while arginine (R) and threonine (T) use decreased among the TCR chains of CD4+ T cells (**Figure 3A**). For asparagine (N), we could not observe any difference within the individual CDR3 amino

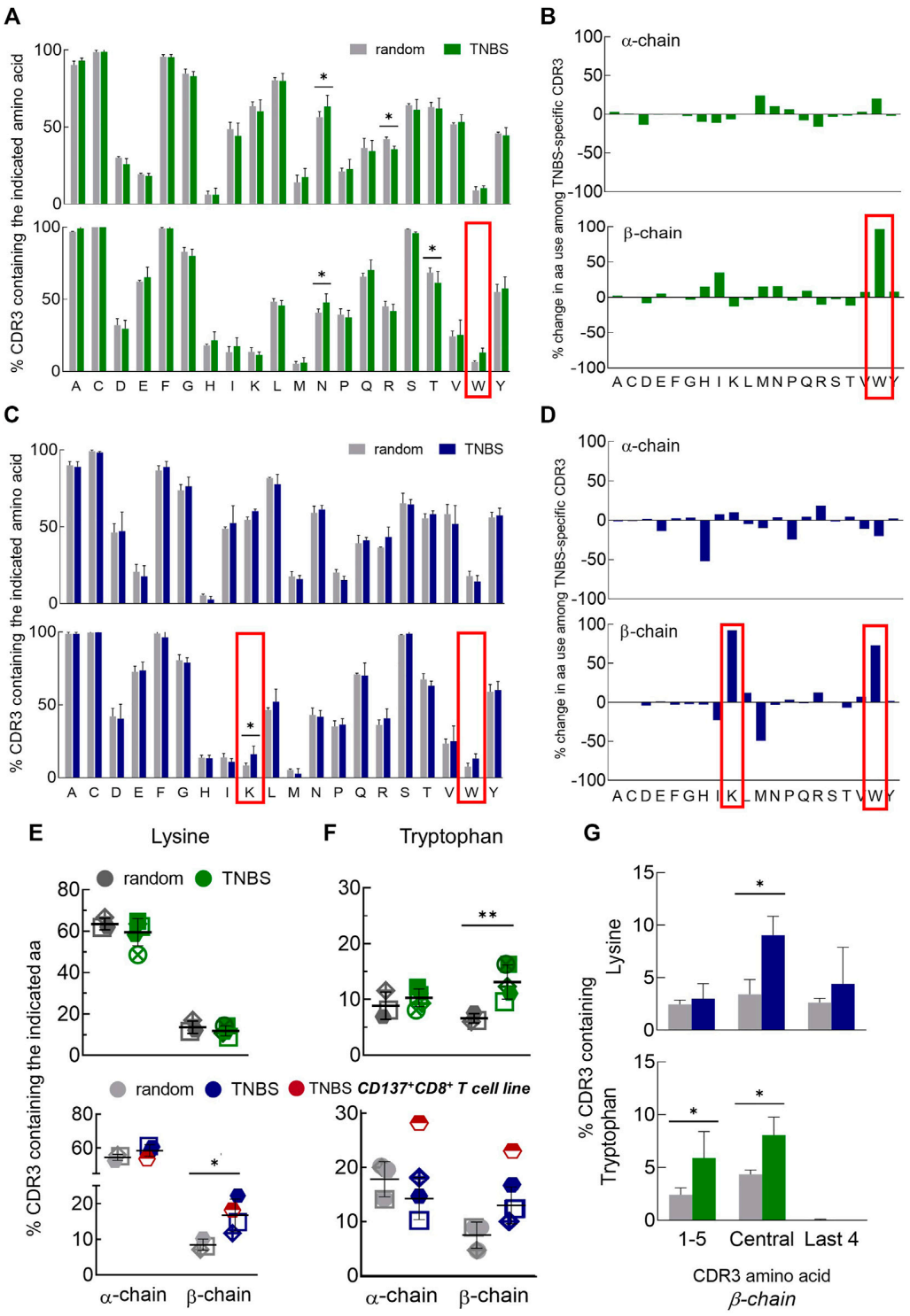

FIGURE 3 | Changes in CDR3 amino acid composition among TNBS-specific TCR. TNBS-specific CD154+CD4+ and CD137+CD8+ memory T cells were sorted from 5 to 16 h experiments, respectively, and their TCR were sequenced (**Supplementary Table S3**). Random CD4+ and CD8+ memory T cells from the same buffy coats served as control. **(A,C)** CDR3 amino acid compositions. The bar charts depict the occurrence of individual amino acids in the CDR3 of TCR α- (upper panel) and β-chains (lower panel) in single letter code for random (white) and TNBS-specific CD154+CD4+ [green, **(A)**] or CD137+CD8+ [blue, **(C)**] T cells, respectively (mean values, TCR diversity, $n = 3–5$). The first C and last F of the CDR3 have been included in the analysis. The red box indicates the most prominent changes. **(B,D)** Percent

(*Continued*)

FIGURE 3 | change in amino acid use. Plotted are relative changes in CDR3 amino acid use among TCR α- and β-chains of TNBS-specific compared to random TCR. Data are based on the mean values shown in **(A,C)**. **(E,F)** Individual donor values showing the overrepresentation of lysine **(E)** and tryptophan **(F)** among TNBS-specific TCR. The red symbol represents data from a TNBS-specific CD137+CD8+ memory T cell line (MLB42a_CD8_1C-50, not included in the statistics). **(G)** Positional CDR3 amino acid analysis. The graphs depict lysine (upper panel) and tryptophan (lower panel) occurrence according to their locations in the β-chain of TNBS-specific TCR (see also **Supplementary Figure S8**). Statistical significance was determined by multiple *t*-test **(A,C,G)** or two-way ANOVA **(E,F)**. Multiple comparisons were corrected according to the Holm-Sidak's method **(E-G)** (n = 3–5, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. random).

acid positions probably due to the prominent expression by random TCR (data not shown). We performed further analysis of the exact amino acid position on the CDR3 of TCR α- and β-chains (**Supplementary Figures S9**). This analysis illustrates the occurrence of conserved flanking amino acids at the beginning of the CDR3 (in 5′-3′ direction, **Supplementary Figures S9A, B**). We found that lysine in position 7 of the TCR β-chain CDR3 may be favorable for TNBS-induced epitope recognition (**Supplementary Figure S9C**). Tryptophan locates closer to the N-terminus of the TCR β-chains. Position 3 for CD4+ T cells and position 5 for CD8+T cells may be favorable for TNBS-induced epitope recognition (**Supplementary Figure S9D**). TCR α-chains were in general more reluctant at changes in the composition and location of CDR3 amino acids (**Figure 3**, **Supplementary Figures S8, S9**).

In summary, these data confirmed that although no major TCR gene segments are involved in the recognition of TNBS, the high frequencies of TNBS-specific T cells are associated with distinct common changes in the CDR3 amino acid composition mainly in the TCR β-chain repertoire.

DISCUSSION

The *in vitro* monitoring of chemical-specific T cells remains challenging. We here introduce short-term CD154/CD137 upregulation for the detection of human TNBS-specific CD4+ and CD8+ naive and memory T cells. We combine a well-established technique for the generation of TNBS-induced T cell epitopes with recently developed AIM assays for the detection of protein antigen and nickel-specific T cells (Shearer et al., 1974; Frentsch et al., 2005; Wolfl et al., 2007; Wehler et al., 2008; Bacher et al., 2013; Richter et al., 2013; Aparicio-Soto et al., 2020).

Due to its reactivity, TNBS covalently binds to free amino groups within 10 min, including ε-amino groups of lysine-containing peptides presented by MHC proteins on the cell surface. Using an anti-TNP antibody staining, we showed an efficient and reproducible cell surface modification, especially for monocytes and B cells, which likely serve as APC in PBMC-based T cell assays. Unfortunately, the availability of hapten-specific antibodies is rather an exception than a rule, making it impossible to monitor cell surface modifications for most chemical allergens.

Incubation of responder PBMC with TNBS-modified autologous PBMC induced an average of ~0.04% TNBS-specific CD154+CD4+ T cells. We observed some individual donor variations, illustrating the need to analyze several donors in T cell based assays. A human leukocyte antigen (HLA) allele association is unknown for TNBS-induced T cell activation. TCR-mediated activation and thus antigen-specificity was confirmed by analyzing the co-expression of CD69 and by restimulation of T cell clones and lines. MHC blocking antibodies prevented activation, proving MHC restriction. We observed equal frequencies of TNBS-specific CD154+CD4+ T cells in the naive and memory compartment, comparable to our study on nickel-specific CD4+ naive and memory T cells from non-allergic individuals (Aparicio-Soto et al., 2020). Thus, cross-reactivity or poly-specificity to TNBS- or nickel-induced T cell epitopes is an intrinsic feature of human TCR repertoires (Bechara et al., 2021; Riedel et al., 2021).

Noteworthy, CD154 function has been associated with systemic autoimmunity and likely contributes to immune cell reactivity in human contact allergy (Mehling et al., 2001; Schonbeck and Libby, 2001; Beissert et al., 2006; Caproni et al., 2007). The interplay between keratinocytes and T cells may affect transcriptional induction of selected genes such as PD1 and CD40 ligands, TNF-α and caspase-1, as shown in co-cultures with CD8+ T cells (Rauschenberger et al., 2019).

TNBS-specific CD137+CD8+ memory T cells were ~10-times more frequent compared to TNBS-specific CD154+CD4+ T cells. The lower frequency of naive TNBS-specific CD8+ T cells is most likely due to a less efficient CD137 upregulation by naive CD8+ T cells (Wolfl et al., 2007). As for CD154+CD4+ T cells, most TNBS-activated CD137+CD8+ memory T cells seem activated *via* their TCR given prominent CD69 co-expression, efficient restimulation of clones and lines from different buffy coats, reduced activation in the presence of MHC blocking antibodies and the occurrence of common TCR repertoire features. Similar to the results of this human study, high frequencies of TNBS-specific CD8+ T cells have been found in mice (Hamann et al., 1983; Iglesias et al., 1992; Martin et al., 2003). Usually, frequencies of protein antigen-specific T cells are orders of magnitude lower in the absence of exposure and adaptive immune responses, e.g., ranging from 1–100 cells per 10 million T cells in the naive or cross-reactive memory compartments (Bacher et al., 2013; Su et al., 2013). Thus, TNBS-induced T cell epitopes interact with unusually large fractions of T cells, similar to nickel ions (Aparicio-Soto et al., 2020), which prompted investigations of the involved TCR.

We here report a first comprehensive and unbiased TCR assessment of human TNBS-specific CD4+ and CD8+ memory T cells. Previous studies on murine TNBS-specific T cells suggested a strong association with certain TCR gene segments or CDR3 amino acids (Hochgeschwender et al., 1986; Hochgeschwender et al., 1987; Kempkes et al., 1991; Martin et al., 1995). However, we did not identify a single dominant feature among human TNBS-specific T cells but rather several moderate changes in the TCR repertoires indicating the existence of diverse

mechanisms for TNBS-related epitope recognition. We observed a moderate, common and significant overrepresentation of TCR with the gene segment TRBV20 among TNBS-specific CD4+ T cells. However, effects were much less prominent compared to the overrepresentation of the TRAV9-2 gene segment among nickel-specific CD4+ T cells (Aparicio-Soto et al., 2020).

TNBS-specific TCR from CD4+ and CD8+ T cells more often contained a lysine and tryptophan in their β-chain CDR3 compared to random TCR, respectively. The involvement of tryptophan in the recognition of TNP-modified amino acids has also been described for antibodies (Little and Eisen, 1967). We did not observe any change in tryptophan or lysine use among the TCR of nickel or cytomegalovirus-specific CD4+ T cells in our prior studies, excluding general effects on the occurrence of these amino acids among antigen-specific TCR (Aparicio-Soto et al., 2020). Instead, TCR of nickel-specific T cells showed a strong overrepresentation of CDR3 histidine (Aparicio-Soto et al., 2020). Interestingly, TNBS-associated TCR repertoire changes mainly occurred in the TCR β-chain while the overrepresentation of histidine among the TCR of nickel-specific CD4+ T cells occurred among both α- and β-chains (Aparicio-Soto et al., 2020). The differences in the general amino acid composition of α- and β-chain CDR3 illustrate the importance of a fine-grained TCR analysis from T cell subpopulations and the sequencing of appropriate control repertoires in order to identify the mechanisms of TCR-mediated chemical allergen recognition.

The TNP moiety may be a dominant interaction partner in T cell activation, as shown for murine TNBS-specific TCR (Burakoff et al., 1976; Martin et al., 1995; Martin et al., 2003). Especially for carrier-peptide independent chemical allergen recognition, PBMC-based assays may represent skin-derived T cell epitopes very well. For other TCR a lack of skin-derived T cell epitopes may prevent antigen-specific T cell activation in PBMC-based assays, as shown for some nickel-specific T cell clones (Kapsenberg et al., 1987). However, representative chemical-reactive T cell clonotypes can be identified by a PBMC-based T cell assay. TNBS-specific TCR were shown to be cross-reactive with dinitrobenzenesulfonic acid (DNBS)-induced T cell epitopes, once confounding effects of a lower or different DNBS reactivity had been excluded (Martin and Weltzien, 1994; Dietz et al., 2010). To fully elucidate details of the multimolecular interactions between chemical, TCR and peptide-MHC complexes, mutation or crystallization studies are needed. Of note, T cells can be activated by a single ligand (Sykulev et al., 1995; Huang et al., 2013), which renders the identification of T cell epitopes challenging until today.

So far, mainly interactions of chemicals with peptides presented by MHC proteins have been studied (Aparicio-Soto et al., 2021). Interactions with TCR residues remain unexplored although they have been theoretically addressed as "p-i TCR" concept (Pichler, 2019). For the drug and chemical allergen sulfamethoxazole, binding to TCR CDR2 and CDR3 regions has been modeled while a functional involvement in drug hypersensitivity remains unknown (Watkins and Pichler, 2013; Pichler, 2019). We here provide experimental evidence for this postulated direct interaction of chemical haptens with the TCR, similar to our recent study on nickel-specific CD4+ T cells.

In general, the "irritant" capacities of a chemical allergen, i.e., its activation of the innate immune system, has been linked to its sensitizing potency (Galbiati et al., 2020; Martin and Esser, 2022). However, for some chemical allergens, an unusually frequent T cell activation may significantly contribute to their sensitization potential. Chemical allergen exposure could activate cross-reactive memory T cells in the skin or in draining lymph nodes upon chemical allergen exposure. This scenario is difficult to investigate since T cells lack somatic hypermutations that would allow tracking of early cross-reactive adaptive immune responses (Giesecke et al., 2018). Interestingly, the activation of pre-existing heterologous skin-resident memory T cells is currently explored in cancer therapy, illustrating the potent *in situ* effector functions of this T cell subset (Rosato et al., 2019). T cell activation represents the final key event (key event 4) in the adverse outcome pathway (AOP) of skin sensitization of the OECD. Thus, the further development of alternative *in vitro* T cell-based assay has not only diagnostic but also regulatory potential. So far, integrated testing strategies (ITS), exemplified by the new OECD guideline 429 on "Defined approaches on skin sensitization", do not comprise T cell activation.

AIM assays offer great potential to contribute to the investigation of chemical-specific T cell responses. Since the efficient *in vitro* generation of chemical allergen-induced T cell epitopes remains the major bottleneck in the development of T cell assays, faster methods could fundamentally accelerate the optimization of assay conditions. One major advantage of AIM assays is their shorter incubation time compared with proliferation-based methods such as the lymphocyte transformation tests (LTT) or amplified T cell libraries (Geiger et al., 2009). In addition, AIM assays are not restricted to the detection of cytokine-producing or proliferating T cell subpopulations but offer the opportunity to follow proliferation patterns and profile cytokine secretion of defined antigen-specific T cell populations. AIM assays are quantitative and compatible with large input cell numbers, e.g., if combined with magnetic enrichment (Bacher and Scheffold, 2015; Reiss et al., 2017; Elias et al., 2020; Saggau et al., 2021). The combination with multi-parameter flow cytometry allows to simultaneously track *in vivo* relevant phenotypic and functional markers in human allergies and the isolation of living T cells for downstream experiments, including TCR HTS (Aparicio-Soto et al., 2020). Besides CD154 and CD137, other activation markers used alone or in combination have been discussed including CD134 (OX40), CD25, CD69, CD71 or HLA-DR (Bacher and Scheffold, 2013). AIM assays are currently extensively used to characterize SARS-CoV-2-specific T cell responses (Bacher et al., 2020; Jung et al., 2021; Tarke et al., 2021). However, for each activation marker and chemical allergen, careful validation to prove TCR-mediated activation, e.g., antigen-specific restimulation of T cell clones, is required. Furthermore, high background expression, e.g., of CD137, CD25 or CD69, on T cells from

some buffy coats, or slow expression kinetics can impede the detection or quantification of rare antigen-specific T cells, respectively.

Taken together, the exploration of frequencies and TCR repertoires of chemical allergen-specific T cell subpopulations can significantly advance our understanding of chemical sensitization pathomechanisms (Villani et al., 2021). Methodological advances in protein antigen-specific T cell assessment may be adapted for the detection of chemical-specific T cells, as shown in this proof-of-principle study with the model allergen TNBS. AIM assays could speed up the development of T cell-based alternative diagnostic and predictive *in vitro* tests. This could pave the way for the inclusion of T cell responses in the emerging era of next-generation risk assessment.

DATA AVAILABILITY STATEMENT

The TCR sequencing datasets presented in this study can be found online European Nucleotide Archive (ENA), accession number PRJEB49381.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Charité's ethics committee, Berlin, Germany (vote EA1_217_19). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KS conceived the study and supervised the project, the experimental work and data analysis. KS, MA-S, and CC planned the experiments. CC, MA-S, IW and AB performed cell culture and flow cytometry experiments and analysed flow cytometry data. CC and FR performed TCR sequencing and analysis. AA contributed to data sequencing analysis. MA-S, CC, and KS interpreted data and wrote the main manuscript. HJ-T and AL revised the manuscript. All authors provided discussion and agreed to the final version of the manuscript.

FUNDING

This work was supported by BfR internal grants SFP 1322-718 and -719.

ACKNOWLEDGMENTS

We thank Burkhard Malorny and Maria Borowiak for their excellent assistance in Illumina MiSeq sequencing and Niels Hempel for helping in analyses involving python.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2022.827109/full#supplementary-material

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3. Discussion

There are three main parts to this thesis. In the first step, the AIM T cell assay was established for the analysed chemical allergens $NiSO_4$, $CoCl_2$, $PdCl_2$ and TNBS. The crucial point for this establishment was the selection of a suitable marker protein to measure the activation of T cells. In addition, the various mechanisms of chemical-induced T cell epitope formation had to be considered, and the toxicity and specificity of the reaction had to be assessed.

In the second step, the high frequencies of allergen-specific T cells were recognised and discussed in the context of high allergy prevalence. Finally, in the third step, the binding mechanisms of allergens to the TCR have been analysed. This was achieved by sequencing the RNA of the allergen-specific TCR using HTS. The analysis revealed common and individual allergen-specific binding mechanisms. Furthermore, sequencing of the allergen-specific TCR additionally enabled the analysis of cross-reactivity.

3.1 Short-term AIM T cell assays as a new approach for the detection of chemical-specific T cells

AIM T cell assays have been extensively used to detect and characterise protein antigen-specific T cells[135, 136, 137, 138]. However, before the present work, the same approach was not applied for the detection of chemical allergen-specific T cells. There are various unique challenges associated with the detection of chemical-specific T cells. For example, chemical-specific T cells are rare, and chemicals can also form epitopes inefficiently *in vitro*. Additionally, the toxicity and solubility of chemicals and their potential interferences with flow cytometry may be issues that should be considered.

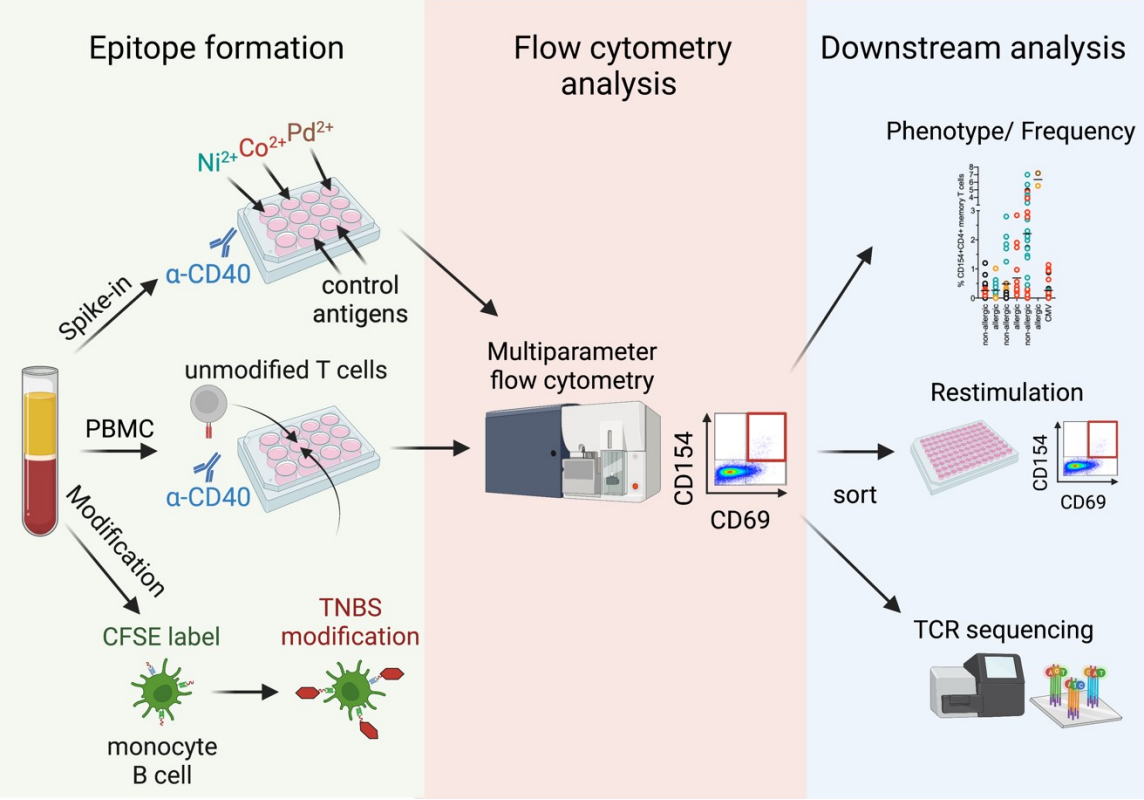

Figure 8: The activation induced marker (AIM) assay as detection method for T cell activation.

AIM T cell assays consist of three key steps. In the first step "Epitope formation", a chemical-induced epitope is generated, which is then recognized by T cells during an incubation period. Different approaches for epitope formation have been applied during this thesis – direct addition to the cell culture ("spike-in") for metal allergens (nickel (Ni^{2+}), cobalt (Co^{2+}), palladium (Pd^{2+}) salts) and separate antigen-presenting cell (APC) modification for 2,4,6-trinitrobenzenesulfonic acid (TNBS). The binding of a specific T cell receptors (TCR) to a peptide-(allergen)-major-histocompatibility complex (MHC) epitope then activates the T cell in the presence of an anti-CD40 blocking antibody (a-CD40). In a second step "Flow cytometry", *de novo* expressed surface proteins on the activated T cells (e.g. CD154, CD137, CD69) are tracked with fluorescently labelled antibodies and analysed by flow cytometry. Large input cell numbers ensure the detection of rare antigen-specific T cells. Magnetic enrichment or sub-gate restricted data acquisition ensures that flow cytometry data processing limits are met. Several read-outs can be utilised in subsequent down-stream analysis. Besides activated cell frequencies, extensive phenotype, or functional characterisation (e.g., intracellular cytokine staining) is possible, depending on the number of available FACS parameters. In case of surface staining, which does not destroy the respective cell, downstream analysis with live cells can be performed in a third step. During this step, the activated T cells may either be taken into culture for later restimulation or sorted for RNA-based sequencing of the T cell receptor. Figure created with BioRender.com

3.1.1 Advantages of the AIM T cell assay

AIM assays rely on the induced expression of activation markers, preferably on the T cell surface, ~5-24 h after the stimulation with an allergen (**Figure 8**). Therefore, one major advantage of this assay is the shorter incubation time compared to proliferation-based assays such as the LTT, which take a minimum of 5 days. In addition, because of the use of activation markers, AIM assays are not restricted to the detection of cytokine-producing or proliferating T cell subpopulations. Instead, AIM assays allow additionally to monitor cytokine secretion from antigen-specific T cell populations for large input cell numbers, which can be combined with, for example, magnetic enrichment[143, 144, 145].

Alternatively, only specific subsets can be analysed during gating to avoid obtaining too extensive data volumes from large cell sets. Surface staining of large input cell numbers allows the analysis of additional subpopulations. The surface staining of activation markers also allows the sorting of living cells for subsequent clone restimulation or TCR sequencing.

Various markers that may be utilised as read-outs in AIM assays have been discussed in the literature. The activation marker CD154 is expressed on all conventional antigen-specific CD4+ T cells. Furthermore, the reaction of CD154 is fast and directly quantitative. CD137 is a marker for CD4+ Treg cells within the first ~5 hours of antigen stimulation[139]. In addition, CD137 is expressed on antigen-specific CD8+ T cells after 16 to 24 h.

In summary, AIM assays are more comprehensive, sensitive, and quantitative than the established LTT. Additionally, AIM assays may also capture naïve antigen-specific T cells and have the potential to work with primary human cells *in vitro* in a reproducible way.

3.1.2 Suitable marker proteins for AIM assays

For each activation marker, careful validation is required. The frequency of each marker in unstimulated T cells and, in comparison, the signal in antigen-specific T cells have to be compared. Only if the signal-to-noise ratio for this marker is high enough to produce a signal distinct from the background level antigen-specific T cells can be detected reliably. On average, the background expression of CD154 on the surface CD4+ memory T cells was minimal[124, 127, 146], whereas the background expression of CD137 on CD8+ memory T cells was higher and more variable among different buffy coats[124]. Nevertheless, the signal of CD137 on CD8+ memory T cells was higher than the background expression level.

CD69, for example, is an early marker of T cell activation, which may also indicate tissue residency and immune response[147]. CD69 is expressed with similar kinetics as CD154 but was unsuitable as a single activation marker since CD69 background expression was higher than CD154 and CD137 background

expression. The high background complicates the detection of rare antigen-specific T cells. As a result, CD154 and CD137 were used as primary activation markers.

The expression kinetics had to be determined to set experimental conditions for the specific and sensitive detection of T cell stimulation for each activation marker. In the present work, the incubation times of AIM assays (5 or 16 h) mirror published times for maximum induced CD154 and CD137 expression among antigen-specific CD4+ T cells and CD8+ T cells, respectively [124, 127, 136, 140]. Short incubation times were possible because epitope formation by metal probably occurs very quickly (complex formation). Similarly, TNBS-modified APC express epitopes from the beginning of the assay. In addition, it is essential to determine the sensitivity of the potential activation marker to non-specific induced upregulation, which may be cytokine or stress-induced.

Furthermore, it was analysed if the marker is expressed on the surface of the cells or if it is internalised over time. The latter aspect concerns, e.g. CD154, which is internalised upon CD40 binding. The addition of an anti-CD40 antibody prevents the ligand-induced down-regulation of CD154[136].

In the first step, this work focused on CD154+ CD4+ T cells. It was shown that CD154 expression is a reliable detection tool for T cell activation by chemical allergens[127, 148]. As an additional activation marker, the surface protein CD137 was examined. It has a higher background activation but, after stimulation, a clear activation signal and was mainly used for the analysis of CD137+ CD8+ T cells. Besides CD154 and CD137, the use of other markers, for example, CD69 and nuclear receptor 4A1 (Nur77), have been discussed[136].

3.1.3 *In vitro* generation of chemical-induced T cell epitopes

The conditions of the AIM assay as a detection method for various chemical-specific T cells critically depend on the allergen-induced epitope formation efficiency. As different allergens, e.g. metal ions and organic chemicals form epitopes in various ways, it is only possible sometimes to use the same approach. The epitope is generally formed by a self-peptide presented by the MHC and an allergen incorporated into this complex. In the presence of high chemical concentrations, complex formation is easily achieved. However, there are limitations like cell toxicity and chemical solubility that limit the possible chemical concentrations used. Metal ions are non-classical haptens as they bind in reversible coordination complexes with proteins[150]. Therefore, protein-bound metal ions mostly do not need to be processed by APC. Metal ions may also directly bind to the self-peptide-MHC complexes, which seems to be the predominant epitope generation mechanism, as shown in this work (Section 1.5.2). Thus, for the epitope generation with metal allergens, it was possible to directly stimulate peripheral blood mononuclear cells (PBMC) with different metal salts dissolved in water (Spike-in, **Figure 8**)[127].

Frequent T cell activation in the presence of high metal ion concentrations was observed, similar to earlier work[151], which is missed in approaches using pulsed APC[135].

Organic chemicals, for example, the model allergen TNBS, may bind covalently to proteins and require processing by the APC for the MHC presentation of hapten-modified self-peptides. In initial experiments, only very weak T cell activation and, thus, inefficient epitope formation was observed by the addition of TNBS to PBMC (unpublished data). Alternatively, TNBS may directly modify self-peptide-MHC complexes on the APC surface. To generate TNBS-specific epitopes, PBMC were labelled using carboxyfluorescein succinimidyl ester (CFSE), modified with TNBS and mixed as APC in a 1:1 ratio with non-modified responder PBMC in the presence of anti-CD40 antibody ("modification" approach, **Figure 8**)[124]. The extracellular surface modification was monitored by anti-TNP staining to analyse the optimal modification concentration of TNBS. Concentrations between 0.8 and 3.1 mM were suited to modify the monocytes and B cells while live cell numbers were preserved. Monocytes and B cells constitute the primary APC in the assay and their surface proteins were modified reliably. In the AIM T cell assay, this approach showed unusually high T cell activation[124]. Thus, the prior modification of APC (or proteins) seems to enhance epitope presentation for sensitisers that require covalent binding. Furthermore, the modification of APCs enables a higher chemical concentration than a direct spike-in of the chemical.

In principle, this approach can also be applied to other organic chemicals. However, the APC modification needs to be adapted for each new chemical allergen. Additionally, other APC that provide better cross-presentation could be used, e.g. monocyte-derived DC (moDC), as shown in the literature[152]. Challenges for the adaptation to other allergens may include solubility issues or pre/pro-hapten formation.

3.1.4 Monitoring of chemical toxicity and effects on T cell function

With the help of buffy coats (PBMC from uncharacterised individuals), non-toxic metal salt concentrations that optimally activate most of the antigen-specific T cell pool in AIM assays were determined. Likewise, the most effective concentrations for modifying APC with TNBS were identified[124, 127, 146].

The Toxic effects of metal ions and TNBS on APC were determined through live cell numbers of CD14+ monocytes and CD19+ B cells (Sections 2.2 and 2.3). The most sensitive read-out concerned the effects of metal ions and TNBS modification on T cell function. A decreased CD154 upregulation on CD4+ T cells and a decreased CD137 upregulation on CD8+ T cells after stimulation with the superantigen SEB were used as indicators. SEB activates a large proportion of T cells by linking certain TCR β-chains with MHC molecules independent of the presented antigen-peptide[153]. The effects on

T cell function extended beyond the more familiar toxicity parameters, such as the number of living cells or apoptosis induction. In the published LTT literature, the control of T cell function in the presence of the tested chemical concentrations is rarely described. Therefore, negative LTT results could be partly due to chemical inhibitory effects on T cell proliferation. Percentages of activated Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific T cells peaked until toxic effects took over (Section 2.3). The short-term AIM assay enables the use of relatively high concentrations of metal ions when compared to proliferation-based assays.

3.1.5 Confirmation of TCR-mediated activation marker upregulation

The specificity of the CD154+CD4+ and CD137+CD8+ memory T cell populations had to be confirmed to exclude the possibility that non-specific activated T cell populations (e.g. induced by stress) influence the frequencies of specific T cells. The specificity was confirmed utilising the successful restimulation of T cell clones and the large CD69 co-expression. Activated allergen-specific T cells are sorted and expanded in cell culture for clone restimulation. Subsequently, these single-cell clone populations are stimulated again with the respective allergen, and the activation frequencies are analysed. Approximately 75% of TNBS-specific CD154+CD4+ and CD137+CD8+ T cell clones were activated through renewed TNBS stimulation (Section 2.3). Comparable results can be seen for the restimulation of metal-specific T cell clones (Sections 2.1 and 2.2).

Not all clones can be restimulated because some T cells are autoreactive. The amount of autoreactive T cells depends on the level of background frequency. Additionally, bystander activation may have occurred, or some T cells may have lost their TCR during *in vitro* culture. The frequency of clones that could be restimulated fits with literature data[154, 155], but the data was greatly expanded in this work by using more clones and looking at multiple metals. This was possible because the AIM assay is significantly faster than proliferation assays.

CD69 is suitable as an additional marker to confirm the specificity of CD154+CD4+ T cells and to distinguish the population from the bystander population[127]. Therefore, the analysis of CD69 on CD154+CD4+ T cells was suggested as a specificity indicator for protein-antigens[149]. The co-expression of CD69 and CD154 was mainly above 80% for non-toxic metal salt and TNBS concentrations, confirming antigen-specificity (Section 2.1, 2.2 and 2.3). The co-expression of CD69 on CD137+CD8+ T cells was not shown before. Although the co-expression of CD69 by CD137+ T cells was less pronounced in TNBS-specific CD8+ T cells, this co-expression indicates antigen-specificity and TCR-mediated TNBS recognition among most CD137+ T cells (Section 2.3).

T cell activation depends on recognising an antigen bound to a particular MHC (MHC restriction). MHC restriction of T cell activation through TNBS and the metal ions was shown by the partial block of CD154

and CD137 upregulation by different MHC I (for CD8+ T cells) and MHC II (for CD4+ T cells) blocking antibody clones (Section 2.1, 2.2 and 2.3). As already shown in the literature, the activation was only partially blocked because of the variability of the HLA of different donors (HLA polymorphism). Besides Ni^{2+}, Co^{2+} and Pd^{2+}, the activation was also blocked in Pt^{2+}-, Cu^{2+}-, and Au^{2+}-specific T cells with anti-MHC II antibodies.

3.1.6 *In vivo* relevance of identified metal-specific T cells

The *in vivo* relevance of the analysed blood-derived T cells for the ACD in the skin was confirmed by the co-expression of other surface markers. The cutaneous lymphocyte-associated antigen (CLA) expression, for example, is associated with skin-directed immune responses and Ni^{2+}-induced ACD. Thereby CLA is an indicator for the *in vivo* relevance of the analysed T cells[156, 157]. In some metal-allergic individuals, an increased CLA expression among CD154+CD4+ memory T cells was detected compared to the total CD4+ memory pool. However, for not skin-associated control antigens like SEB, CMV and TT, no increased CLA expression was observed (Sections 2.1 and 2.2). In the literature, it has been suggested that all Ni^{2+}-specific T cells are CLA+[156]. In this thesis, it was shown that this is not the case. A possible explanation for this would be that not all Ni^{2+}-specific T cells are skin homing but can also be systemic.

The increased co-expression of CLA by some metal-specific T cells indicates *in vivo* relevance of at least a part of the identified T cell populations. To investigate the *in vivo* relevance of CLA- T cells, the TCR of CLA+ and CLA- activated T cells could be sequenced individually in future work. The sequencing would enable the analysis of congruent TCR between the two populations. A high congruence would argue for T cell plasticity, where CD4+ T cells with the same TCR express different surface markers and, therefore, represent several T cell subtypes at the same time[158]. This analysis would allow a conclusion about the relevance of the CLA- T cell population. TNBS is a model allergen. Therefore there are no allergic individuals in the human population for this allergen.

CD154 and CD137 were suitable activation markers for chemical allergens in AIM assays (Section 3.1.2). Based on titrations, non-toxic concentrations of the investigated chemical allergens were identified, which activates a large part of the specific T cell repertoire (Section 3.1.4). Other markers such as CD69, HLA-DR and CLA confirmed the *in vivo* relevance of the analysed cells. Clone restimulation verified the specificity of the reaction. Additionally, MHC restriction of the reaction could be detected (Section 3.1.5). These results first hint that the AIM assay may be suitable to investigate chemical-specific T cell responses for strong T cell activating chemicals. Therefore, the AIM assay could be utilised as a predictive *in vitro* test for next-generation risk assessment of new chemicals in consumer products.

3.2 Connecting strong chemical-specific T cell responses with allergy detection and prevalence

3.2.1 Implication of high frequencies of metal-specific T cells in non-allergic individuals for allergy detection

The present work has demonstrated that the frequencies of allergen-specific CD154+CD4+ and CD137+CD8+ T cells depend on the concentration of the chemical allergen applied. The strongest T cell activation was observed at the highest non-toxic chemical concentrations. Therefore, a concentration of 200 and 400 µM metal salts for direct stimulation and three mM TNBS for the modification of PBMC were chosen for the analysis. After stimulation, allergen-specific CD4+ and CD8+ T cells showed high frequencies of induced CD154+ or CD137+ T cells, respectively[124, 127, 146].

At the highest tolerated chemical concentrations in the AIM assay, TNBS-specific CD137+CD8+ memory T cells were ~10-times more frequent compared to TNBS-specific CD154+CD4+ T cells (Section 2.3). The frequencies of metal-specific CD4+ memory T cells were even higher than that of TNBS-specific T cells (Sections 2.1 and 2.2). Several other metal allergens were investigated in preliminary experiments as part of this work. Among the tested metals, two groups could be identified. Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} solutions activate only about 0.5% of all T cells. On the other hand, activation of T cells by Pd^{2+}, Pt^{2+} and Au^{3+} is considerably more pronounced and is closer to approximately 3%. A possible reason for this difference could be that the larger ions of Pd^{2+}, Pt^{2+} and Au^{3+} would more effectively undergo coordinative binding by free electron pairs present in the TCR and the peptide-MHC complex (see section 1.5.2 for discussion on TCR-allergen-self-peptide MHC interactions).

Because of the high non-toxic concentrations of metal salts used in the assay, we likely captured the complete reactive T cell pool, including clonotypes with low-affinity TCR. However, a high chemical concentration may also activate T cells with no or negligible *in vivo* relevance.

Interestingly, the frequencies of allergen-specific T cells in non-allergic and allergic individuals were very similar. The activation of the T cells, measured using activation markers, may thus not be decisive for the expression of an allergy. The possible implications of this finding will be discussed further down. An exception to this is the population of recent patch-tested nickel-allergic individuals with an ongoing acute allergy. The cells of this patient group could be reliably distinguished from the non-allergic and former patch-tested populations when tested with 200 µM Ni^{2+} (Section 2.1). The threshold to distinguish non-allergic individuals from recently patch-tested allergic ones in this study was determined at 0.53% Ni^{2+}-specific CD4+ memory T cells. For patients that show an especially high Ni^{2+}-specific T cell activation above the background, allergy detection *in vitro,* as an addition to the

patch test, could be possible. However, more research is necessary to elucidate the process of T cell activation in ACD.

The proportion of antigen-specific human memory T cells in the total T cell pool is typically less than 0.05%, as shown for measles virus, TT, and food allergens[159, 160]. Even during effector immune responses like infections, this proportion usually increases to 5%[149]. On average, the frequencies of Pd^{2+}-specific memory T cells in non-allergic individuals measured with the AIM assay were on average 3.0% (Section 2.3). In general, the frequencies of metal-specific T cells in allergic individuals are not likely to exceed this high background T cell activation. This comprises one major problem of establishing *in vitro* T cell assays as an allergy detection method. Future development work could test lower metal ion concentrations to determine if they might better differentiate between allergic and non-allergic individuals. Kapp et al., for example, could distinguish implant patients with an acute allergic reaction from non-allergic patients using the LTT[161].

The differentiation between allergic and non-allergic people seems especially feasible in patients with an acute allergic reaction[162]. Therefore, different methods, such as the LTT and the AIM assay with different concentrations, must be compared using the same patient. Otherwise, the performance of the AIM assay compared to the LTT is fraught with great uncertainties due to the high interindividual donor variation.

One uncertainty regarding the allergen concentration is that, *in vivo,* the actual local metal ion concentration in the skin in nickel-induced ACD is unknown.

In human serum, ~3 nM Ni^{2+} was measured, and the standard nickel patch test substance contains 190 mM Ni^{2+}. The analysis of allergen-specific T cells in affected tissues, further T cell subpopulations or cytokines could be included in the analysis to investigate this uncertainty. The *in vitro* concentration selected for the AIM assay (200-400 µM) is in between metal ion concentrations in human serum and patch test substance concentrations. The metal ion concentration was shown to significantly affect the outcome due to activation, solubility, and toxicity issues. High metal ion concentrations might also occur *in vivo* if the skin is damaged and metal ions get released locally, e.g. from piercings. Local exposure doses in different individuals may also vary widely. Lower metal ion concentrations in AIM assays could improve allergy detection for some patients comparable to observations in the LTT[161].

The AIM assay, as presented here, can identify allergic individuals with an acute immune response to chemical allergens by the induced expression of the activation marker CD154 CD4+ T cells. In addition, several approaches can be developed further to identify non-acute allergic immune responses.

Pathogens elicit a polarised immune response. Type I allergies, e.g. food and respiratory protein allergens, elicit mostly a Th2 response. So far, only a few papers analyse cytokines at the single cell level in combination with antigen-specific T cell activation[163, 164, 165]. In the literature, an increased Th2

response is mainly shown in the case of contact allergens[165]. However, this could also be because a Th2 response is easier to detect because of a lower background expression.

For Ni^{2+}-specific T cells, the cytokine patterns indicating the involvement of different Th cell subpopulations were analysed in this work. Many donors showed a dominant Th1 Ni^{2+}-specific T cell population through the expression of IFNγ. However, in some donors, an additional Th2 (IL-4) or Th17 (IL-17A) response was detected. Hence, Ni^{2+}-specific CD154+CD4+ T cells of different donors had no uniform polarisation. Instead, different Th cell subpopulations were expressed among this rather limited set of patients.

3.2.2 High frequencies of allergen-specific T cells do not result in comparably high numbers of allergic individuals in the general population

Frequencies of metal-specific CD4+ T cells were found already high in non-allergic individuals compared to conventional antigen-specific T cell levels directed towards, e.g. CMV and TT. Due to this strong T cell activation, the question arises why not all people react allergically to metals. The lack of exposure to a sufficient high metal ion concentration, combined with the absence of other heterologous immunostimulatory triggers, might explain that not all people develop a metal contact allergy. For example, a supporting innate activation could be necessary for an allergy to develop, e.g. through an injury. In addition, humans have very efficient barriers to the outside world, such as the skin, which the metal ions must first penetrate by before initiating the immune response.

On the other hand, strong T cell activation could also activate Tregs, which could regulate and curb the immune response and result in tolerance induction[82]. An alternative explanation might be that the local concentration of tissue-resident memory T cells contributes to allergy development[96]. The results of this thesis show that the presence of allergen-specific memory T cells alone does not necessarily lead to an allergic reaction in the respective individual.

The frequencies of Pd^{2+}-specific memory T cells in non-allergic individuals were exceptionally high. However, significantly fewer people are diagnosed with palladium than nickel or cobalt allergies[166, 167]. The few diagnosed palladium-allergic individuals may be due to the general population being less likely to encounter palladium. Since palladium is a noble metal, fewer metal ions are formed by oxidation that can dissolve out of alloys. In addition, Pd^{2+} forms strong complexes with organic and inorganic ligands, which can make migration through the skin much more difficult because of the size of the complexes[168]. Therefore, fewer Pd^{2+} ions could reach the viable skin layers, activating DC and triggering sensitisation (**Figure 9**).

Palladium is not integrated into the standard baseline patch test series but only into the dental series. Only allergens that induce a positive patch test reaction in ~1% of the patients are usually included in

the German recommended standard series of the Deutsche Kontaktallergiegruppe (DKG)[120]. Palladium is the most frequent allergen (3%) in this series among dental technicians with occupational ACD[161]. In the patch test, the water-insoluble $PdCl_2$ (1%), emulsified in petrolatum, is used. However, $PdCl_2$ could not release enough Pd^{2+} ions during the patch test to elicit eczema.

These circumstances might result in underestimating of the actual palladium allergy prevalence in the general population. In the literature, positive patch test reactions using $PdCl_2$ were compared with the results of tests using sodium tetrachloropalladate (Na_2PdCl_4). The tests with Na_2PdCl_4 revealed more allergic patients than those with $PdCl_2$, and patch tests could be repeated to yield more reliable results compared to $PdCl_2$[169, 170].

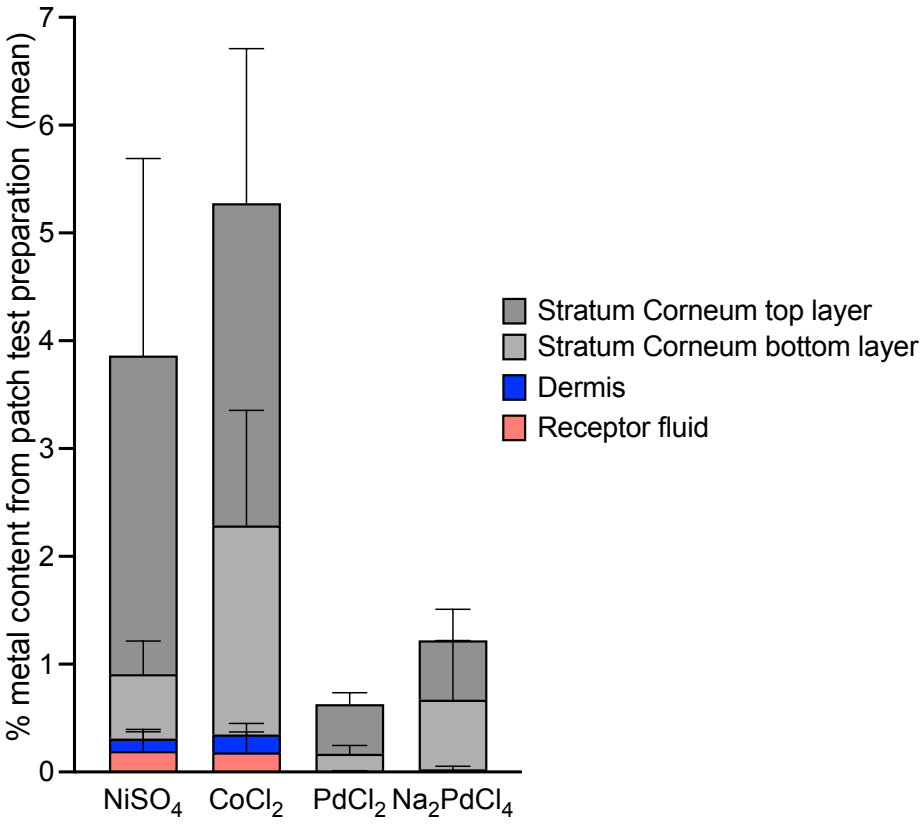

Figure 9: $PdCl_2$ and Na_2PdCl_4 patch test substances hardly penetrate the skin.

In a Franz cell diffusion test setup, 5% $NiSO_4$, 1% $CoCl_2$, 2% $PdCl_2$, 3% Na_2PdCl_4, all in petrolatum, were each applied onto thawed pig skin. After 48 h (analogue to human patch testing) the different compartments were separated (remaining patch test substance, *stratum corneum* layers by tape stripping, dermis, receptor fluid). The different matrices were destroyed by microwave digestion and analysed by ICP-MS (n=6). The reason for the rare diagnosis of palladium allergy could be the low exposure of the population to palladium ions in combination with an inadequate diagnosis due the infrequent patch test of palladium. Additionally, patch testing with water insoluble $PdCl_2$ underestimates the true number of allergic individuals, because not enough Pd^{2+} ions are released from the patch test emulsion during the 48 h of patch testing.

Based on these assumptions and observations, the migration of metal ions through the skin was tested in a Franz cell diffusion assay as part of this thesis' experimental work. Franz cell chambers were prepared with a sample of thawed pig skin with a diameter of 25×25 mm and a thickness of 500 µm. The cells were filled with 12 ml Bovine serum albumin (BSA) and heated to 32°C. 20 mg of 5% $NiSO_4$,

1% $CoCl_2$, 2% $PdCl_2$, 3% Na_2PdCl_4 patch test solution were applied to the pig skin. After an incubation time of 48 h, the different compartments were separated. The skin was stripped 20 times with tape stripes to separate the *stratum corneum* layers. The different matrices of the compartments were destroyed by microwave disruption, and the metal ion contents were analysed by ICP-MS (**Figure 9**).
Most of the metal ions remained in the patch test preparations. Most metal ions that migrated into the skin from the patch test preparations remained suspended in the uppermost layer of the *stratum corneum*. Less than <1% reached the receptor fluid except for the palladium patch test preparations $PdCl_2$ and Na_2PdCl_4, which hardly migrated. Compared to $PdCl_2$, however, a significantly higher amount of the water-soluble Na_2PdCl_4 migrated to the lower *stratum corneum* layers, though it did not reach the viable skin in significant amounts (**Figure 9**). However, DC are known to be capable of reaching into the lowest layer of the *stratum corneum*[171]. Therefore, our findings might explain the higher frequencies of palladium sensitisation detected with Na_2PdCl4 patch tests. These data suggest a call for an exchange of the hardly soluble $PdCl_2$ in current patch test preparations since it may lead to an underestimation of the frequency of palladium-allergic individuals[172].

3.3 Chemical-specific TCR repertoire features inform on allergen binding mechanisms

Here, unusually high metal- and TNBS-specific T cell frequencies were also observed in non-allergic individuals. This prompted the question of how metal ions and organic chemical allergens interact with the TCR. The prediction of T cell epitopes from TCR sequences remains one of the greatest challenges due to the diversity of possible binding mechanisms and antigen-peptide amino acid sequences. The issue is further complicated for chemical-specific TCR as hardly any structural data exist.
This project advanced the TCR HTS, generated the first TCR HTS data on chemical-specific TCR, and ultimately developed the associated data analysis. The nucleotide and amino acid sequences of various chemical-specific TCR were investigated. The aim was to determine the use of gene segments and the positionally accurate CDR3 amino acid composition of the TCR's α- and β-chain. In addition, cross-reactivity (intra-individual antigen-specific TCR) was also analysed.

3.3.1 The choice of the TCR HTS protocol determines the repertoire data

TCR sequencing can be DNA- or RNA-based. RNA-based sequencing has several advantages. It can utilise the conserved constant region for primer annealing, avoiding the need for multiplex primers that circumvent the intron in front of the constant region in genomic DNA. It also allows the introduction of a UMI by template switching[173]. Given these advantages, a protocol developed by C. Chudakov's group was adapted for this work[174]. A disadvantage of RNA-based sequencing is that RNA

is more sensitive to degradation. Therefore, no analysis of tissues can be performed upon their fixation (conservation). In addition, a bias in the analysis may occur, as a cell may have multiple RNA copies in its cytoplasm, especially during proliferation. The overrepresentation of proliferating cells may foster the detection of relevant T cells with slightly increased TCR mRNA numbers.

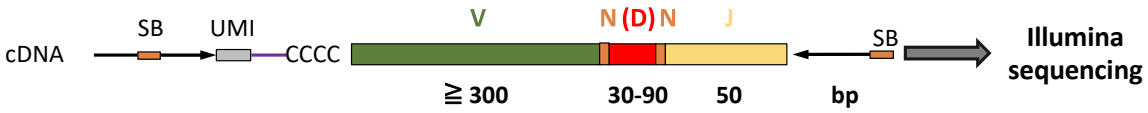

Figure 10: Identifier attached on the cDNA for high quality multiplexed sequencing of T cell receptors (TCR)

During this thesis work, TCR high-throughput sequencing (HTS) of chemical-specific T cells was based on a unique molecular identifier (UMI) and sample specific barcode (SB) approach. The UMI is added during reverse transcription of RNA to cDNA by a template switch mechanism[173, 174]. The SB is attached to both forward and reverse primers and added during two consecutive step-out PCR runs. The graph shows the final PCR product that is further processed by Illumina indices ligation and MiSeq sequencing. V – variable gene segment; J – joining gene segment; D – diversifying gene segment, N – nucleotide indels.

For TCR HTS, RNA was isolated from sorted allergen-specific T cells and control cells (e.g. protein antigen-specific T cells, unstimulated T cells). A SMART adapter is attached to each mRNA strand in the following reverse transcription. The SMART adapter consists of a poly-g RNA tail for the template switch, the UMI with 12 random nucleotides (diversity 4^{12}/ 17 million sequences) and a fixed anchor sequence for subsequent PCR (**Figure 10**). Through the UMI, individual mRNA molecules can be identified after sequencing. In addition, PCR and sequencing errors can be corrected. Usually, we sequenced ≤ 100,000 cells, obtaining < 100,000 TCR sequences. Therefore, the likelihood of the same UMI in one sample is low and diverse sequences with identical UMI are removed during data analysis. To verify the efficient TCR sequencing error correction, Jurkat cells, an immortalised T cell line with a single known TCR, were sequenced, and only the correct α- and β-chains were found[127]. Thus, the majority of the identified chemical-specific TCR presumably have correct sequences. The RNA- and template-switch-based TCR sequencing also captures most of the variable region, allowing to uniquely identify V-gene segments, which are usually not being identified by protocols based on multiplex primers. Historically, UMI-based approaches were not practical for TCR HTS until Illumina introduced the MiSeq reagent kit v3 in late 2013. This kit enabled read lengths of 250 - 300 bp and thus full TCR CDR3 and extended V-gene sequence coverage. At the start of this thesis work, existing multiplex primer-based protocols were rather elaborate, while the chosen approach is applicable to both TCR α- and β-chain[175]. Most TCR sequencing data only address TCR β-chains and are derived from multiplex primer-based protocols that lack unambiguous reliable V-gene segment identification. Although a new generation of HTS devices and protocols have been introduced, the approach chosen here still represents state of the art and is now in commercial use. Single-cell sequencing, e.g. via 10x' chromium

controller, lacks high-throughput, although it offers the potential to identify the pairing TCR α- and β-chains.

A further advantage of the chosen PCR protocol is its compatibility with introducing of sample-specific barcodes using PCR primer sets that carry unique barcodes (**Figure 10**). During this project, a set of 12 barcodes was used. Each barcode is six nucleotides long, with at least two nucleotide differences between the different barcodes. Thus, multiplexing of up to 24 PCR products (12 x α-chain, 12 x β-chain) was possible before the alignment of an Illumina index adapter. Several Illumina indices could be sequenced together to utilise the flow cell capacity fully (~20 million reads for ~1 million cells, at least 3-4 reads/UMI).

3.3.2 TCR HTS data analysis

The employed RNA- and UMI-based TCR HTS protocol and the intended analysis of repertoire features (i.e., gene segment use, CDR3 amino acid composition) required a suitable customised data analysis. To this end, existing programs were used, and complementing tools were developed.

Data analysis was based on the free programs MIGEC[176], MiXCR[177], and VDJtools[178], designed specifically for adaptive immune receptor repertoire (AIRR) HTS data analysis. During the analysis routine of MIGEC, the HTS data set is de-multiplexed according to the sample-specific barcodes, and the TCR α- or β-chain and the UMI are extracted. We chose standard settings while further customisation according to mismatch alignment is possible. Next, the distribution of reads per UMI is statistically evaluated. One of the critical control parameters, the over-sequencing threshold, is calculated during this step. The over-sequencing threshold equals the number of reads per UMI. For this thesis work, a minimum over-sequencing threshold of 3-4 reads per UMI was required to allow error correction (assembly of a consensus read). This step also removes similar UMI, e.g. with only one nucleotide difference, which may have been derived from sequencing errors. Subsequently, the sequences with the same UMI were coupled, assembled and error corrected considering sequence quality and abundance. For each UMI, one high-quality sequence count was obtained. The resulting consensus read was then used in the program MiXCR. MiXCR identifies the V(D)J-gene segments. In addition, the program extracts the CDR3 of the TCR based on identifying of a conserved cysteine residue at the 3' end of the V-gene segment and a conserved phenylalanine residue at the 5' end of the J-gene segment. The software utilises paired read information and was applied using default settings. TCR with identical V(D)J segments and CDR3 nucleotide sequences are considered one TCR clonotype. Thus, one TCR clonotype may be represented with several sequence counts within a sample.

The program VDJtools filters the non-coding clonotypes containing a stop codon and analyses basic statistic parameters, such as the number of individual TCR in each sample.

While MIGEC, MiXCR and VDJtools were available, their combined use was rather tedious and time intensive. All routines always had to be adapted manually for each sample, e.g. input/output directory, over-sequencing-threshold, and barcode assignment. Therefore, within the context of bioinformatics internships, the individual analysis steps were streamlined in a pipeline ("Seq_pipe"). Additionally, the pipeline extracts and reports control values from the report files generated by the software during the consensus sequence generation to control the sequencing and data analysis steps.

A final sensitive quality control parameter for the whole TCR HTS process is the number of TCR sequences obtained above the over-sequencing threshold (≥3) per sorted T cell. This parameter informs on the reverse transcription efficiency as a key step. The number of sequence counts of TCR per cell was usually in the range of 0.1 – 0.2 (mean), comparable to literature data. Lower values may be due to SMART adapter degradation. The low efficiency of the template-switching Moloney murine leukaemia virus reverse transcriptase is another limiting factor.

The subsequent in-depth data analysis can be based on TCR clonotypes (diversity frequency) or count number per TCR clonotype (count frequencies). The diversity frequencies were chosen for data publication during this thesis work because no differences in the clonal expansion were detected for different TCR repertoires. This was expected due to the frequent interaction of chemicals with many T cells, but the count frequency parameters are still continuously reviewed.

"Seq_pipe" was custom-built during this thesis work. The pipeline yields a list of TCR clonotypes (diversity) characterised by a given V(D)J and CDR3 nucleotide sequence and the number of sequence counts for each clonotype.

The additional analysis program "TCR features" was programmed to analyse gene segment use in the data and joined in one pipeline. First, the percentage of each V- and J-gene segment used by the TCR clonotypes in each sample was examined. In addition, the prevalence of amino acids at each position of the TCR α- or β-chain CDR3 were analysed. The cysteine located at position 104 (in the international immunogenetics information system (IMGT)) of the variable region is used as the start of the CDR3 and a conserved phenylalanine as an end. The CDR3 was examined in both directions because it can be of different lengths. This work focused on the analysis of individual CDR3 amino acids because chemical allergens may react with individual TCR amino acid residues, which is different from conventional peptide antigens. An alternative analysis approach would be to cluster TCR with identical or similar αβCDR3 amino acid sequences to search for amino acid motifs with a few allowed miss-matches (k-mer-analysis)[179, 180, 181].

In addition, the TCR congruence among different antigen-specific TCR was studied. The intra-donor analysis captures the cross-reactivity of a TCR specific for one allergen to another allergen. The inter-donor analysis captures public clonotypes, which are very rare. No sophisticated software for intra-individual TCR congruence analysis exists, but concepts and first proof-of-principle data have been generated during this thesis work.

3.3.3 Superantigen-like TCR activation by chemical allergens

Analysis of metal- and TNBS-specific TCR elucidated characteristic repertoire features that inform on antigen recognition mechanisms and explain the frequent T cell activation.

Prior information on Ni^{2+}-specific TCR suggested the association with Vb17 (TRBV19 in IMGT nomenclature) or Va22 (TRAV9-2 in IMGT nomenclature) sequences[2, 182, 183]. Therefore, in the first step, TCR repertoires of Ni^{2+}-specific T cells were analysed. However, no association with Vb17 could be shown. Instead, in the α-chains of Ni^{2+}-specific TCR, an overrepresentation of the gene segment TRAV9-2 was detected (Section 2.1), and the results were reproduced in a subsequent publication (Section 2.2). The TRAV9-2 segment is one of 47 functional α-chain V-gene segments and is expressed by ~5% random CD4+ T cells[127, 146].

The TRAV9-2 gene segment appeared in combination with different J-gene segments[127]. In total, between 1 in 80[146] and 1 in 100[127] TRAV9-2+ T cells were activated by Ni^{2+}. The binding of Ni^{2+} to amino acids of the protein segment encoded by the TRAV9-2 gene segment was previously described for one single TRAV9-2+ Ni^{2+}-specific T cell clone termed "SE9"[2, 184]. A mutation study showed the complexation of Ni^{2+} through the conserved tyrosine at position 36 in the CDR1 of the protein segment encoded by TRAV9-2 and the histidine at position 81 in the β-chain of the HLA complex (**Figure 11**) [2]. These two amino acid residues are in close proximity and constitute a significant HLA contact site in the usual docking mode of the TCR[58].

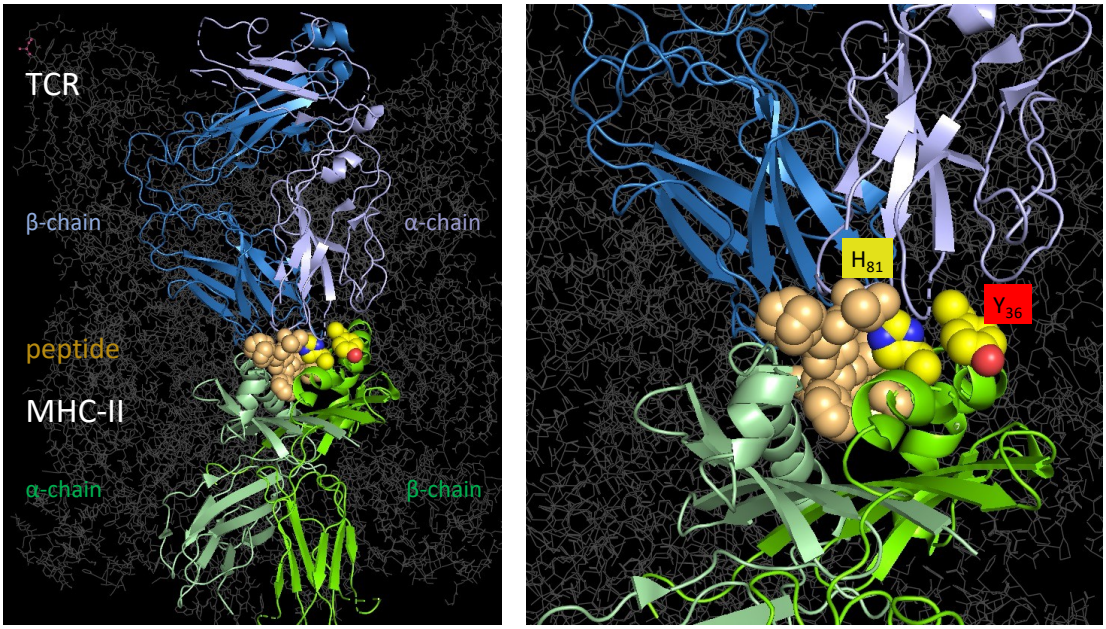

Figure 11: Suspected Ni^{2+} complexation site for TRAV9-2+ T cell receptor (TCR).

The only spatial structures of a TRAV9-2+TCR that is currently known, stems from clone "3A6". A Ni^{2+} specificity of "3A6" is unlikely (only 1 in 80 to 100 TRAV9-2+ TCR recognise Ni^{2+}) but the close proximity of tyrosine36 (Y36) encoded by TRAV9-2 and histidine81 (H81) in the human major histocompatibility complex (MHC) II (human leukocyte antigen; HLA) β-chain suggest the existence of a Ni^{2+} complexation site as depicted[2]. Left panel – overview structure, right panel zoom-in. Bright yellow spheres: carbon atoms, blue spheres: nitrogen atoms, red spheres: oxygen atoms. Created with Pymol. Crystal structure from Li, et al. 2005[185]

For the CDR1 of TRAV9-2, no polymorphisms are known. Furthermore, the HLA β-chain histidine at position 81 is expressed by most HLA-DRB1 alleles, which fits with the observation that nickel allergy is not associated with a particular HLA haplotype[186]. This identified binding mechanism explains the high frequencies of Ni^{2+}-specific TCR in non-allergic individuals. Since residues involved in TRAV9-2-mediated Ni^{2+} recognition are conserved, the interaction could occur in most individuals, depending on individual gene segment use and metal ion concentration. The binding of Ni^{2+} could strengthen the complex of TCR-self-peptide and HLA and therefore surpass the threshold of T cell activation.

Other metal allergies, like beryllium disease, are HLA allele associated, and the beryllium ion (Be^{2+}) is bound in the cleft between HLA-DP2 and the presented self-peptide. Therefore, Be^{2+} is not in direct contact with the TCR, but the TCR recognises changes in the surface of the HLA-presented peptide[187].

Besides Ni^{2+}-specific TCR, gene segment usage was analysed for Co^{2+}- and Pd^{2+}-specific TCR.

A significant increase in TRAV2-expressing TCR was observed in Co^{2+}-specific T cells compared with random TCR (Section 2.2). Pd^{2+}-specific TCR also showed a trend of increased TRAV2 expression, but most prominently, the three gene segments of the TRBV4 family (TRBV4-1, 4-2 and 4-3) were more abundant compared to control TCR (Section 2.2). The over-representation of different gene segments for different metal ions indicates unique metal allergen binding characteristics. The spatial

conformation of amino acid residues in the TRBV4 gene segments strongly favours the complexation of Pd^{2+} ions. As a result, 1 in 7 TRBV4-expressing TCR allow complexation of Pd^{2+} and subsequent activation, whereas only 1 in 80 to 1 in 100 TRAV9-2+ TCR become activated by Ni^{2+} and 1 in 75 TRAV2+ TCR by Co^{2+}, respectively.

Several indications for the *in vivo* relevance of metal-specific TCR expressing the overrepresented gene segments were observed during this thesis work. It was possible to restimulate TRAV9-2+ T cell clones with 2 µM Ni^{2+} (Sections 2.1 and 2.2). The activation is, therefore, not only an effect of high Ni^{2+} concentrations. Already low *in vivo* Ni^{2+} concentrations can engage with TRAV9-2+ TCR. Additionally, the increased TRAV9-2 gene segment use was also observed among Ni^{2+}-specific T cells in donors that strongly exceeded background frequencies and showed increased co-expression of CLA, HLA-DR and Ki-67[127].

For TNBS-specific CD4+ T cells, only a slight increase in the expression of the TRBV20-1 gene segment was observed. A similar small increase in expression was shown for various gene segments of TNBS-specific CD8+ T cells (e.g. TRAV26-1 and TRBV28), but all of these increases were not statistically significant[124]. The lack of gene segment associations with TNBS-induced T cell epitopes suggests preferential binding within the CDR3.

The interaction of metal ions with distinct gene segments is reminiscent of the activation of TCR by superantigens[188]. Superantigens directly induce T cell activation by binding to specific TCR V-gene segments independently from an antigen peptide. The most recent example of a superantigen is the SARS-CoV-2 spike protein, which may underlie recent reports on increased hepatitis or autoimmunity cases post infection[153, 189, 190]. Since only a fraction of T cells expressing the corresponding gene segment responded to Ni^{2+}, Co^{2+}, and Pd^{2+} ions, a superantigen-like interaction seems to be involved. The MHC-presented self-peptide may provide crucial additional contact sites for metal-specific TCR activation or could interfere with metal ion binding for non-responding TRAV9-2+/TRAV2+/TRBV4+ TCR. In the mutation study of clone "SE9", activation decreased when $tyrosine_{108}$ in the CDR3 of the α-chain was mutated to histidine, suggesting a role for tyrosine in peptide but not Ni^{2+} binding[2]. Many Ni^{2+}-specific T cell clones have been described that depend on specific APC, and thus the presence of certain endogenous peptide-MHC complexes for the activation. Other clones are even activated by allogenic APC[127, 146, 191, 192]. Therefore, a spectrum of metal-induced epitopes with a varying dependency on the MHC-presented self-peptide exists.

Nickel, cobalt, and palladium are transition metals with partially filled d-orbitals. They generate cations that form complexes with nitrogen-containing lewis-bases[193]. The coordination complexes with amino acid residues are formed through the interaction of free electron pairs of nitrogen or oxygen atoms with respective s-, p- and d-orbitals of the metal ion[194]. This complicates the characterisation of

metal-ion interaction with the TCR-peptide-MHC complex and the identification of the geometric (configurational) layout of the resulting complex. Further mutation and crystallisation studies would be mandatory to identify the precise binding site of metal ions to the TCR-self-peptide-MHC interface.

3.3.4 Individual CDR3 amino acids are major binding points for chemical-specific TCR

In search for additional chemical allergen-TCR binding mechanisms, the amino acid composition of the CDR3, the most variable binding site of the TCR, was analysed. The amino acid composition of the CDR3 was similar between CD4+ and CD8+ T cells but quite different between TCR α- and β-chains (Section 2.3). Histidine, for example, occurs more frequently in the β-chain compared to the α-chain of CDR3. The identified differences in CDR3 amino acid composition between CD4+ and CD8+ T cells, as well as between TCR α- and β-chains, show the importance of analysing T cell subpopulations and appropriate control repertoires. The combination of both enables the identification of the mechanisms of TCR-mediated allergen recognition.

Among the analysed Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific CD4+ memory T cells, an increased abundance of histidine in the TCR CDR3α or CDR3β sequence was observed, but no other amino acids were similarly changed. One histidine residue seems sufficient for metal ion recognition (Sections 2.1 and 2.2). However, since metal ion complexation requires several ligands besides the histidine residue, electronegative oxygen atoms from other amino acid residues nearby may contribute to the complex formation (Section 2.2). The increase in histidine expression was comparable for all three metals studied. However, Pd^{2+} interacts with histidine in the CDR3 more often than the other metal salts because the stimulation with Pd^{2+} results in higher frequencies of activated T cells.

Both allergic and non-allergic individuals showed an increased expression of histidine in metal-specific T cells (Sections 2.1 and 2.2). This implies that allergic individuals cannot be identified by a CDR3 histidine alone. However, some individuals with elevated CDR3 histidine topped the background and co-expressed CLA, HLA-DR and Ki-67. This indicates that histidine binding could nevertheless be a relevant binding mechanism for metal contact allergies. For further verification, the local T cell enrichment was analysed in tissues affected by allergic reactions. Preliminary results suggest the enrichment of histidine in the CDR3 of allergen-specific T cells, indicating the relevance of this binding mechanism for the contact allergy.

It was also investigated whether the binding of metals to histidine supports binding to TCR encoded by a specific gene segment (e.g. TRAV9-2) or whether it is an independent binding mechanism. As a CDR3 histidine was less abundant among metal-specific TCR expressing the gene segments associated

with metal ion binding (TRAV9-2, TRAV2, TRBV4 family), histidine binding of metal ions represents an independent binding mechanism.

The amino acid occurrence at individual positions was analysed to search for a preferred histidine position in the CDR3 for the binding of metal ions. The histidine positions of the CDR3 in the random repertoire were comparable to those in the metal-specific TCR, arguing against a preferred metal ion binding site along the CDR3 (Section 2.2).

For TNBS-specific TCR, relatively small changes in the CDR3 amino acid compositions were observed compared to those for metal-specific TCR. In TNBS-specific TCR, a lysine at position 7 of the β-chain CDR3 seems to recognise TNBS-induced epitopes preferentially. Additionally, tryptophan occurred more frequently at position 3 in the CDR3 of TNBS-specific CD4+ T cells and position 5 in CD8+ T cells (Section 2.1). On the side of the MHC-presented self-peptide, TNBS binds preferably to free amino groups and thus mainly to lysine[195]. The binding suggests that these positions are the preferred interaction sites of the TCR to TNBS-modified epitopes and that the TNBS recognition mainly occurs via these amino acids.

TCR sequencing from metal-specific T cell clones supported the results obtained from bulk sequencing and allowed analysis of pairing α- and β-chains. The gene segment TRAV9-2 was expressed by 27% of Ni^{2+}-specific CD4+ T cell clones, and 41% expressed a histidine in either their α- or β-chain CDR3. The latter results indicate that the histidine binding may occur independently in either α- or β-chain CDR3. Most of the histidine was located in the β-chain CDR3[127], which is in line with the observation that a higher percentage of histidine in the β-chain occurs in the random repertoire.

The complexation of metal ions or the binding of TNBS by specific amino acid residues at the TCR self-peptide MHC interface may exceed the activation threshold of a physiological weak TCR-self-peptide-MHC interaction required for T cell survival[196]. Exceeding the activation threshold would explain the high frequencies of allergen-specific T cells, especially Pd^{2+}-specific T cell frequencies.

3.3.5 Cross-reactivity of chemical-specific TCR

The shared binding of Ni^{2+}, Co^{2+} and Pd^{2+} ions to a histidine residue in the CDR3 sequence suggests that cross-reactivity of different metal-specific TCR may exist. The analysed metal ions are close in the periodic table of the elements and carry identical ion charges. Similar chemical properties may likely lead to cross-reactivity in biological systems.

Through HTS of Ni^{2+}-, Co^{2+}-, Pd^{2+}- and control-antigen-specific TCR from the same individual, cross-reactive TCR were identified in our experimental studies. However, the sampling effect is a major limitation of the analysis, which reduces the chance of identifying cross-reactive TCR. The sampling effect occurs because only a subset of a person's TCR repertoire can be sequenced. Therefore, the TCR

differ in each PBMC sample from a person analysed by the AIM assay. Hence, the congruence between several samples of the same antigen specificity must first be determined before it can be compared with samples of different antigen specificity.

For α- and β-chains, cross-reactivity was increased between metal-specific TCR compared to metal- and protein-specific TCR. Although the gene segments TRAV9-2, TRAV2 and the TRBV4 family are associated with the respective metals, they showed increased frequencies among cross-reactive compared to unstimulated T cells (Section 2.2). The increased frequencies indicate a cross-reactive T cell subpopulation within all the individual metal-specific T cells. In addition, the frequency of histidine within the CDR3 of metal-metal cross-reactive TCR was comparable to the frequency in the individual metal-specific TCR (Section 2.2). These results suggest that the metal ions can bind interchangeably in the cross-reactive T cell population.

Specific T cells were sorted, expanded, and restimulated with their respective and other metal ions to confirm the results of the congruence analysis of the sequencing data.

The analysis of single metal-specific T cell clones supported the tendencies observed by TCR bulk sequencing. A very high degree of cross-reactivity was observed for Ni^{2+}-specific clones when restimulated with equimolar concentrations of Pd^{2+}. The other metal-specific T cell clones also showed distinct cross-reactivity of 10 to 30%[146]. Because of the sampling effect, the cross-reactivity is underestimated in bulk sequencing. Only the expanded clones can be analysed for cross-reactivity in the future to counteract the sampling effect. The analysis of the expanded clones is a new form of intra-individual congruence analysis that has yet to be established for TCR HTS data analysis. However, preliminary results indicate that cross-reactivity analysis may be possible by either focusing on expanded clonotypes reliably re-identified in replicates or by analysis of larger sample data sets and appropriate controls.

Only a few Ni^{2+}-specific T cell clones were tested for cross-reactivity in the literature. Most clones could be activated additionally only by Pd^{2+}, except for two Ni^{2+}-specific T cell clones that also weakly cross-reacted to Co^{2+}, in line with our results[154, 155]. For Pd^{2+}-specific T cell clones, a cross-reactivity could only be observed for Ni^{2+} [116]. However, this thesis work also observed cross-reactivity of Pd^{2+}-specific T cell clones to Co^{2+}. Since Pd^{2+}-specific T cells are much more abundant than Co^{2+}- or Ni^{2+}-specific ones, cross-reactivity is expected to be detected less frequently.

It has been shown that TNBS-specific TCR can also be activated by dinitrobenzene sulfonic acid (DNBS)-induced T cell epitopes, but this was not further addressed in the present work[133].

4. Conclusion and Outlook

During this thesis work, the AIM T cell assay was established in combination with multi-parameter flow cytometry and TCR HTS for chemical allergens. The metal salts $NiSO_4$, $CoCl_2$, $PdCl_2$ and the model chemical allergen TNBS were applied to test the value of this new approach. AIM assays offer tremendous advantages over conventional methods that mostly rely on proliferation measurements or cytokine secretion. Therefore, AIM assays may overcome the limitations that prevented the development of *in vitro* diagnostic and regulatory T cell-based tests in the past.

The flow cytometric analysis was mainly established with the activation marker CD154 for conventional CD4+ T cells with some pilot data on the marker CD137 on CD8+ T cells. The analysis allows simultaneous tracking of *in vivo* relevant phenotypic and functional markers in immune responses. CD154 can be stained on the cell surface if combined with an anti-CD40 blocking antibody and enables down-stream experiments, including RNA-based TCR HTS and clone restimulation[124, 127, 146].

The AIM T cell assay confirms previous evidence from proliferation and cytokine secretion-based assays for the existence of strong T cell activation by chemical allergens. Moreover, the frequent T cell activation by chemicals may be more common than thought. Activation may have been previously missed due to low sensitivity of read-outs, inefficient epitope formation of chemicals and possible toxic effects that inhibit proliferation. Testing of additional, especially organic chemical allergens with the AIM assay will show, how frequent strong T cell activation occurs. In AIM assays, blood as liquid biopsy specimen can be used to interrogate the circulating T cell pool directly *ex vivo*.

Allergic individuals can be detected ex vivo using the AIM assay if the frequencies of allergen-specific T cells exceed the background activation level of T cells in non-allergic individuals, as memory T cells have a strong overall effector response. A reliable diagnosis would enable population monitoring and alert regulators to potential problems. However, due to high background activation, only some allergic individuals with distinctively increased frequencies of allergen-specific T cells could be identified in the present work. As another obstacle, the number of allergen-specific T cells does not determine whether a person develops an allergy or not. Nevertheless, the identified T cells were specific as seen by the expression of additional markers (e.g. CD69, CLA, HLA-DR) and the restimulation of T cell clones.

An intrinsic feature of the human TCR repertoires is cross-reactivity or polyspecifity[116, 124, 127, 197]. The extensive activation of chemical-specific TCR indicates cross-reactive binding to self-peptide-MHC complexes which includes pre-formed memory T cells from unrelated immune responses. The frequent T cell activation found upon treatment with the model allergen TNBS could also be explained by the cross-reactive recognition of T cells originally activated by a different T cell epitope during an unrelated

immune response. Based on these data, future risk assessment must evaluate the contribution of the cross-reactive memory T cell population to the sensitisation potential of chemicals more precisely.

Human-centred research will become increasingly important in the future, especially in the frame of chemical regulation. Animal testing is not efficient, reliable, or reproducible with respect to effects in humans. It should be avoided whenever possible in accordance with ethical, economical, and ecological principles.

At present, the LLNA is performed in mice and dominates as regulatory test to evaluate potential skin sensitisers. The test captures T cell proliferation which represents the final key event 4 in the adverse outcome pathway for skin sensitisation, as defined by the Organisation for Economic Cooperation and Development (OECD)[198]. None of the existing new approach methodologies (NAM) required for next generation risk assessment (NGRA) addresses this key event. To replace the LLNA, a validated predictive *in vitro* T cell assay would be key. In addition, not even the existing *in vivo* assays reliably predict the sensitising potential of metal allergens[199]. Furthermore, the strong T cell activation and possible cross-reactivity currently represent a black box in the regulatory admission process of new chemicals. A superantigen-like T cell activation, as being observed for Ni^{2+}, Co^{2+}, Pd^{2+} and TNBS in this thesis work, might also be relevant for other chemicals and may have been overlooked by previous T cell assays due to inefficient epitope formation. Strong T cell activation may contribute to the sensitising potency of a chemical. This can lead to regretful substitutions if a chemical is replaced by an equally sensitising one.

For further development of AIM T cell assays, the analysis of additional metal allergens such as platinum, copper or gold are currently being addressed. Moreover, a vast number of other organic allergens starting with PPD and BB as relevant sensitisers in humans, chemicals leaching from nanomaterials, mixtures, and food allergens could be investigated using the now established methods and the corresponding tools. Novel methods of chemical delivery are also being tested, such as nanoparticle encapsulation for hydrophobic non-soluble chemicals. To get further insights on the epitope configuration required for optimised T cell responses, chemically modified proteins could be tested. For each new allergen and adapted approach, the optimal chemical concentration, toxicity, T cell functionality, and fluorescence interferences need to be considered. In the past, these aspects were extremely work intense in proliferation-based assays but now could be more efficiently optimised when applying the AIM assay. In addition to the CD154+ CD4+ T cells that have been mainly studied so far, other T cell subpopulations should also be analysed in the future, in particular CD137+ CD8+ T cells or Tregs. To improve the diagnostic capabilities of the AIM assay and to overcome the limitations posed by the high background frequencies of metal-specific T cells, effector and memory T cell subpopulations could be separately analysed using additional markers in flow

cytometry. Additionally, different chemical allergen concentrations should be compared performing the AIM assay and the LTT in parallel with PBMC from the same donor. This would exclude interindividual variations and inform on the performance of both methods and the optimal chemical concentrations for allergy detection.

Within the scope of this thesis, data on chemical-specific T cells were generated for the first time using TCR HTS. This allows the analysis of binding mechanisms related to the complete repertoire. Coupling the AIM assay with TCR HTS elucidated major mechanisms of chemical allergen recognition by the TCR. Both observations, the binding of metal ions to specific conserved TCR protein segments or a particular histidine residue within the TCR CDR3 sequence as main antigen binding region provide a reasonable explanation for the high frequencies of allergen-specific T cells that were identified (**Figure 12**). In addition, extensive cross-reactivity of allergen-specific TCR and conserved repertoire features among these cross-reactive TCR have been confirmed.

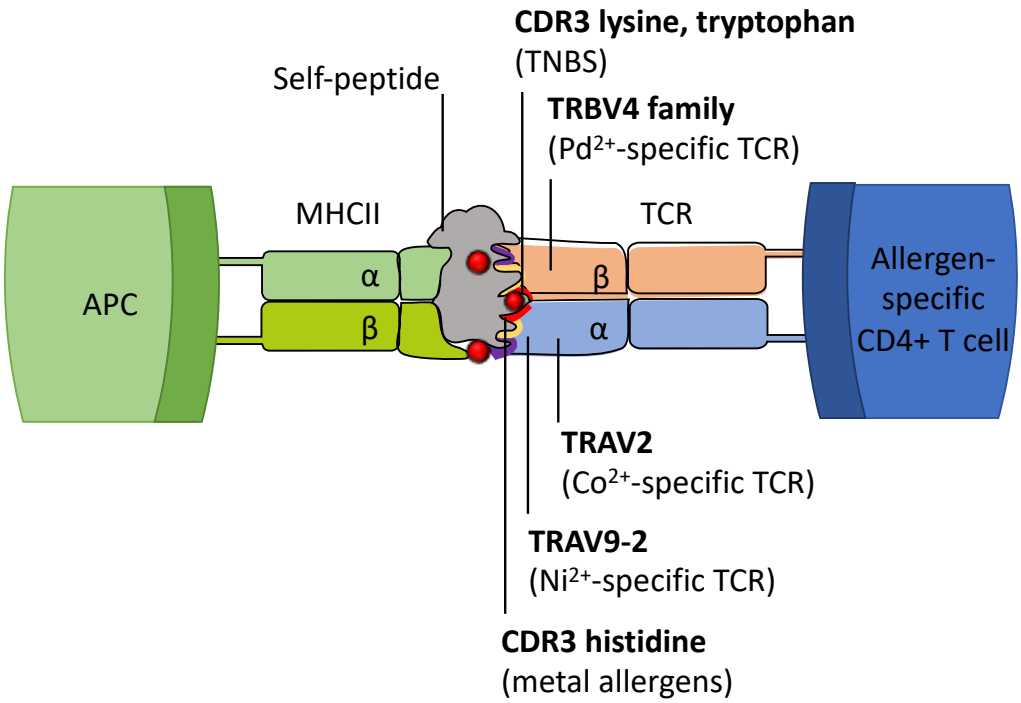

Figure 12: Chemical allergen binding sites at the T cell receptor (TCR) self-peptide major histocompatibility complex (MHC) interface for CD4+ T cells.

This work identified chemical allergen interaction with certain conserved TCR gene segments and complementary determining regions (CDR) 3 regions (coloured in lilac, yellow and red). The red dots represent chemical allergens (metal ions Ni^{2+}, Co^{2+}, Pd^{2+}, or the organic contact allergen 2,4,6-trinitrobenzenesulfonic acid (TNBS)).

Regarding bioinformatics TCR data, the software tools developed in this thesis work can be used to comprehensively investigate cross-reactivity. This could be used to study the cross-reactivity of different metal allergens or of novel foods to conventional foods.

An understanding of cross-reactive metal-specific T cell subpopulations may also be important for the choice of implant materials to avoid implant rejection reactions. Furthermore, the newly developed analysis programs could be published in compliance with AIRR community standards e.g. on GitHub.

The combination of the AIM assay and HTS enables the comprehensive analysis of the TCR repertoire of allergen-specific T cells and therefore contributes to the elucidation of basic immunological mechanisms. Until today, restricting the exposure to metal allergens is the most effective prevention to avoid the elicitation of compound-driven allergic dermatitis. Fostering the knowledge on the basic binding mechanisms may open the path to develop treatment options in the future. To achieve this goal, the *in vivo* relevance of the identified *in vitro* mechanisms must be further elucidated. This can be achieved by HTS analysis of enriched tissue-specific memory T cells. In addition, the penetration of metal ions through the skin should be assessed to obtain deeper insights into ACD.

An immunome-based diagnostics has been recently developed for CMV and SARS-CoV-2[200]. By sequencing T cell pools of several hundred people, public clonotypes could also be identified for metal allergens.

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6. Publication Record

Original research articles

1. **Riedel, F.***; Aparicio Soto, M.*; Leddermann, M.; Bacher, P.; Scheffold, A.; Kuhl, H.; Timmermann, B.; Chudakov, D.M.; Molin, S.; Worm, M.; Heine, G.; Thierse, H.-J.; Luch, A.; Siewert, K., TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells. Allergy 2020, 75, 2574-2586, doi:10.1111/all.14322
2. Curato, C.*; Aparicio Soto, M.*; **Riedel, F.**; Wehl, I.; Basaran, A.; Abbas, A.; Thierse, H.-J.; Luch, A.; Siewert, K., Frequencies and TCR Repertoires of Human 2,4,6-Trinitrobenzene-sulfonic Acid-specific T Cells. Front Toxicol 2021, 4, 827109, doi: 10.3389/ftox.2022.827109
3. **Riedel, F.**; Aparicio Soto, M.; Curato, C.; Münch, L.; Abbas, A.; Thierse, H.-J.; Peitsch, W.K.; Luch, A.; Siewert, K., Unique and common TCR repertoire features of Ni2+-, Co2+- and Pd2+-specific human CD154+CD4+ T cells. Allergy. 2022; 00: 1–13, doi: 10.1111/all.15494

*Equal contribution

Review

1. **Riedel, F.**; Aparicio-Soto, M.; Curato, C.; Thierse, H-J.; Siewert, K.; Luch, A. Immunological Mechanisms of Metal Allergies and the Nickel-Specific TCR-pMHC Interface. Int J Environ Health Res 2021, 18(20), 10867; doi:10.3390/ijerph182010867
2. Aparicio-Soto, M*; Curato, C*; **Riedel, F**; Thierse, HJ; Luch, A; Siewert, K. In Vitro Monitoring of Human T Cell Responses to Skin Sensitizing Chemicals - A Systematic Review. Cells 2021, 11, 83, doi: 10.3390/cells11010083

*Equal contribution

Conferences (published abstracts)

1. **Riedel, F**.; Aparicio-Soto, M.; Leddermann, M.; Bacher, P.; Scheffold, A.; Heine, G.; Thierse, H-J.; Luch, A.; Siewert, K. Clonal expansion of nickel-specific CD154+ memory Th cell clones in peripheral blood of a donor with acute allergic contact dermatitis compared to a non-allergic donor. DGPT Jahrestagung (25-28.2.2019, Stuttgart) / Naunyn-Schmiedeberg's Archives of Pharmacology, DOI: 10.1007/s00210-019-01621-6
2. Aparicio-Soto, M.; Leddermann, M.; **Riedel, F**.; Bacher, P.; Scheffold, A.; Heine, G.; Thierse, H-J.; Luch, A.; Siewert, K. Increased frequencies of specific memory CD4+ T cells in acute nickel contact allergy quantified by CD154 (CD40L) expression". Abstract 85th Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), DGPT Jahrestagung (25-28.2.2019, Stuttgart) / Naunyn-Schmiedeberg's Archives of Pharmacology, DOI: 10.1007/s00210-019-01621-6
3. Aparicio-Soto, M.; Leddermann, M.; **Riedel, F.**; Bacher, P; Scheffold, A.; Heine, G.; Thierse, H-J.; Luch, A.; Siewert, K. Direct quantification of nickel-specific naïve and memory Th cells in peripheral blood of allergic and non-allergic donors. EAACI Congress 2019, 01.06.2019 / Allergy, DOI: 10.1111/all.13962
4. Curato, C.; Aparicio-Soto, M.; **Riedel, F.**; Wehl, I.; Basaran, A.; Leddermann, M.; Hillen, U.; Thierse, H.; Luch, A.; Siewert, K. Direct quantification of human 2,4,6-trinitrobenzensulfonic acid (TNBS)-specific CD154+CD4+ and CD137+CD8+ naïve and memory T cells. Allergy 76, 583-637 (2021).
5. **Riedel, F.**; M., L.; Aparicio Soto, M.; Peitsch, WK.; Thierse, H.; Luch, A.; Siewert, K. ab TCR repertoire characteristics of nickel-, cobalt-, and palladium-specific CD154+CD4+ T cells. Allergy 76, 23-423 (2021).
6. **Riedel, F.**; Aparicio Soto, M; Leddermann, M; Peitsch, W-.; Thierse, H-J.; Luch, A.; Siewert, K. Metal contact allergen-specific CD4+ T cell responses and the involved αβ T cell receptor repertoires. Congress of the European Society of Contact Dermatitis, 8.–10.6.2022

Annex I: Supplementary Material

Research paper: TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells

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Published online on 16 April 2020 in:
Allergy. 2020; 75: 2574– 2586
Link: https://doi.org/10.1111/all.14322

Appendix S1

Supporting methods

Blood samples and PBMC isolation

Buffy coats were purchased from Deutsches Rotes Kreuz. Serum cytomegalovirus (CMV) IgG titers for all samples were determined at Institut für Laboratoriumsmedizin Berlin (IFLb). PBMCs were isolated by standard density gradient centrifugation within 2 h of a fresh blood collection (Ficoll Paque Plus, GE-Healthcare).

Antigen stimulation assays

PBMCs were cultured in complete RPMI 1640 medium supplemented with 5% (v/v) human AB serum (both PAN-Biotech) or autologous serum (fresh blood samples) at $2.5x10^6$ cells/cm^2 in flat bottom tissue culture plates. For antigen stimulation assays 200 μM $NiSO_4 \cdot 6H_2O$ (Sigma-Aldrich, 40 mM stock dissolved in H_2O) or control antigens CMV pp65 (1:100, Miltenyi Biotec), staphylococcal enterotoxin B (SEB, 1 μg/mL), PMA-Ionomycin (PMA-I, 10 ng/ml and 1 μg/ml, all Sigma-Aldrich) were added to the PBMC cultures in the presence of CD40 blocking antibody to prevent contact-dependent downregulation of CD154[1]. For MHC block experiments, anti-HLA-DR, DP, DQ (10 μg/ml, tu39, AB_395938), anti-HLA-DR (10 μg/ml, ac122, AB_2661330) or an isotype control antibody (10 μg/ml, eBM2a, eBioscience) were added 30 min prior to stimulation. In case of intracellular cytokine staining, Brefeldin A (1 μg/ml, Merck) and BD GolgiStop™ (1:1000, BD Biosciences) were added for an additional 2 h of incubation. We used 200 μM $NiSO_4$ because this concentration was not toxic as assessed by only marginal reductions in monocyte, B cell, and T cell numbers (<20%, mean)[2] and the absence of inhibitory capacity of CD154 expression on CD4+ T cells in SEB-stimulated cultures (data not shown). Frequencies of Ni-specific CD154+ naïve and memory CD4+ T cells and co-expression of cytokines and activation markers was assessed on 96-well plate format. For TCR sequencing, 12-well plates were used with $10x10^6$ PBMCs per sample.

Antibody staining and FACS analysis

Cells were stained with different combinations of the following reagents: LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fischer Scientific), CD4-PerCP (VIT4), CD154-PE (5C8, both Miltenyi Biotec), CD3-Alexa Fluor 700 (UCHT1), CD4-PerCp-Cy5.5 (SK3), CD8-BV510

(SK1), CD45RA-PE/Dazzle™ 594 (HI100), CD45RO-PE-Cy7 (UCHL1), CCR7-PE-Cy7 (G043H7), CCR7-PE-CF594 (G043H7), CLA-FITC (HECA-452), CCR9-BV421 (L053E8), CD69-BV711 (FN50, all Biolegend) and HLA-DR-V500 (G46-6, BD Biosciences). In some experiments PBMCs were incubated with anti-CD154-PE followed by incubation with anti-PE beads (Miltenyi Biotec) to enrich CD154+ cells prior to the sort for TCR sequencing by two consecutive MS columns[3] (Miltenyi Biotech; Table S2). For intracellular cytokine or nuclear staining, cells were fixed and permeabilized with Inside Stain Kit (Miltenyi Biotec) or Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific), respectively, and incubated with combinations of the following antibodies: TNFα-BV421 (MAb11), IL-2-BV711 (MQ1-17H12), GM-CSF-APC (BVD2-2 1C11, all Biolegend), IL-22-FITC (22URTI, eBiosciences), IFNγ-PE-CF594 (B27), IL-4 PE-Cy7 (8D4-8), IL-17-BV510 (N49653), Ki-67-BV421 (B56, all BD Biosciences), nur77-APC (REA704) and CD154-PE (both Miltenyi Biotec). Cells were analyzed and sorted on a BD FACSAria III sorter (11 colors, BD FACS Diva Software V7.0, BD Biosciences). All conjugates were titrated to determine their optimal dilution and staining volume. All events of a 96-well sample or a magnetically enriched sample were acquired. For TCR sequencing, only cell surface staining was employed to maintain RNA integrity until the sort. Data were analyzed with FlowJo software V.10.4.2 (BD Biosciences).

Bead-based cytokine analysis

Cell culture supernatants were collected from parallel antigen stimulation assays on 96-well plates 16 h after antigen stimulation. Cytokines were analyzed with the LEGENDplex™ HU Th Cytokine Panel and LEGENDplex™ data software V.8.0 (both Biolegend) according to the instructions of the manufacturer.

Clone restimulation assays

Single, antigen-specific CD154+ memory T cells were sorted into 200 μl cloning medium (complete medium without extra $CaCl_2$, 15% human AB serum, 200 U/ml IL-2 [IS grade, Miltenyi Biotech], 30 ng/ml a-CD3 [OKT-3, pure, Miltenyi Biotech] and 100 000 allogeneic mitomycin C-treated PBMCs as feeder cells) on 96-well flat bottom plates. Clones were further expanded in medium without feeder cells and OKT-3 antibody and, if required, with Dynabeads Human T-Activator CD3/CD28 beads (Thermo Fisher Scientific). Cloning efficiency was typically ~30%. Clones were rested for 3 days in medium without IL-2 in 10% human AB serum

and restimulated with 50 000 autologous PBMCs or CD3-depleted PBMCs (CD3 MicroBeads, human, and LD columns, Miltenyi Biotech) in a 1:1 ratio in 384-well plates in 100 μl medium under the same conditions as used for bulk culture antigen stimulation assays plus CD28 antibody (1 μg/ml, functional grade, 15E8, Miltenyi Biotech). Specific restimulation was assumed if the frequency of CD154+ cells was 3-times higher in antigen-stimulated samples compared to samples without antigen.

TCR sequencing

Cells were sorted directly into 1 ml buffer RLT (Qiagen, Hilden, Germany) and stored at -80°C. RNA was extracted with RNeasy Micro Kit (Qiagen). Reverse transcription, PCR amplification, library preparation, and Illumina sequencing for TCR αβ chains was performed as described[4] (Table S3). PCR products were purified with QIAquick PCR Purification Kit (Qiagen), pooled, size selected to ~300 – 600 bp with beads (0.65 ratio, CleanPCRNA kit, GC Biotech) and Illumina sequencing adapters were annealed with TruSeq DNA PCR-Free LT Library Prep Kit (Illumina). Libraries were quantified with KAPA Library Quantification Kit (Roche, Basel, Switzerland), Qubit (Qubit™ dsDNA HS Assay Kit, Thermo Fischer Scientific), and Agilent Bioanalyzer (DNA 7500 Kit, Agilent, Santa Clara, CA). Sequencing was done with MiSeq Reagent Kit v3 (2x250 bp paired-end sequencing, Illumina). Raw data were demultiplexed and error corrected with MIGEC (v1.2.9; over sequencing threshold: ≥ 4 reads per UMI[5]). TCRs were extracted with MIXCR (v3.0.2; library repseqio.v1.5[6]) and sequences with identical V, (D), CDR3 and J -segments were considered as one TCR clone. Further analysis was performed with VDJtools v1.2.1 (e.g. removal of non-functional clonotypes[7]), Galaxy v.19.01.rc1, Geneious v7.1.9 , and R. Origin 2018b, and Prism v.7 and 8 were used for visualization. International ImMunoGeneTics information system nomenclature (IMGT v1.2.9[8]) is used throughout the manuscript (Arden nomenclature in brackets[9]). αβ TCRs of clones were determined with a simplified protocol using 50 000 cells directly lysed in 25 μl distilled water supplemented with 1 mM DTT and 12.5 U recombinant RNAse inhibitor (Takara, Kusatsu, Japan). After a freeze thaw cycle, 3 μl lysate were directly used for reverse transcription. cDNA was amplified by the first PCR separately for α- and β-chains and sequenced with reverse PCR primers (Eurofins Genomics GmbHs, Ebersberg, Germany).

Statistics

Prism (GraphPad Software, San Diego, CA) was used for visualization and statistical analysis as indicated in the figure legends. To estimate the diversity of TCR repertoires, the normalized Shannon-Index[10] was calculated as a measurement of diversity according to the following equation:

$$E_H = \frac{-\sum_{i=1}^{S} \frac{n_i}{N} * \log_2(\frac{n_i}{N})}{\log_2 S}$$

where E_H is the normalized Shannon- Index, n_i is the frequency of a TCR, N is the sum of all counts normalized to the TCR diversity S of a TCR repertoire in a sample.

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Fig. S1

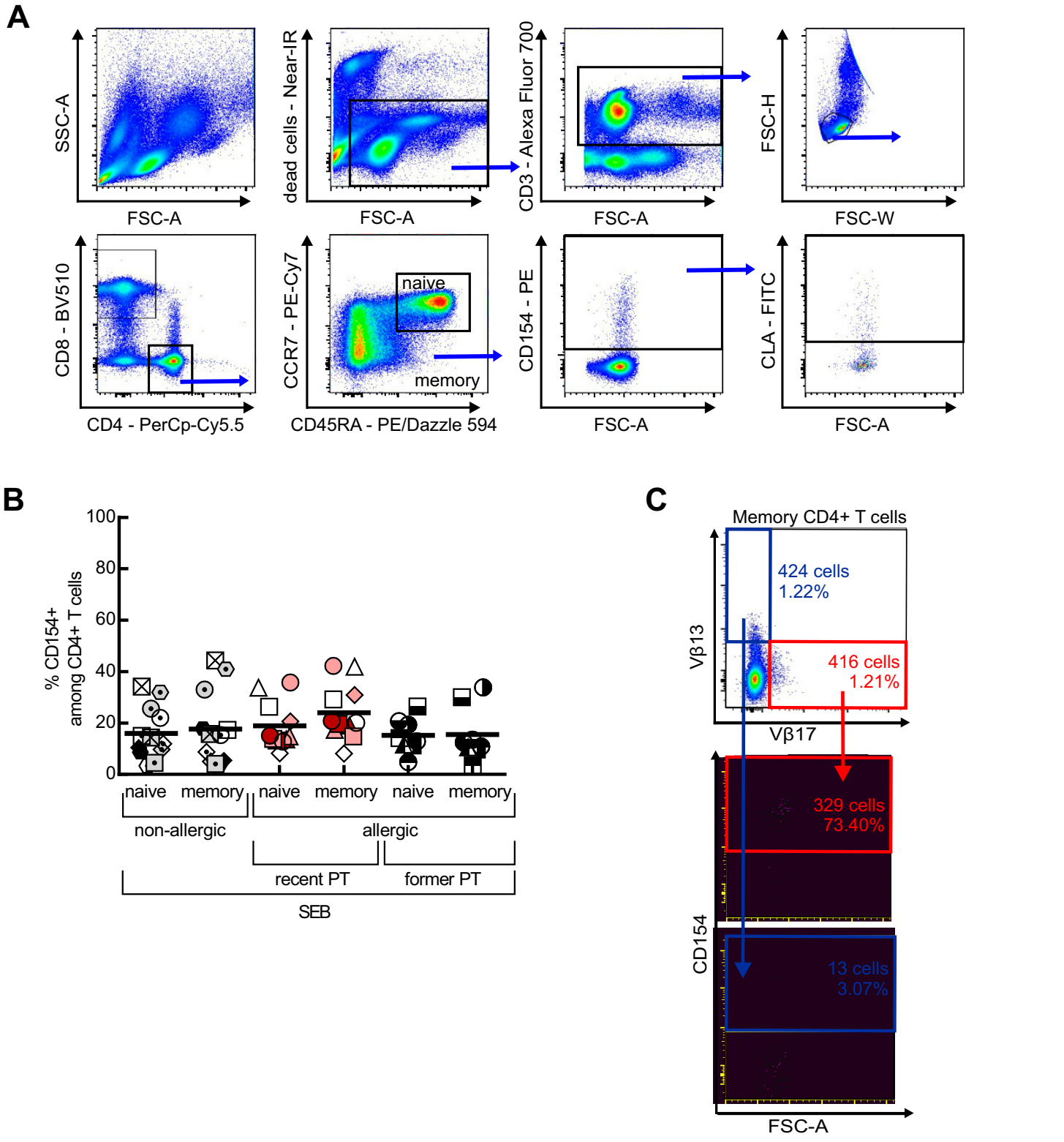

Figure S1: **(A) Gating strategy**. PBMCs were gated on live, CD3+, single, CD4+, naïve (CCR7+, CD45RA+) or memory (non-naïve) T cells to analyze antigen-induced CD154 expression (example from donor Ch23). Expression of further activation markers, e.g. CLA, was assessed among total and CD154+ T cells. **(B) SEB stimulation.** Frequencies of CD154+ naive and memory CD4+ T cells after stimulation with SEB for 5 h for all donors from the different groups (Table S1). Frequency values from samples without antigen stimulation were subtracted. Lines indicate the means. One-way nonparametric ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparison was used to exclude differences between groups. **(C) Lack of bystander CD154 expression**. CD154 upregulation was monitored on SEB-stimulated CD4+ memory T cells expressing the SEB-interacting TCR β-chain TRBV19 (Vβ17) or the non-SEB-interacting β-chain TRBV6-5 (Vβ13) (one representative experiment out of three).

Fig. S1, cont. 1

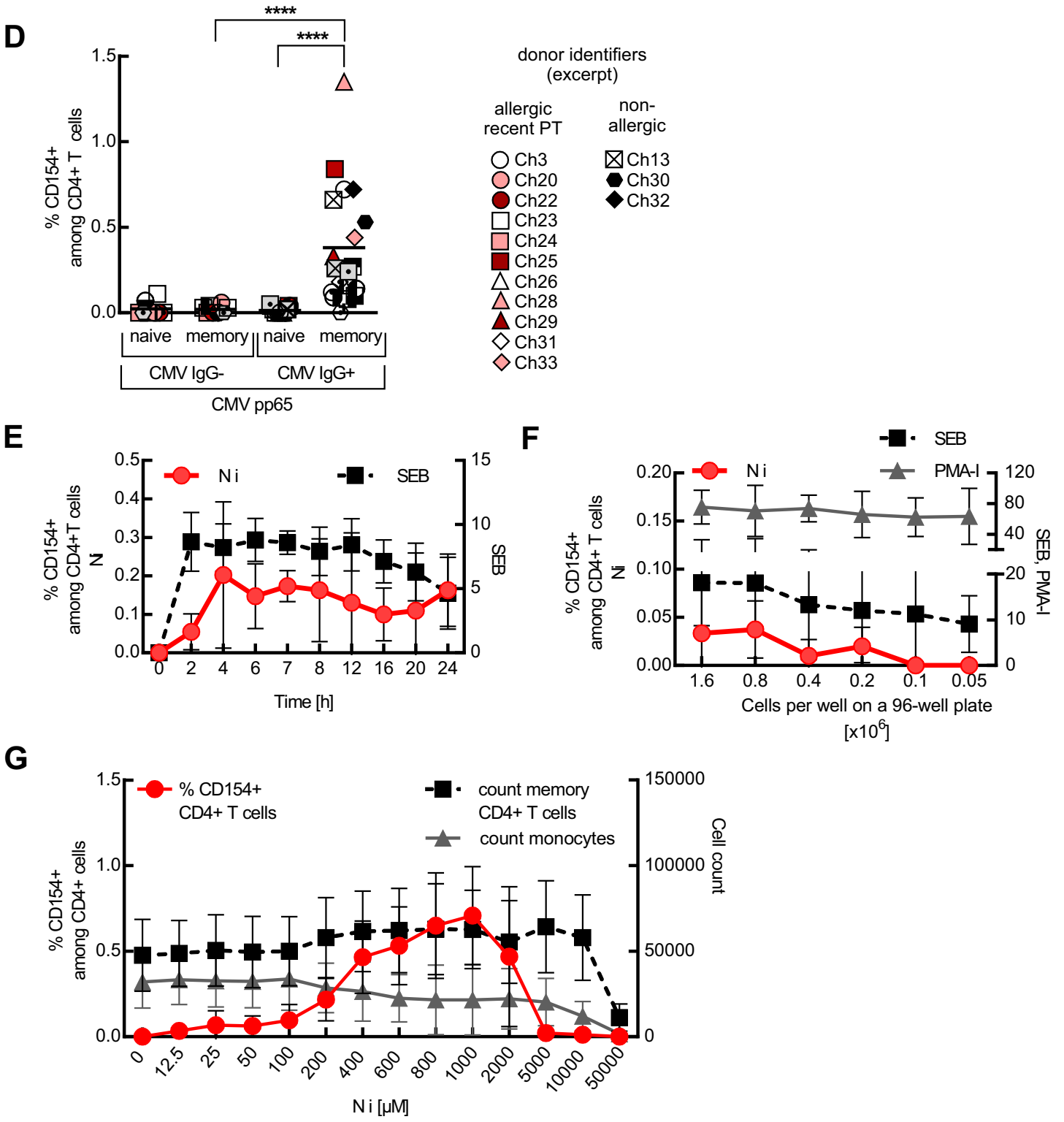

Figure S1 cont. 1:**(D) CMV pp65 stimulation.** Frequencies of CD154+ naive and memory CD4+ T cells after stimulation with CMV pp65 for 5 h for donors with negative (CMV IgG-, n = 9) and positive (CMV IgG+, n = 19) CMV titer (Table S1, also lists all donor identifiers). Lines indicate the means. One-way nonparametric ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparison was used to assess differences between groups (****p<0.0001). **(E) Time curve.** PBMCs from buffy coats (likely non-allergic donors) were stimulated for the indicated time points and CD154 expression was analyzed by flow cytometry on CD4+ T cells after Ni- (left y-axis) or SEB- (right y-axis) stimulation (n = 4 donors). **(F) APC dependency.** PBMCs were seeded in different densities and stimulated with Ni- (left y-axis), SEB-, or PMA-I (right y-axis). CD154 expression on CD4+ T cells was analyzed after 5 h (n = 3 donors). **(G) Ni-titration curve.** CD154 expression on CD4+ T cells (left y-axis) and total T cell and monocyte cell count (right y-axis) after 5 h of stimulation with different concentrations of $NiSO_4$ (n ≥3 donors). (E) - (G) Means +/- SD, frequencies of CD154+ T cells without antigen stimulation were subtracted.

Fig. S1, cont. 2

H

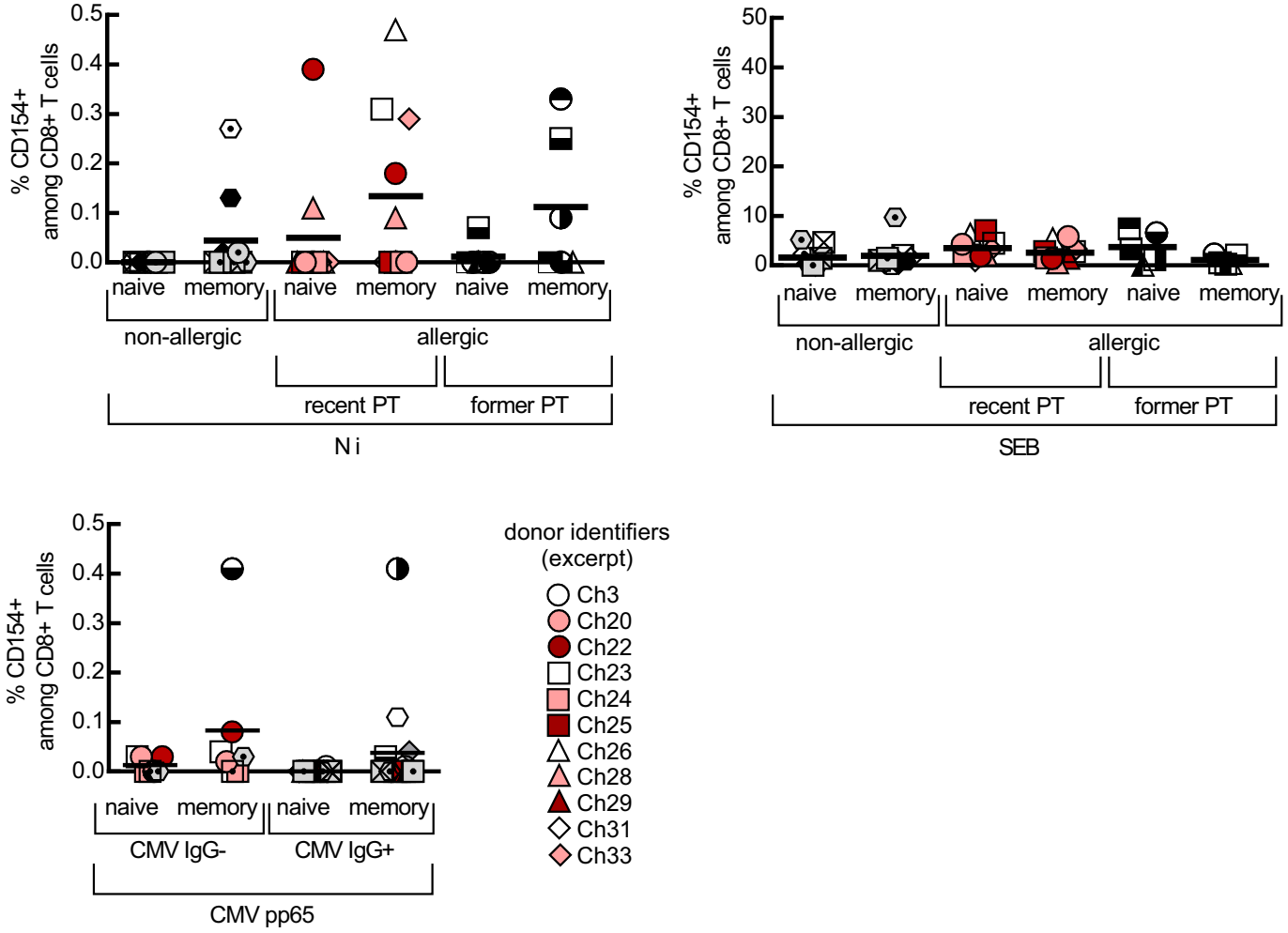

Figure S1 cont. 2: **(H) Ni-specific CD154+ CD8+ T cells.** Frequencies of CD154+ CD8+ T cells after stimulation of PBMCs with the indicated antigens for 5 h. Frequency values from samples without antigen stimulation were subtracted. Lines indicate the means. One-way nonparametric ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparison was used to exclude differences between groups. For all donor identifiers see Table S1. Non-allergic n = 11, recent patch test (PT) allergic n = 11, former PT allergic n = 7, CMV IgG- n=7, CMV IgG+ n=16.

Fig. S2

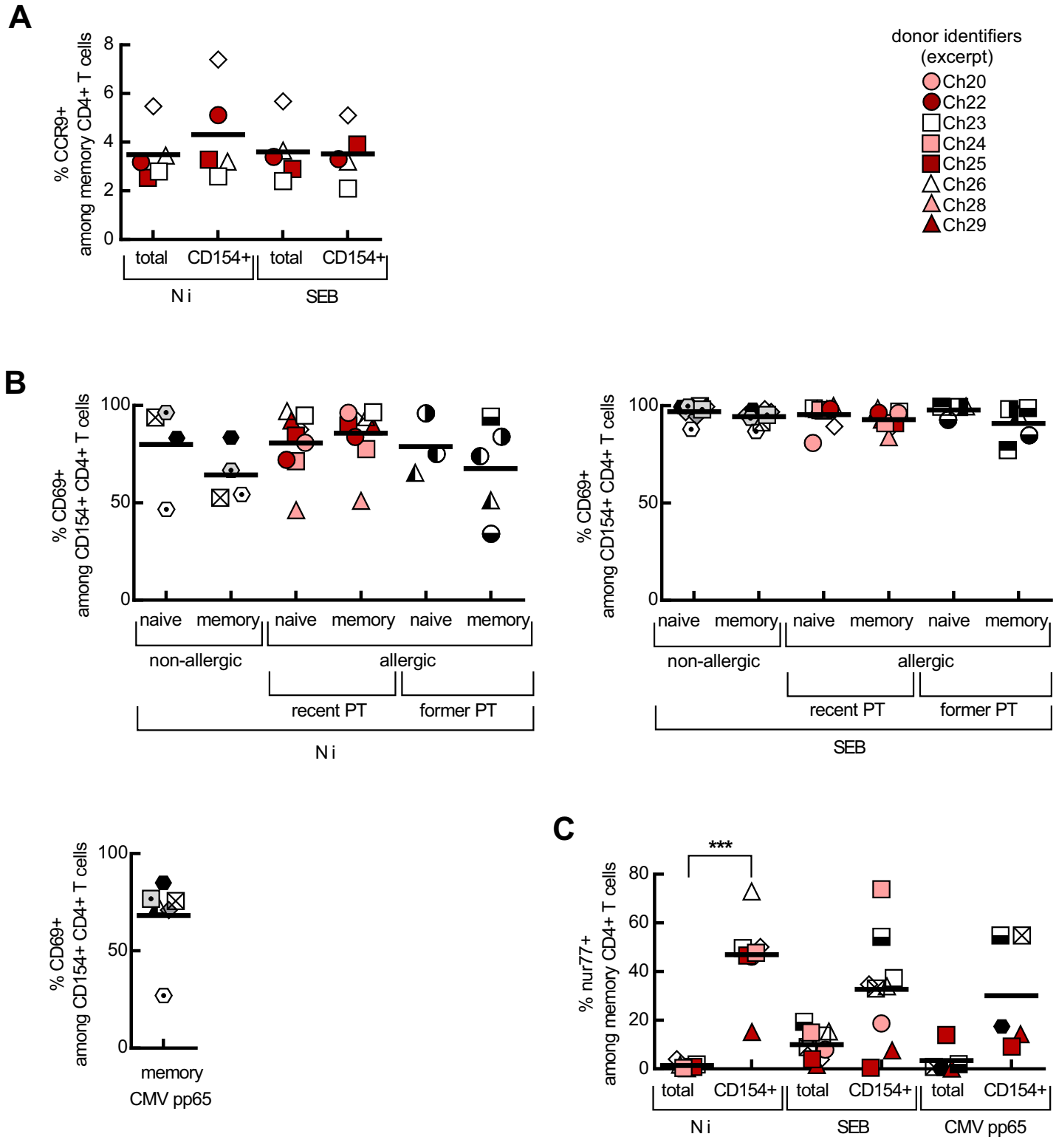

Figure S2: **Co-expression of activation marker by Ni-specific CD4+ T cells**. PBMCs were incubated with 200 µM $NiSO_4$ or with the indicated antigens for 5 h and the expression of **(A)** CCR9, **(B)** CD69, and **(C)** nur77 was analyzed for total or CD154+ T cells by flow cytometry. Only populations with ≥20 cells were analyzed. Lines indicate the means. For all donor identifiers see Table S1. Wilcoxon signed-rank test was used to observe paired differences in the same group (***p<0.001).

Fig. S3

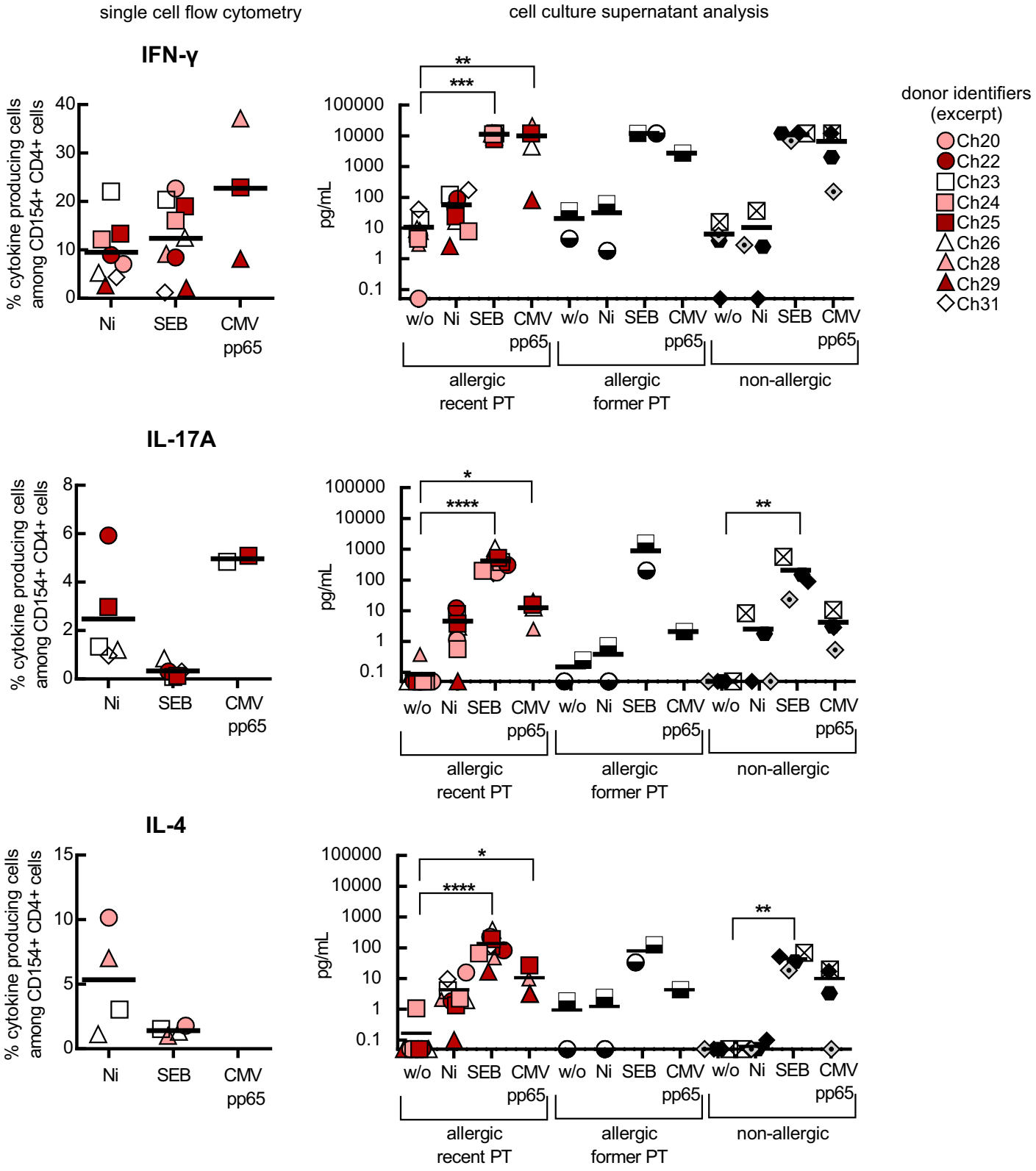

Figure S3: **Cytokine expression by Ni-specific T cells**. Left panels: Cytokine expression of CD154+ CD4+ T cells was analyzed by flow cytometry. PBMCs were stimulated with the indicated antigens in CD154 upregulation assays. Only populations with ≥20 cells are shown. For Ni-specific cells, this detection limit was only reached for samples from allergic donors with recent positive patch test. For samples without antigen stimulation cytokine and/or CD154-expressing cells were below the detection limit. Right panels: Cytokine content of cell culture supernatants as assessed by multiplex bead-based assay after 16 h of stimulation with the indicated antigens. Values below the detection limit were set to 0.05 pg/ml for graphical purposes. Lines indicate the means. For all donor identifiers see Table S1. One-way ANOVA analysis (Kruskal-Wallis) with Dunn's test for multiple comparisons was used to assess differences between groups (*p<0.05; **p< 0.01, ***p<0.001; ****p<0.0001). For IL-13, IL-21, IL-9, IL-5, IL-10, and IL-6 only supernatant was analyzed, for GM-CSF only single cells.

Fig. S3 cont. 1

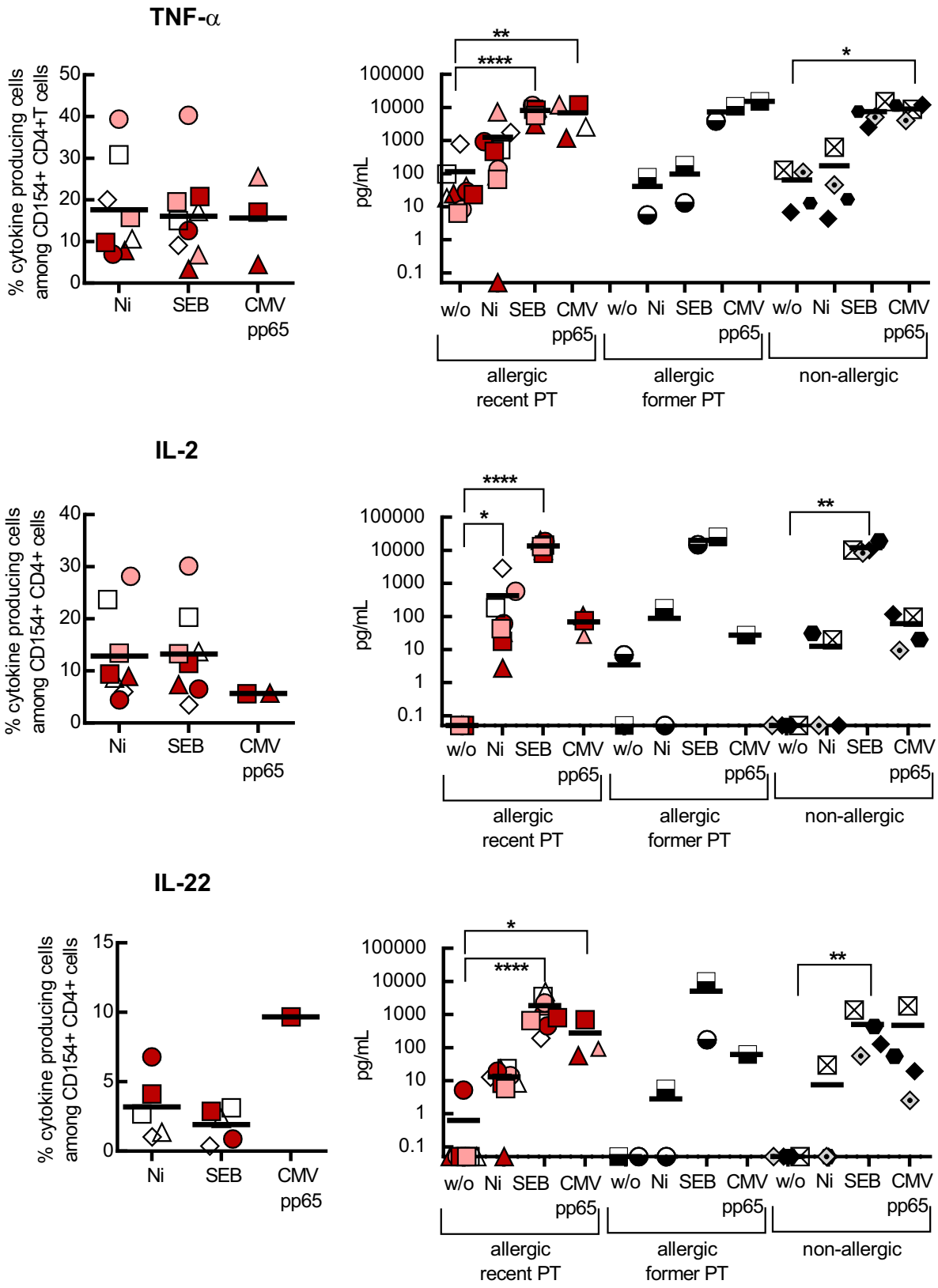

Figure S3 cont. 1: see legend of Fig. S3

Fig. S3 cont. 2

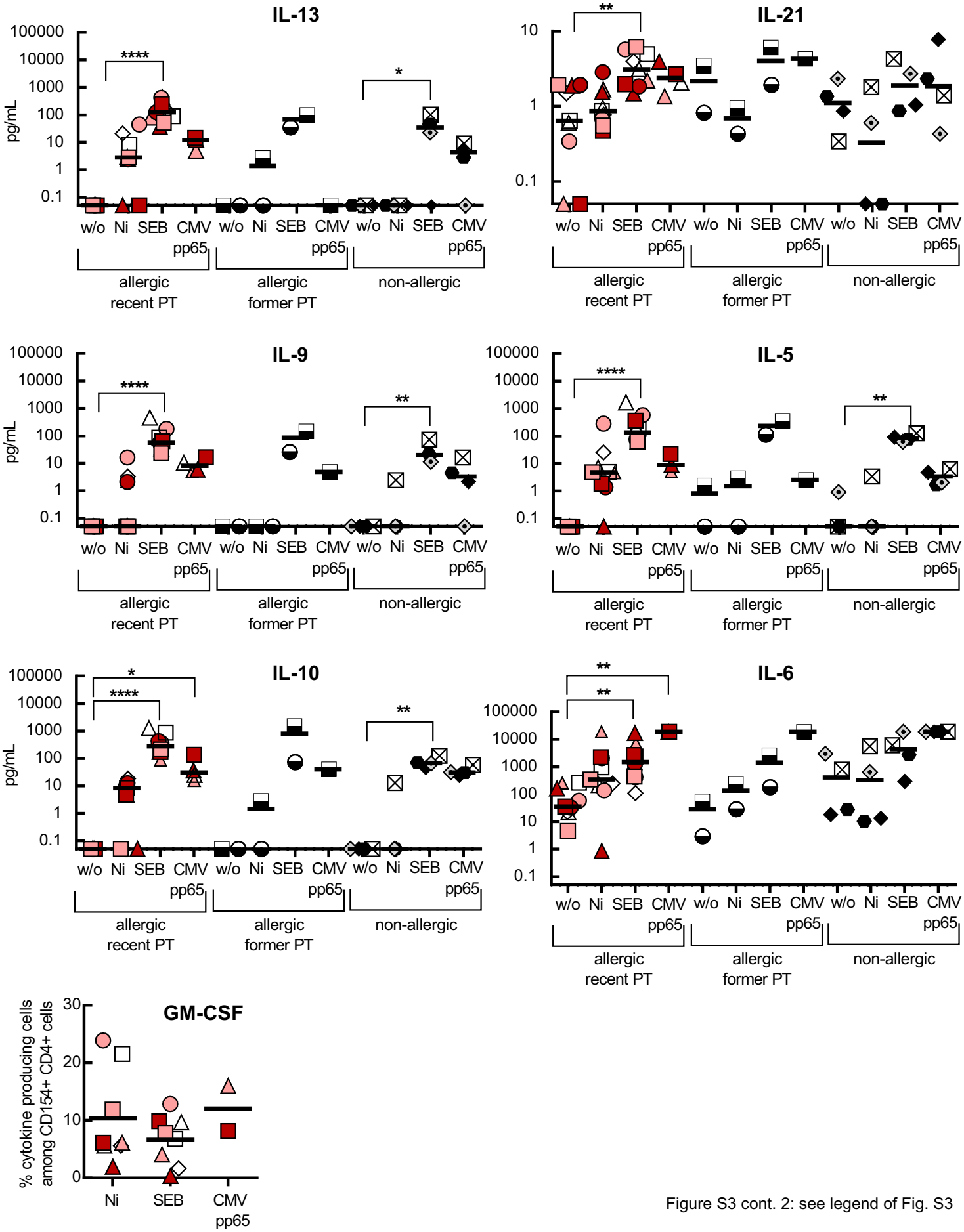

Figure S3 cont. 2: see legend of Fig. S3

Fig. S4

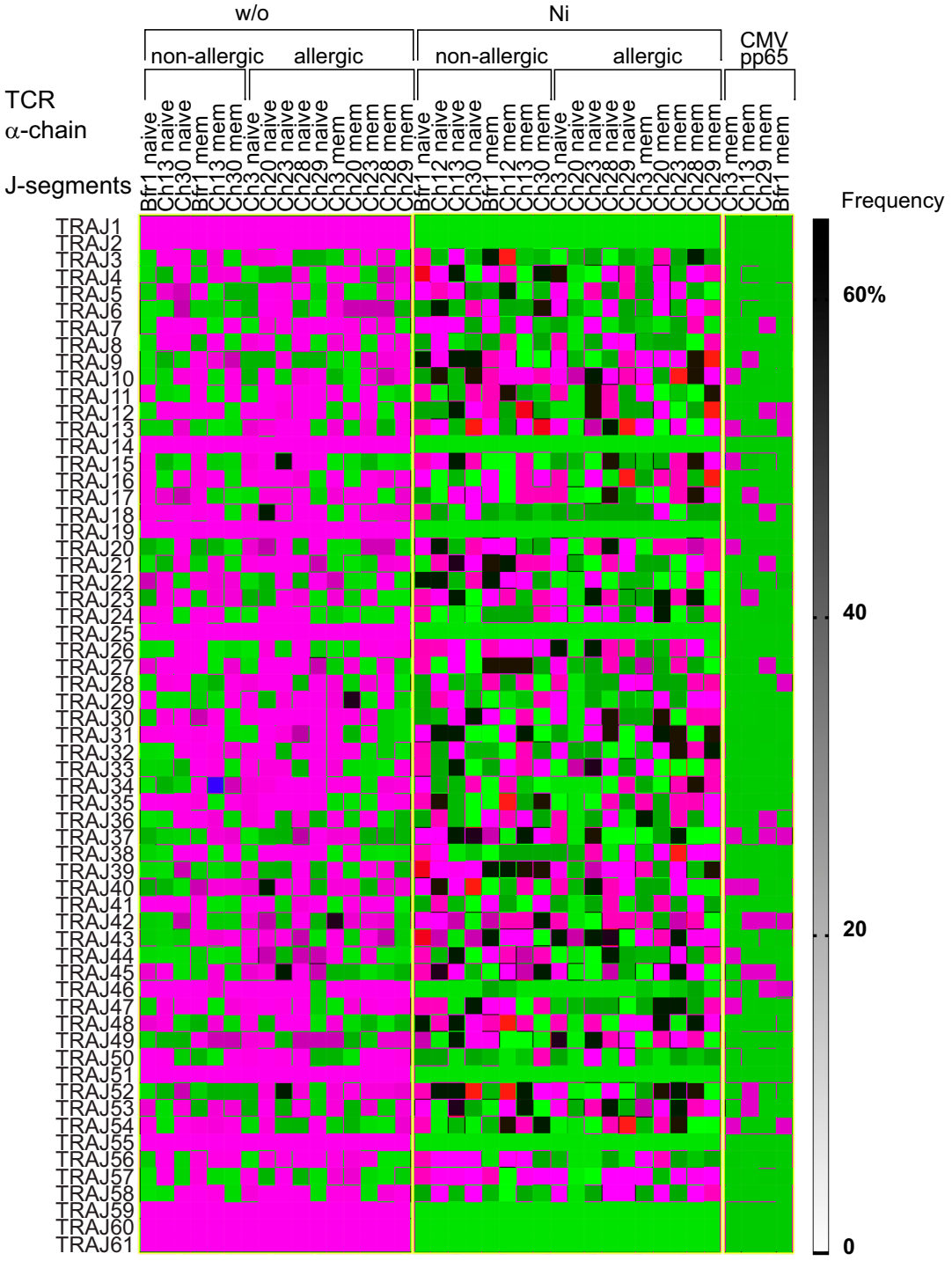

Figure S4: **TRAJ, TRBV, and TRBJ segment use.** Heat plot depicting segment use among randomly sorted CD4+ T cells (black), Ni-specific CD154+ CD4+ T cells (red), and CMV pp65-specific CD154+ CD4+ T cells (blue, frequencies). Cells were sorted from CD154 upregulation assays (Table S2) and their αβ TCRs were analyzed by high throughput sequencing.

Fig. S4 cont.

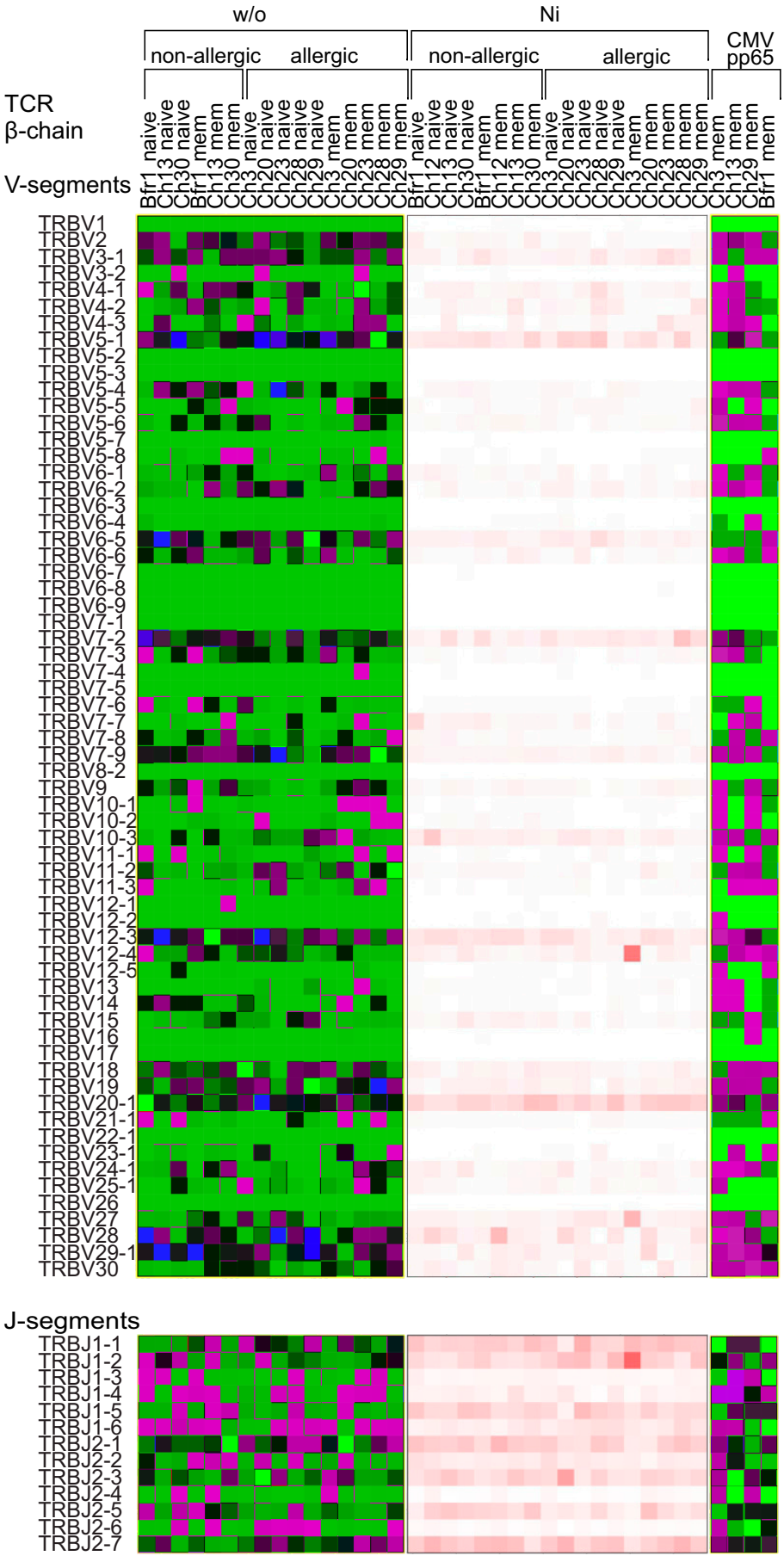

Figure S4 cont.: see legend of Fig. S4

Fig. S5

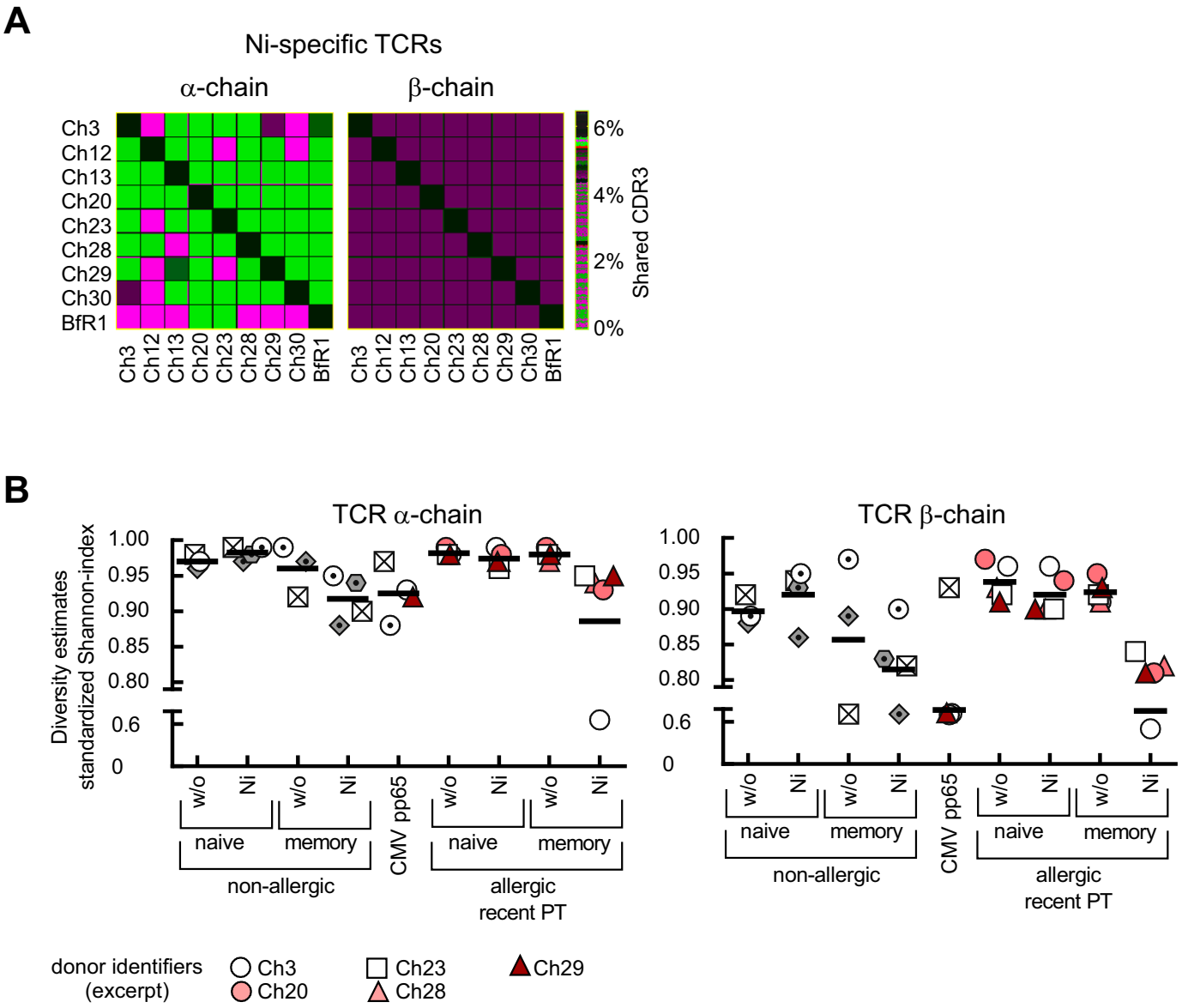

Figure S5: **(A) Clonal congruence of Ni-specific TCR**. The heat plots depict overlap of TCR α- and β-chains from different donors (frequencies, nucleotide level). Ni-specific CD154+ CD4+ memory T cells were identified by CD154 upregulation assay and their TCRs were analysed by high throughput sequencing (Table S2). **(B) TCR diversity.** Shannon index diversity estimation for the indicated CD4+ T cell populations. For all donor identifiers see Table S1. w/o – without antigen, randomly sampled CD4+ T cells; Ni - Ni-specific CD154+ CD4+ T cells, CMV pp65 - CMV pp65 - specific CD154+ CD4+ T cells.

Fig. S5 cont.

C

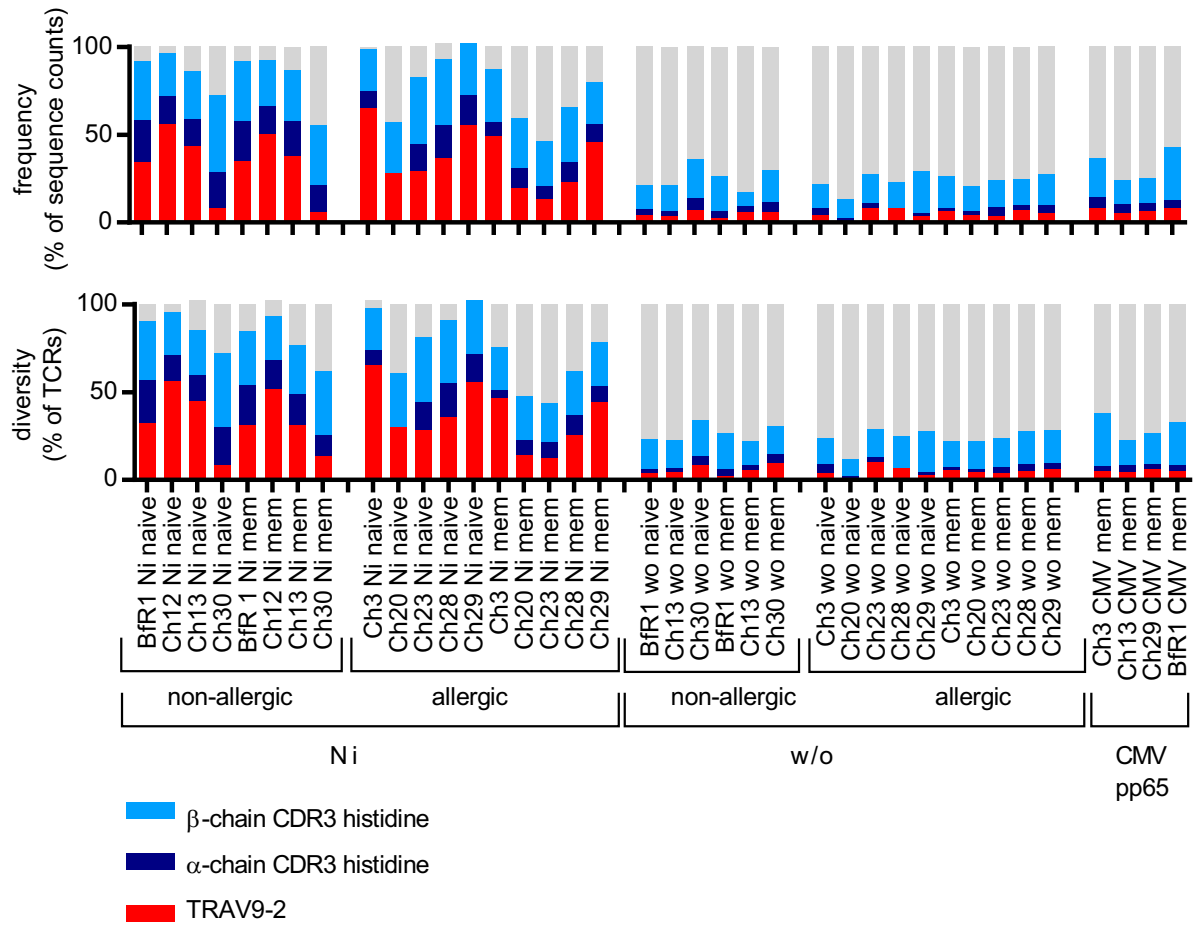

Figure S5 cont: **(C) Frequency and diversity of TCR repertoires features**. Frequency (percentages of sequence counts, upper panel) and diversity (percentages of TCR clonotypes, lower panel) of TCRs expressing TRAV9-2 (red), α-chain CDR3 histidine (dark blue) or β-chain CDR3 histidine (light blue) among the indicated samples.

Table S1 **Donor demographic and clinical characteristics**

Ni-allergic donors (recent positive patch test[†], ≤2 weeks between patch test and blood draw)													
Donor	**Ni patch test**[‡] (72h)	**Time** (days)[§]	**Age**	**Sex**	**Atopic status**[¶]	**Smoking** (ciga-rettes/ day)	**CMV IgG titer** [AE/ml]	**ID** [††]	**Frequencies** (%)				
									CD154+ naïve / memory CD4+ T cells		Co-expression by total CD4+ / CD154+ CD4+ memory T cells (Ni-stimulation[¶¶])		
									w/o	Ni[§§] (% w/o subtracted)	CLA	Ki-67	HLA-DR
Ch3	++	14	55	f	IgE	10	112	○	0.02/0.04	0.68/2.23	n.d.	n.d.	n.d.
Ch20	++	3	30	f	neg.	5-10	neg.	●	0.03/0.01	0.05/0.80	14.6/22.8	2.9/12.3	3.3/28.7
Ch22	++	3	56	m	IgE	0	neg.	●	0.02/0.02	0.68/0.64	26.6/24.2	-	-
Ch23	++	7	40	f	neg.	0	neg.	□	0.03/0.17	0.53/2.15	22.2/34.3	1.8/2.8	6.8/44.8
Ch24	++	3	51	f	neg.	0	neg.	■	0.02/0.01	0.14/0.32	27.0/29.0	1.8/5.6	2.0/7.0
Ch25	++	3	46	f	neg.	0	635	■	0.01/0.01	0.85/0.90	14.0/14.4	2.0/5.4	2.2/8.1
Ch26	+	3	30	m	A	0	neg.	△	0.02/0.02	0.46/1.15	20.5/19.3	2.5/2.8	2.5/9.4
Ch28	++	7	34	f	IgE, X	0	237	▲	0.04/0.21	0.18/0.71	25.3/36.4	-	7.1/33.3
Ch29	+++	3	65	f	neg.	0	65	▲	0.06/0.06	0.59/1.30	20.8/17.3	1.0/1.8	4.5/22.6
Ch31	++	3	28	f	IgE, A	12-15	neg.	◇	0.04/0.15	1.14/3.28	25.0/20.0	4.6/4.4	4.4/10.4
Ch33	+++	3	26	f	neg.	10	67	◆	0.01/0.02	0.18/0.70	-	-	-
Donors, n			11										
Age (year), mean (SD)			42 (13)										
Sex: female, n (%)			9 (82)										
male, n (%)			2 (18)										
Atopic, n (%)			5 (45)										

Non-allergic donors (negative Ni patch test)										
Donor	**Ni patch test**‡ (72h)	**Time** (days)§	**Age**	**Sex**	**Atopic status**¶	**Smoking** (ciga-rettes/ day)	**CMV IgG titer** [AE/ml]	**ID** ††	**Frequencies** (%) CD154+ naïve / memory CD4+ T cells	
									w/o	Ni§§ (% w/o subtracted)
BfR1	-	years	26	m	neg.	0	neg.		0.05/0.08	0.23/0.25
BfR12	-	3	34	m	neg.	0	29		0.00/0.12	0/0.00
Ch1	-	14	37	f	neg.	0	neg.		0.04/0.05	0.12/0.16
Ch2	-	3	77	m	IgE	0	neg.		0.04/0.06	0.06/0.09
Ch10	-	3	74	m	neg.	10	667		0.04/0.04	0/0.01
Ch11	-	3	67	m	IgE	0	537		0.14/0.34	0/0.35
Ch12	-	3	54	f	A	0	neg.		0.09/0.08	0.17/0.43
Ch13	-	3	33	m	IgE	2	183		0.02/0.03	0.15/0.32
Ch16	-	3	36	f	neg.	0	661		0.01/0.08	0/0.00
Ch19	-	3	30	f	neg.	0	22		0.02/0.03	0.00/0.00
Ch30	-	3	33	f	neg.	0	1025		0.01/0.05	0.27/0.34
Ch32	-	3	70	f	neg.	10	479		0.02/0.07	0/0.03
Donors, n			12							
Age (year), mean (SD)			48 (19)							
Sex: female, n (%)			6 (50)							
male, n (%)			6 (50)							
Atopic, n (%)			4 (33)							

Ni-allergic donors (former positive patch test, ≥ 3 weeks between patch test and blood draw)										
Donor	**Ni patch test**‡ (72h)	**Time** (days)§	**Age**	**Sex**	**Atopic status**¶	**Smoking** (ciga-rettes/ day)	**CMV IgG titer** [AE/ml]	**ID** ††	**Frequencies** (%) CD154+ naïve / memory CD4+ T cells	
									w/o	Ni§§ (% w/o subtracted)
BfR6	+	years	58	f	neg.	0	n.t.		0.05/0.11	0.02/0.23
Ch7	++	106	45	f	neg.	10	146		0.02/0.02	0/0.04
Ch8	++	97d	53	m	neg.	0	126		0.03/0.01	0.11/0.26
Ch14	+	n.d.	51	f	IgE	0	neg.		0.07/0.04	0.22/0.08
Ch17	+	21	79	m	neg.	0	79		0.01/0.02	0.11/0.10
Ch18	+	21	39	f	IgE	0	158		0.02/0.01	0/0.02
Ch21	+	28	58	f	IgE	0	1050		0.01/0.02	0/0.00
Ch27	++	years	36	f	IgE	0	672		0.03/0.01	0.41/0.39
Donors, n			8							
Age (year), mean (SD)			52 (13)							
Sex: female, n (%)			6 (75)							
male, n (%)			2 (25)							
Atopic, n (%)			4 (50)							

†Ni-allergic donors were split according to the time interval between positive patch test and blood draw (≤2 weeks versus ≥3 weeks, respectively) based on available donors und the assumption, that donors with a former positive patch test are less likely to have ongoing Ni-related effector immune responses. ‡Patch tests were performed with the standard series suggested by the "Informationsverbund Dermatologischer Kliniken" (IVDK, Germany) with 5% $NiSO_4$ in petrolatum (Smart Practice) in Finn Chambers for 48 h. The scoring system of the International Contact Dermatitis Research Group (ICDRG) was used. §Days between patch test and blood draw. ¶Hints for serum elevated IgE, atopic dermatitis (A), and/or allergic asthma (X), ††ID - donor identifier. Shaded rows indicate donors whose TCRs have been sequenced. §§Shown are frequencies of Ni-stimulated samples after subtraction of background frequencies from samples without antigen stimulation (w/o). ¶¶Only populations with ≥20 cells have been analyzed for co-expression of activation markers by CD4+ memory T cells and by activated, CD154+ fraction. For CD154+ cells, the detection limit was not reached for Ni-stimulated samples from

non-allergic donors or for allergic donors with former positive patch test on a 96-well plate format (data not shown). n.d. – not determined, neg. - negative.

Table S2 **Summary of αβ TCR sequencing data**

Summary of sorted CD4+ T cells for αβ TCR high throughput sequencing. One sequence count represents a single transcribed cDNA identified by one distinct UMI. Counts with identical V, (D), CDR3 and J segments represent one TCR clone (diversity). Only functional recombination events are included.

Donor	antigen	CD4+ (CD154+[§]) population	number of sorted cells		counts (UMI)	diversity
Ch3	w/o	memory	2000	α	193	162
				β	394	280
		naïve	2000	α	179	144
				β	274	231
	Ni[†]	memory	3000[‡]	α	1964	438
			3000	β	2235	445
			3000			
		naïve	1457[‡]	α	409	351
			1308	β	733	609
			1816			
	CMV pp65[†]	memory	3000[‡]	α	598	304
			1083	β	1098	343
			739			
Ch20	w/o	memory	3000	α	178	150
				β	204	172
		naïve	1000	α	51	44
				β	55	50
	Ni	memory	3000	α	377	207
				β	290	150
		naïve	1000	α	57	46
				β	63	52
Ch23	w/o	memory	3000	α	505	355
				β	707	478
		naïve	1000	α	100	75
				β	153	113
	Ni	memory	3000	α	994	468
				β	910	434
		naïve	1000	α	327	192
				β	324	208
Ch28	w/o	memory	3000	α	141	101
				β	347	240
		naïve	809	α	45	37
				β	100	78
	Ni	memory	3000	α	1235	535

				β	1175	505
		naïve	809	α	328	204
				β	440	267
Ch29	w/o	memory	3000	α	375	266
				β	544	384
		naïve	1000	α	69	57
				β	164	115
	Ni	memory	3000	α	1347	587
				β	1682	622
		naïve	1000	α	299	192
				β	421	267
	CMV pp65	memory	3000	α	767	284
				β	1152	323
BfR1	w/o	memory	2000	α	103	92
				β	202	179
		naïve	2000	α	174	141
				β	232	178
	Ni†	memory	138‡	α	368	256
			1228	β	471	313
			988			
		naïve	613‡	α	152	142
			739	β	274	225
			699			
	CMV†	memory	738‡	α	251	113
			1836	β	340	136
			1130			
Ch12	Ni	memory	3000	α	1367	653
				β	1111	523
		naïve	3000	α	754	539
				β	676	488
Ch13	w/o	memory	3000	α	194	140
				β	543	281
		naïve	3000	α	203	171
				β	442	329
	Ni	memory	3000	α	840	389
				β	799	391
		naïve	3000	α	123	101
				β	186	147
	CMV pp65	memory	2577	α	281	198
				β	221	166
Ch30	w/o	memory	2000	α	449	285
				β	553	327
		naïve	1000	α	210	125
				β	268	161

	Ni	memory	2000	α	1961	460
				β	1980	457
		naïve	1000	α	628	338
				β	767	385
Jurkat T cells	w/o		3000	α	1381	2
				β	206	1

[†]Magnetic enrichment of CD154+ cells prior to sort for TCR sequencing. [‡]Triplicate samples. TCR sequences of all three samples were pooled with VDJtools for further analysis.
[§]CD154+ T cells for were sorted to obtain Ni- and CMV pp65-specific cells. Samples without antigen stimulation (w/o), were used to sort random CD4+ T cells.

Table S3 **Primers and PCR conditions for high throughput sequencing of TCR α- and β-chains**

Reverse transcription	Primers and PCR condition
SMART adapter bc1R ACR_st4	AAGCAGUGGTAUCAACGCAGAGU NNNN U NNNN U NNNN UCTT(rG)4 CAGTATCTGGAGTCATTGA GTCTAGCACAGTTTTGTC
Reaction mixture (setup according to manufacturer)	3 µl AcR St4/bc1R (2 µM each, final dilution 0.2 µM) 3 µl Smart Adapter (2 µM, final dilution 0.2 µM 9 µl RNA 6 µl 5X First-Strand Buffer 3 µl dNTP (10 mM, final dilution, 1 mM) 3 µl DTT (20 mM, final dilution 2 mM) 3 µl SMARTScribe RT (Takara, final dilution 10 U/µl) 1.5 µl Recombinant RNase Inhibitor (Takara)
Temperature program	72°C 3 min 4°C 2 min 42°C 90 min 70°C 15 min 37°C 42 minutes, + 3 µl Uracil-DNA Glycosylase (UDG, NEB) 4°C ∞ Purification of cDNA with MinElute PCR Purification Kit (Qiagen)
1st PCR	
bc2R ac2R Na-SBX-M1_V2	TGCTTCTGATGGCTCAAACAC TACACGGCAGGGTCAGGGT CGAGCGTGACGACGACAG XXXXXX GTGGTATCAACGCAGAGT
Reaction mixture (setup according to[17])	10.5µl H_2O 10 µl Q5® Reaction Buffer 1 µl dNTP (final dilution 0.2 mM) 2 µl ac2R/bc2R-mix (final dilution 0.4 µM) 2 µl Na-SBx-M1_V2 (final dilution 0.4 µM) 0.5 µl Q5® High-Fidelity DNA Polymerase (NEB) 10 µl purified cDNA
Temperature program	95°C 2 min 18 cycles ○ 95°C 10 s ○ 61°C 20 s ○ 72°C 50 s 72°C 4 min 4°C ∞ Purification of PCR product with QIAquick PCR Purification Kit (Qiagen)

2nd PCR	
N2Na N3Na N4Na ac3R-SBX bc3R-SBX	NNCGAGCGTGACGACGACAG NNNCGAGCGTGACGACGACAG NNNNCGAGCGTGACGACGACAG XXXXXX GGGTCAGGGTTCTGGATAT XXXXXX ACACSTTKTTCAGGTCCTC
Reaction mixture (setup according to[17]; separate for TCR α- and β-chain)	30.5 µl H2O 10 µl Q5® Reaction Buffer 1 µl dNTP (final dilution 0.2 mM) 2 µl NNa-mix (final dilution 0.4 µM) 2 µl ac3R-SBX or bc3R-SBX (final dilution 0.4 µM) 0.5 µl Q5® High-Fidelity DNA Polymerase (NEB) 4 µl purified product of 1st PCR
Temperature program	95°C 2 min ~19 cycles (optimal cycle number determined for each sample): ○ 95°C 10 s ○ 61°C 20 s ○ 72°C 50 s 72°C 4 min 4°C ∞
Sample barcodes (SB)	
SB8 SB9 SB10 SB11 SB12 SB13 SB14 SB16 SB17 SB18 SB19 SB20	ACTTCA ATCCTA CAACTT CCTAAT CGGTCT CTCGGT GCTCTG TAATCC TCTGGC TGGCTC TTCAAC GTCTCG

Table S4 **Matching αβ TCRs of Ni-specific CD4+ T cell clones**

V- und J-segment use and CDR3 amino acid sequences of Ni-specific CD4+ T cell clones. Clones were derived from single CD154+ CD4+ memory T cells were sorted from a CD154 upregulation assay with Ni-stimulated PBMCs. Ni-specificity of clones was confirmed by a Ni restimulation assay. TCRs with TRAV9-2 segments and CDR3 histidines are colored in red and orange.

	Clone	Chain	TRAV/BV	TRAJ/BJ	CDR3 amino acid sequence
1	BfR2Ni1C	α	TRAV41	TRAJ53	CAGGSGGSNYKLTF
		β	TRBV28	TRBJ2-4	CASSAGRKNIQYF
2	Ch5Ni5D	α	TRAV8-3	TRAJ6	CAVLSSGGSYIPTL
		β	TRBV5-6	TRBJ2-7	CASSLRLAGGYEQYF
3	**Ch13Ni3B**	α	TRAV29/DV5	TRAJ52	CAAKGTYAGGTSYGKLTF
		β	TRBV12-4	TRBJ2-1	CASS**H**PTGYNEQF
4	**Ch13Ni3C**	α	TRAV3	TRAJ30	CAVRDMGRDDKIIL
		β	TRBV28	TRBJ2-7	CASS**H**GT**H**SYEQYF
5	**Ch13Ni8F**	α	TRAV2	TRAJ40	CAVD**H**SGTYKYIL
		β	TRBV19	TRBJ2-7	CASSTRDFNEQYF
6	**Ch13Ni11F**	α	TRAV13-2	TRAJ31	VQRISRAGTMPDSV
		β	TRBV7-7	TRBJ1-5	CASSLDPQGPQ**H**F
7	**Ch32 Ni5E**	α	**TRAV9-2**	TRAJ6	CALTSAGGSYIPTL
		β	TRBV20-1	TRBJ1-1	CSAGDAGQGRTEAFF
8	**Ch32Ni8C**	α	TRAV24	TRAJ39	CAFRDAGNMLTL
		β	TRBV5-6	TRBJ1-5	CSSSLARDVSTQPQ**H**F
9	**Ch33Ni2F**	α	**TRAV9-2**	TRAJ27	CALSEWNTNAGKSTF
		β	TRBV20-1	TRBJ2-7	CSASRDRFSYEQYF
10	Ch33Ni2H	α	TRAV29/DV5	TRAJ44	CAASALNTGTASKLTF
		β	TRBV11-3	TRBJ2-3	CASSFASTDTQYF
11	Ch33Ni3A	α	TRAV3	TRAJ4	CAVPTSGGYNKLIF
		β	TRBV7-2	TRBJ2-7	CASSLVLTPPGYEQYF
12	**Ch33Ni4E**	α	TRAV20	TRAJ58	CAVGETSGSRLTF
		β	TRBV6-6	TRBJ1-6	CASSYRGAENSPL**H**F

13	**Ch33Ni4G**	α	TRAV20	TRAJ58	CAVGETSGSRLTF
		β	TRBV6-6	TRBJ1-6	CASSYRGAENSPL**H**F
14	Ch33Ni6A	α	TRAV8-1	TRAJ39	CAVNEADAGNMLTL
		β	TRBV10-3	TRBJ2-3	CATSSGGVTDTQYF
15	**Ch33Ni6E**	α	TRAV20	TRAJ5	SAVQVNQFYF
		β	TRBV12-5	TRBJ2-3	CASG**H**DSTTDTQYF
16	Ch33Ni6G	α	TRAV21	TRAJ22	CAVRPGSARQLTF
		β	TRBV6-6	TRBJ2-3	CASSYRLAGDSTDTQYF
17	**Ch33Ni7C**	α	**TRAV9-2**	TRAJ37	CALSESNTGKLIF
		β	TRBV7-2	TRBJ2-5	CASSLNAGRETQYF
18	**Ch33Ni8G**	α	**TRAV9-2**	TRAJ3	CALSGSSASKIIL
		β	TRBV29-1	TRBJ2-1	CSVLAGSSYNEQFF
19	**Ch33Ni9B**	α	**TRAV9-2**	TRAJ58	CALKETSGSRLTF
		β	TRBV3-1	TRBJ2-5	CASSQWASGGAETQYF
20	Ch33Ni9H	α	TRAV3	TRAJ34	CAVRPPSGGGFKTIF
		β	TRBV10-3	TRBJ2-3	CAISPGGGRGTDTQYF
21	**Ch33Ni11A**	α	TRAV30	TRAJ28	CGTEIRSGAGSYQLTS
		β	TRBV6-6	TRBJ1-5	CASSYRGSNQPQ**H**F
22	**Ch33Ni11B**	α	**TRAV9-2**	TRAJ57	CALRTQGGSEKLVL
		β	TRBV27	TRBJ1-1	CASSLFGGTEAFF

Annex II: Supplementary Material

Research paper: Unique and common TCR repertoire features of Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific human CD154+CD4+ T cells

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Published online on 25 August 2022 in:
Allergy. 2022; 00: 1–13
Link: https://doi.org/10.1111/all.15494

Appendix S1

Supporting methods

Blood samples and PBMC isolation

In addition to fresh blood samples from patients (S-Monovette CPDA1, Sarstedt), buffy coats from Deutsches Rotes Kreuz were analyzed. Serum cytomegalovirus (CMV) IgG titers were determined at Institut für Laboratoriumsmedizin Berlin (IFLb). Peripheral blood mononuclear cells (PBMC) were isolated within 2 hours of a fresh blood collection using Ficoll Paque Plus (GE-Healthcare) and cultured in T cell media (TCM). TCM consisted of RPMI 1640 medium supplemented with 5% (v/v) human AB serum (both PAN Biotech) or autologous serum in case of fresh blood samples, 2 mM glutamine (GlutaMAX), 55 µM β-mercaptoethanol (both Thermo Fisher Scientific), 1x non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (all PAN Biotech), 1x Penicillin/Streptomycin (Capricorn Scientific) and 1.5 mM $CaCl_2$ (Sigma Aldrich) for optimal T cell responses[1].

Antigen stimulation assays

Depending on the available cell numbers, antigen stimulation assays were performed on 96- to 12-well flat-bottom tissue culture plates in the presence of CD40 blocking antibody to prevent CD40 binding-induced downregulation of freshly expressed CD154[2,3]. For metal salt titration experiments, PBMC from buffy coats (likely non-allergic individuals) provided sufficient input cell numbers. For MHC block experiments, functional antibodies were added 30 min prior to antigen stimulation. Reagents and antigens are listed in **Supplementary Methods Table 1.**

Supplementary methods Table 1: Reagents and antigens for antigen stimulation assays

Reagent	Clone	Concentration stock / final	Company	Catalog number and/or identifier
CD40	HB14	100 µg/m 1 µg/ml	Miltenyi Biotec	Cat#: 130-094-133 RRID: AB_10839704
CD28	15E8	100 µg/ml 1 µg/ml	Miltenyi Biotec	Cat#: 130-093-375 RRID: AB_1036134
$NiSO_4$ x $6H_2O$ (Ni^{2+})		40 mM 400 µM	Sigma-Aldrich	Cat#: 31483-100G
$CoCl_2$ x $6H_2O$ (Co^{2+})		40 mM 400 µM	Sigma-Aldrich	Cat#: C8661-25G
$PdCL_2$* (Pd^{2+})		80 mM 400 µM	Sigma-Aldrich	Cat#: 205885-1G
$PtCl_2$ (Pt^{2+})		40 mM 400 µM	Sigma-Aldrich	Cat#: 206091-1G
$CrCl_3$ x $6H_2O$ (Cr^{3+})		40 mM 400 µM	Sigma-Aldrich	Cat#: 27096-100G-F
$HAuCl_4$ x $3H_2O$ (Au^{3+})		40 mM 400 µM	Sigma-Aldrich	Cat#: 520918-5G
$CuSO_4$ x $5H_2O$ (Cu^{2+})		40 mM 400 µM	Sigma-Aldrich	Cat#: C-8027
$BeSO_4$ x $4H_2O$ (Be^{2+})		40 mM 400 µM	Sigma-Aldrich	Cat#: 202789-50G
Tetanus toxoid (TT)		5 mg/ml 5 µg/ml	NIBSC	Cat#: 02/232
Cytomegalovirus CMV lysate (CMV)		1 mg/ml 1 µg/ml	Biozol	Cat#: NAC-CMV-CL-100

Reagent	Clone	Concentration stock / final	Company	Catalog number and/or identifier
HLA-DR, -DP, -DQ	Tu39	500 µg/ml 10 µg/ml	BD Biosciences	Cat# 555556 RRID: AB_395938
HLA-DR	Ac122	1 mg/ml 10 µg/ml	Miltenyi Biotec	Cat# 130-108-056 RRID: AB_2661330
HLA-DR	L243	1 mg/ml 10 µg/ml	BioLegend	Cat# 307602 RRID: AB_314680
IgG2a isotype control	MOPC-173	1 mg/ml 10 µg/ml	BioLegend	Cat#: 400224 RRID: AB_2861018
Staphylococcus enterotoxin B (SEB)		1 mg/ml 1 µg/ml	Sigma-Aldrich	Cat#: S4881-1MG

*At concentrations >800 µM, $PdCl_2$ was insoluble.

Antibody staining, flow cytometry analysis and cell sorting

Antibodies were titrated using SEB-stimulated PBMC from buffy coats to determine optimal dilutions and staining mix volumes according to input cell numbers (96-well plates - 15 µl, 48- and 24-well plates 20 µl, 12-well plates 40 µl). Antibody staining mixes were prepared in FACS buffer (PBS, 2 mM EDTA, 0.5% FCS). In case of Annexin V labeling, staining, washing and final resuspension steps were performed using FACS buffer with additional $CaCl_2$ (2.5 mM). Cells were stained for 20 min at room temperature in the dark. Information on antibodies used for flow cytometry analysis is listed in **Supplementary Methods Table 2.**

Supplementary Methods Table 2: Antibodies and reagents for flow cytometry analysis

Antigen	Clone	Fluorochrome conjugate	Dilution	Company	Catalog number and/or identifier
CD3	UCHT1	AlexFluor® 700	1:160	BioLegend	Cat#: 300424 RRID: AB_493741
CD4	SK3	BB700	1:80	BD Biosciences	Cat#: 566392 RRID: AB_2744421
CD8	SK1	BV510	1:320	BioLegend	Cat#: 344732 RRID: AB_2564624
CD45RA	HI100	PE/Dazzle™ 594	1:320	BioLegend	Cat#: 304146 RRID: AB_2564079
CCR7	G043H7	PE-Cy7	1:80	BioLegend	Cat#: 353226 RRID: AB_11126145
CD154	5C8	PE	1:320	Miltenyi Biotec	Cat#: 130-113-607 RRID: AB_2751142
CD69	FN50	BV711	1.160	BioLegend	Cat#: 310944 RRID: AB_2566466
CD14	M5E2	BV421	1:320	BioLegend	Cat# 301830 RRID: AB_10959324
CD19	HIB19	APC	1:160	BD Biosciences	Cat#: 555415 RRID: AB_398597
CLA	HECA-452	FITC	1:40	Biolegend	Cat#: 321306 RRID: AB_492898
AnnexinV		FITC	1:80	Biolegend	Cat#: 640906

Dead cell stain	-	*		Thermo Fisher Scientific	Cat#: L10119

Sample analysis was done on a BD FACSAria III equipped with BD Diva 9.0.1 software (BD Biosciences) and three lasers (405 nm, 488 nm and 633 nm) and mirror/filter combinations for the simultaneous detection of 11 colors. For samples from 96-well plates, all events were acquired. For samples from 48-well to 12-well plates, a smaller number of total events (typically 100,000 or 1 million total events) was acquired and for the remaining cells all events within the SSC single cell gate (see gating strategy, **Figure S1**). Thus, data for all CD4+ T cells within a sample were acquired. Since metal-specific CD154+CD4+ T cells were relatively frequent, no magnetic enrichment was required in this study[4]. Data were further analyzed and visualized with FlowJo (V.10.7.1, BD Biosciences). Cells were sorted in single-cell mode for the expansion of antigen-specific T cell clones or in purity mode for the collection of T cells for TCR sequencing.

Generation of T cell clones and restimulation assays

Single CD154+CD4+ memory T cells were sorted into 200 µl of cloning medium (TCM without extra $CaCl_2$, with 15% human AB serum, 200 U/ml interleukin (IL)-2 (IS grade, Miltenyi Biotec), 30 ng/ml a-CD3 (OKT-3, pure, Miltenyi Biotec) and 200,000 allogeneic mitomycin C-treated PBMC, as feeder cells, on 96-well flat bottom plates. Clones were further expanded in medium without feeder cells and OKT-3 antibody and, if required, with Dynabeads Human T Activator CD3/CD28 beads (Thermo Fisher Scientific). Cloning expansion efficiency was typical ~30% of sorted cells. Before restimulation, clones were rested for 3 days in medium without IL-2 in 10% human AB serum. Restimulation experiments were performed in the presence of the indicated antigens using cryopreserved, thawed and overnight rested autologous CD3-depleted PBMC (human CD3 MicroBeads and LD columns, Miltenyi Biotec) in 384-well plates. For cryopreservation, cells were resuspended in 10% DMSO in fetal calf serum (FCS) slowly cooled to -80°C and transferred to liquid nitrogen the next day for long term storage. Restimulation conditions were the same as for bulk culture experiments with the additional costimulatory CD28 antibody (**Supplementary Methods Table 1**).
Feeder cells were prepared by incubation of $50x10^6$ PBMC/ml with 50 µg/ml mitomycin C (Abcam) in PBS and 10% FCS (Biochrome) for 30 minutes at 37°C with occasional mixing and extensive washing afterwards. Cells were kept in liquid nitrogen until use.
Clones were considered positively restimulated when the following conditions were fulfilled: (i) the frequency of CD154+CD4+ cells were 3-times higher in antigen-stimulated samples compared to samples without antigen, (ii) the frequency of CD154+CD4+ T cells after phorbol-12-myristate-13-acetate (PMA)-ionomycin stimulation was >30%.

TCR sequencing and data analysis

All antigen-specific CD154+CD4+ memory T cells in a sample or a defined number of random CD4+ memory T cells from samples without antigen stimulation (usually 100,000 cells) were sorted directly into 1 ml buffer RLT (Qiagen) and stored at -80°C. RNA was extracted with RNeasy Micro Kit (Qiagen). Reverse transcription with the introduction of a unique molecular identifier (UMI)-containing SMART adapter, PCR amplification, library preparation and Illumina sequencing for TCR αβ chains were performed as described[5]. During PCR amplification, sample barcodes were introduced at both ends of the amplicons. PCR products were purified with QIAquick PCR Purification Kit (Qiagen). Sequencing adapters were annealed to pooled, ~300-600 bp size selected PCR products with beads (0.65 ratio, CleanPCRNA kit, GC Biotech) using TruSeq DNA PCR-Free Low Throughput Library Prep Kit (Illumina). Libraries were quantified using a Qubit device (Qubit™ dsDNA HS Assay Kit, Thermo Fisher Scientific). Sequencing was performed with MiSeq Reagent Kit v3 (2x250 bp paired-end sequencing, Illumina). Raw data were demultiplexed and error corrected with MIGEC (v1.2.9; over sequencing threshold ≥ 4 reads per UMI)[6]. TCR were extracted with MIXCR (v3.0.13; library repseqio.v1.6)[7]. Further analysis was performed with VDJtools v1.2.1 (e.g., removal of non-functional clonotypes)[8]. Sequences with identical

V-, (D-) and J-gene segments and CDR3 nucleotide sequence were considered as one TCR clonotype. The CDR3 amino acid occurrence and TCR sharing between samples were assessed using custom Python software (available upon reasonable request). In case of replicate samples, all samples were processed independently and mean values were used for TCR further repertoire analysis. International ImMunoGeneTics information system nomenclature (IMGT) is used throughout the manuscript.

Quantification and statistical analysis

Data were analyzed using GraphPad Prism 8. Statistical tests are indicated in the respective figure legends. A p-value <0.05 was considered statistically significant. For flow cytometry data, only populations with more than 20 antigen-specific CD154+CD4+ T cells per well were considered for data analysis (except for metal titration experiments shown in **Figure S2**). In the case of replicate data, we calculated the mean.

Supplementary References

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Figure S1

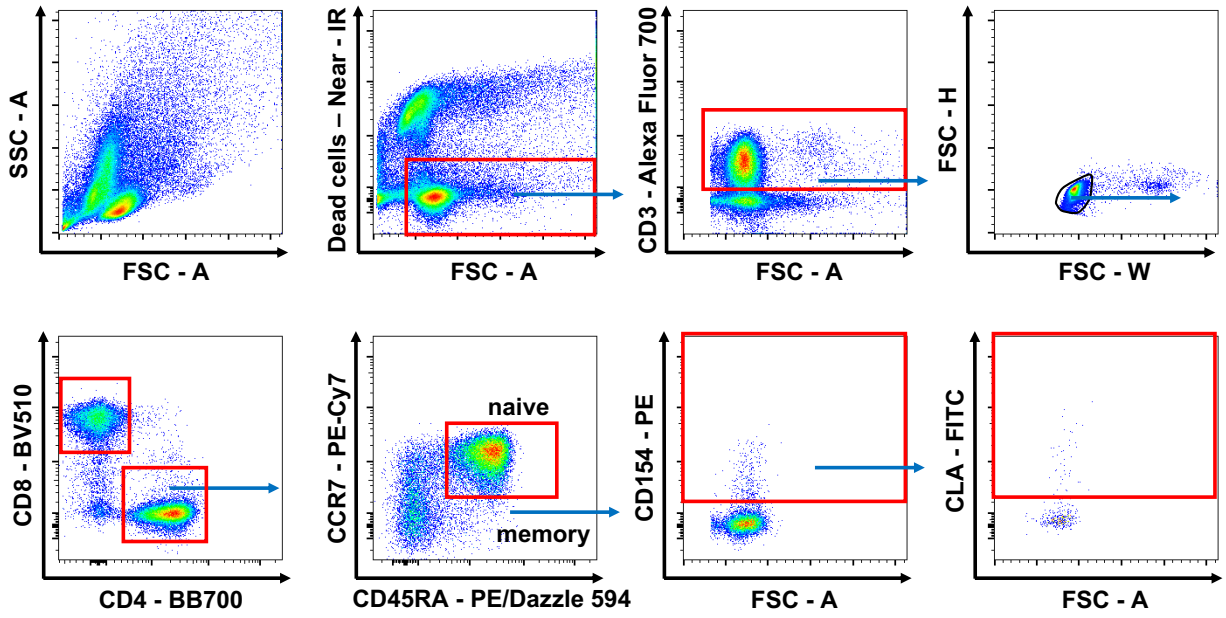

Figure S1. **Gating strategy**. PBMC were gated on live, CD3+, single, CD4+, naïve (CCR7+CD45RA+) or memory (non-naïve) T cells to assess antigen-induced CD154 upregulation (representative dot plots from donor VF12, Pd^{2+} 400 µM, 48-well plate, 100.000 total events acquired). Expression of CLA (or CD69, data not shown) was assessed among total CD4+ memory T cells and CD154+CD4+ memory T cells.

Figure S2

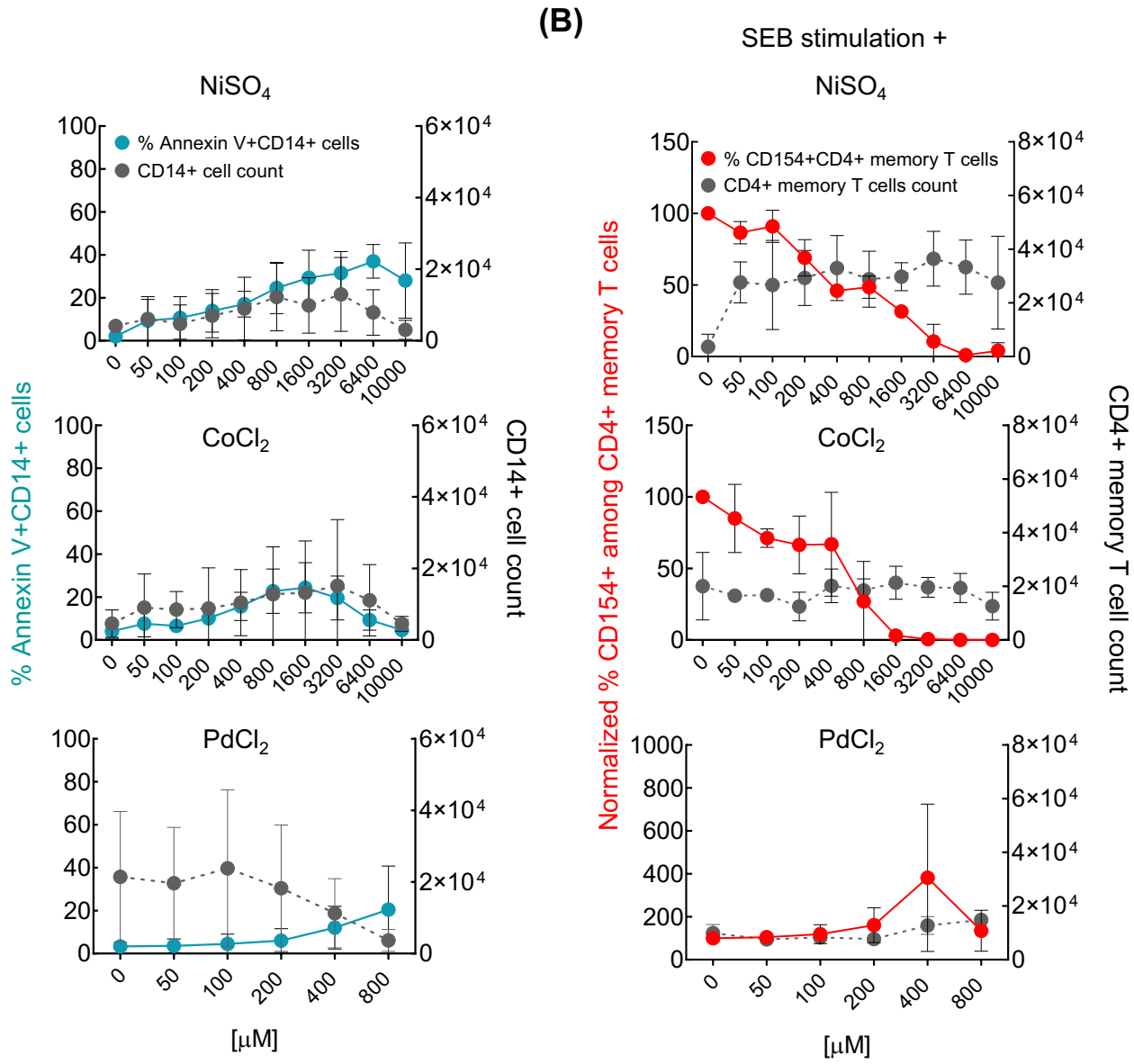

Figure S2. **Metal salt toxicity in the CD154 upregulation assay. (A) Toxic effects on antigen presenting cells** Depicted are percentages of apoptotic CD14+ monocytes (left y-axis) and total live CD14+ monocyte cell numbers (right y-axis) after 5 hours of incubation with the indicated metal salt concentrations. **(B) Inhibition of SEB-induced T cell activation**. Frequencies of CD154+CD4+ memory T cells in response to SEB stimulation (left y-axis) and total CD4+ memory T cell numbers after 5 hours of incubation with the different metal salt concentrations. SEB-induced CD154 upregulation was normalized to values without metal salt (set to 100%). The gating strategy is depicted in **Figure S1**. Horizontal lines indicate mean values, vertical lines represent the SD (n = 2 - 3, buffy coats).

Figure S3

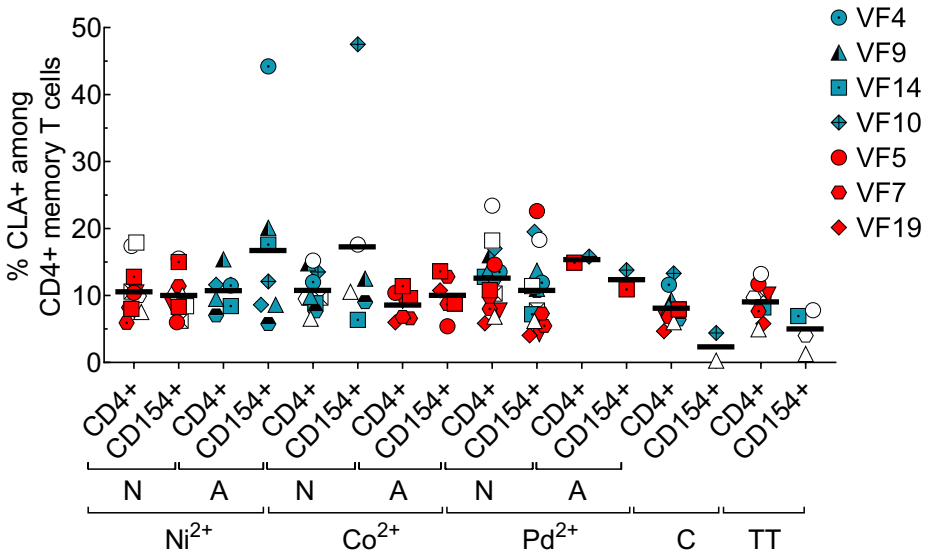

Figure S3. **CLA expression by antigen-specific T cells**. PBMC were incubated with 400 µM of $NiSO_4$ (Ni^{2+}), $CoCl_2$ (Co^{2+}) or $PdCl_2$ (Pd^{2+}) or with the control antigens CMV lysate (C) or TT for 5 hours, stained and analyzed by flow cytometry. Depicted is the expression of CLA+ cells among total CD4+ memory T cells (CD4+) and antigen-specific CD154+CD4+ memory T cells (CD154+). The gating strategy is depicted in **Figure S1**. Horizontal lines indicate the mean values. Each symbol identifies one donor (**Table S1**), raw values are listed in **Table S2**. N, non-allergic; A, allergic donor. Ni^{2+}: n = 13 (N) and 7 (A); Co^{2+}: n = 10 (N) and n =6 (A); Pd^{2+}: n = 16 (N) and 2 (A); C: n = 11; TT: n = 9).

Figure S4

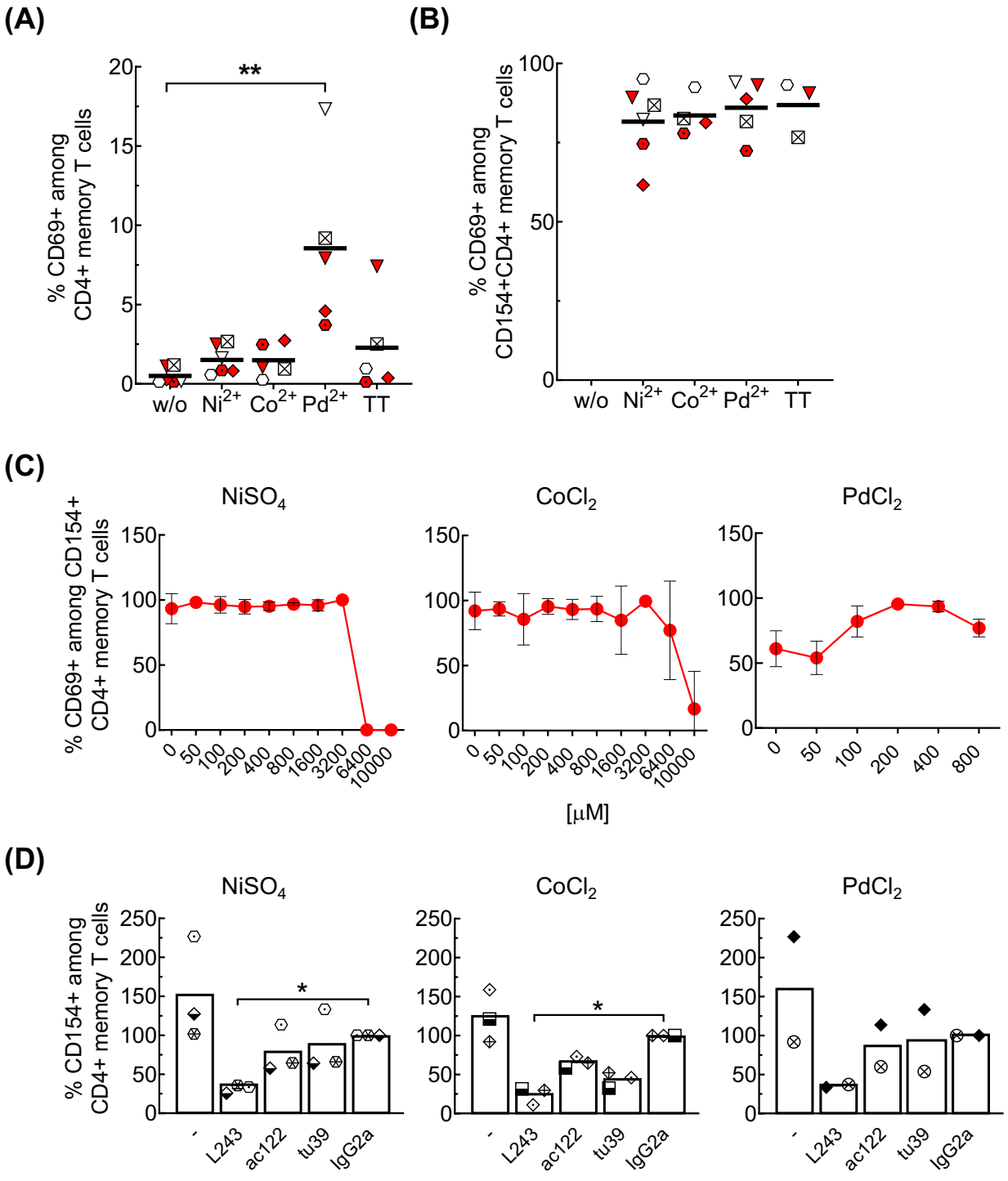

Figure S4. **CD69 co-expression by metal-specific CD4+ T cells and MHC restriction**. PBMC were incubated without antigen (w/o) or with 400 μM $NiSO_4$ (Ni^{2+}), $CoCl_2$ (Co^{2+}), $PdCl_2$ (Pd^{2+}), CMV lysate (C) or TT for 5 hours, stained and analyzed by flow cytometry. **(A) CD69 expression.** CD69 upregulation on metal-stimulated CD4+ memory T cells. **(B) CD69 co-expression.** Co-expression of CD69 by CD154+CD4+ memory T cells (n = 3 - 6 donors, **A, B**, **Table S2**). **(C) Metal salt titration.** Analysis of CD69+ co-expression by CD154+CD4+ memory T cells after the incubation with different metal salt concentrations (n = 4 - 6, buffy coats). **(D) MHC II block.** Relative inhibition of induced CD154 expression in the presence of MHC II blocking antibodies (clone 243 - anti-HLA-DR; clone ac122 - anti-HLA-DR; clone tu39 - anti-HLA-DR/DP/DQ; n = 2 - 3, Sample with isotype control antibody ("IgG2a") was set to 100%. Lines **(A-C)** and white bars **(D)** indicate the mean values. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal-Wallis) with Dunn's post-hoc test (**A**) (** $p < 0.01$ vs. w/o); (**D**) (* $p < 0.05$ vs. "IgG2a" samples).

Figure S5

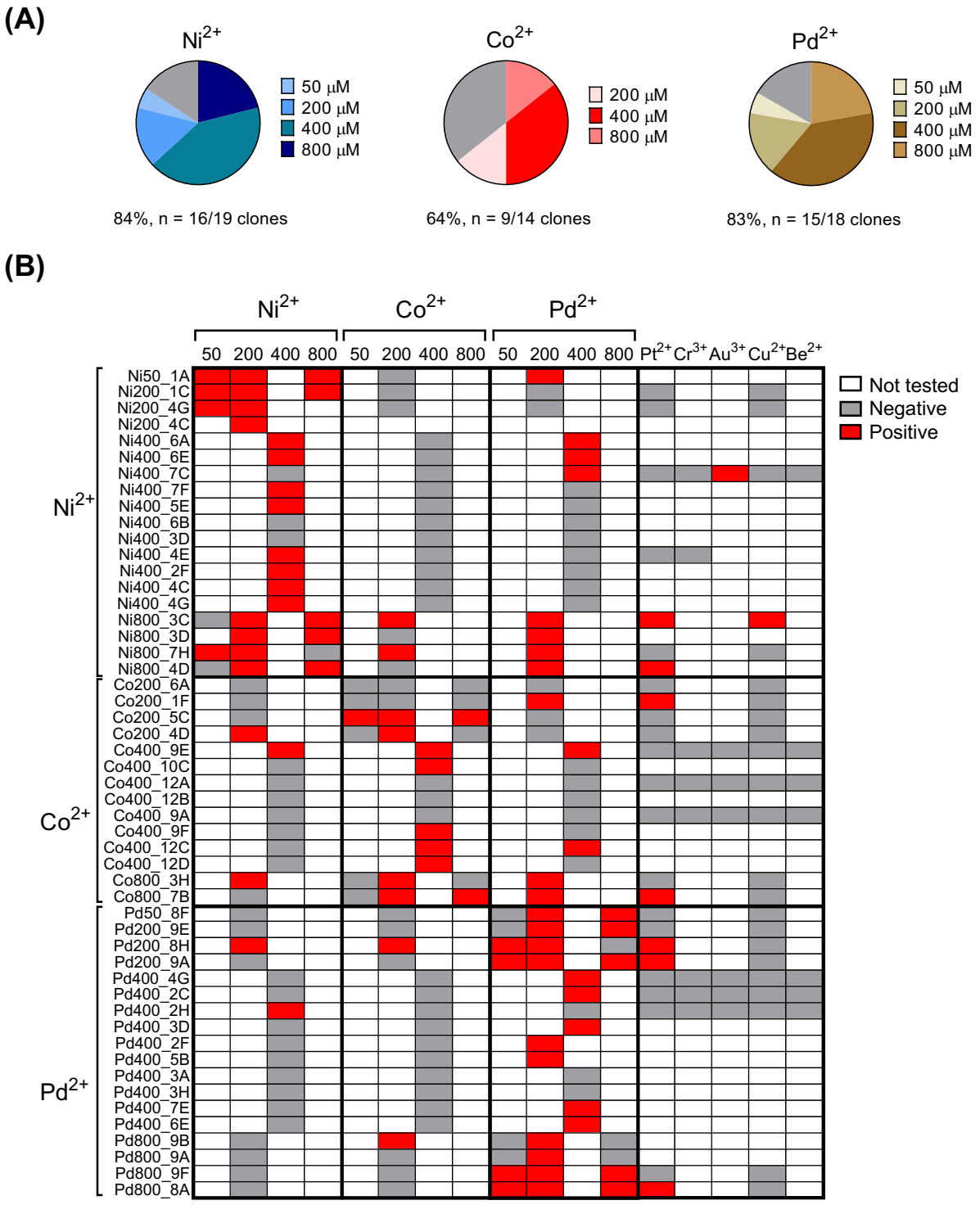

Figure S5. **Restimulation of Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific T cell clones**. Single activated CD154+CD4+ memory T cells were sorted from CD154 upregulation assays upon stimulation with 50, 200, 400 or 800 µM $NiSO_4$ (Ni^{2+}), $CoCl_2$ (Co^{2+}) or $PdCl_2$ (Pd^{2+}) and expanded for 2 - 3 weeks. **(A) Restimulation efficiencies.** Specific restimulation was tested for clones isolated from assays with the indicated metal salt concentrations. Grey color - absent (negative) restimulation. **(B) Cross-reactivity.** The heat map summarizes results from restimulation assays with the original antigen (original metal salt concentration indicated in the clone name) and potentially cross-reactive antigens including $PtCl_2$ (Pt^{2+}), $CrCl^3$ (Cr^{3+}), $HAuCl_4$ (Au^{3+}), $CuSO_4$ (Cu^{2+}) or $BeSO_4$ (Be^{2+}) at 200 or 400 µM. Clone names are listed on the left and indicate the original antigen and metal salt concentration (µM). **Table S5** lists all clones and original donor PBMC. Gated on live, CD3+, single, CD4+ T cells (gating strategy in **Figure S1**).

Figure S6

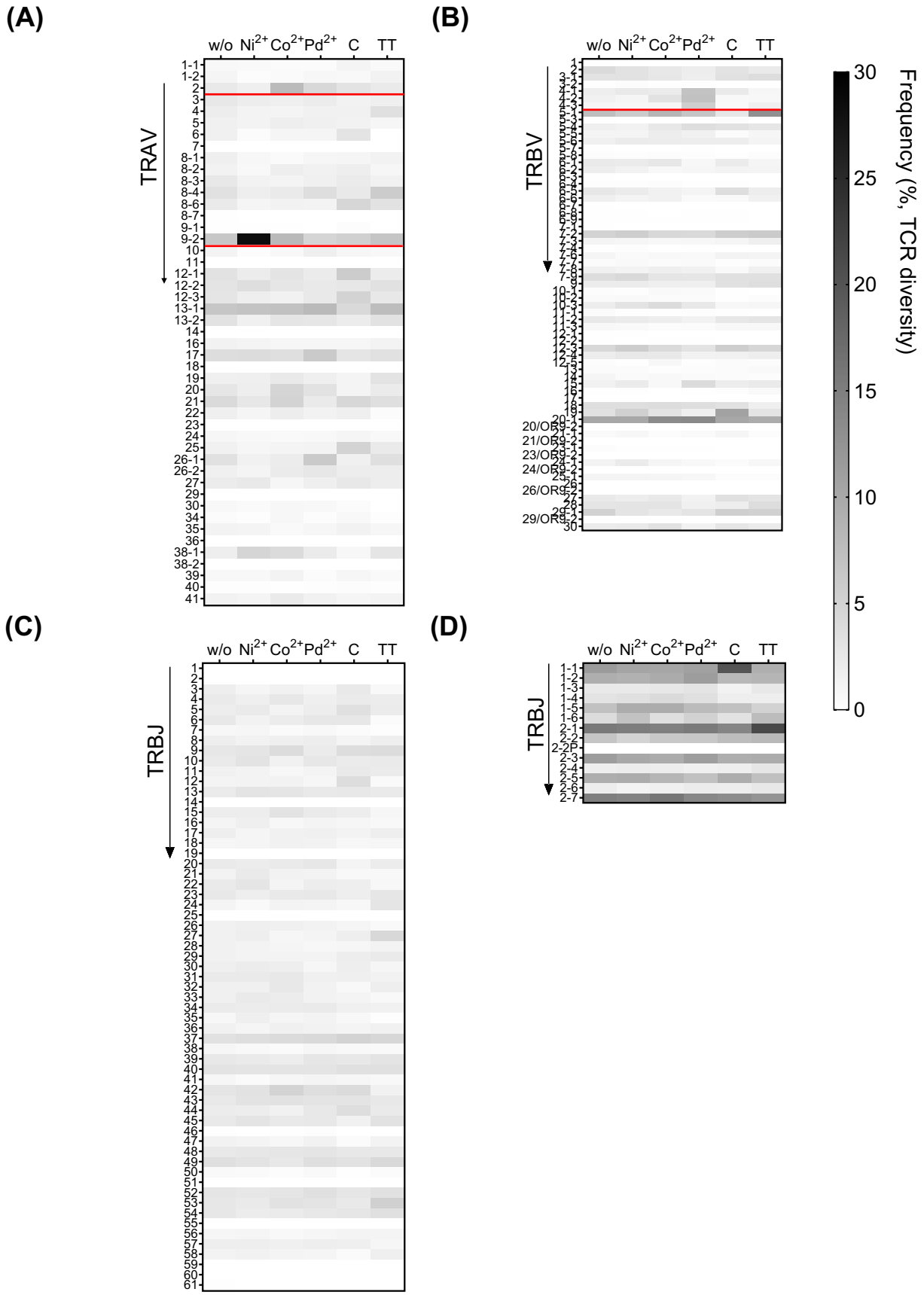

Figure S6. **Gene segment use by metal-specific TCR.** Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells (w/o) were sorted after 5 hours of antigen stimulation and their TCR were sequenced (**Table S4**). The heatmaps depict TCR α- **(A, C)** and β-chain **(B, D)** V- and J- segment use, respectively (diversity percentages).

Figure S7

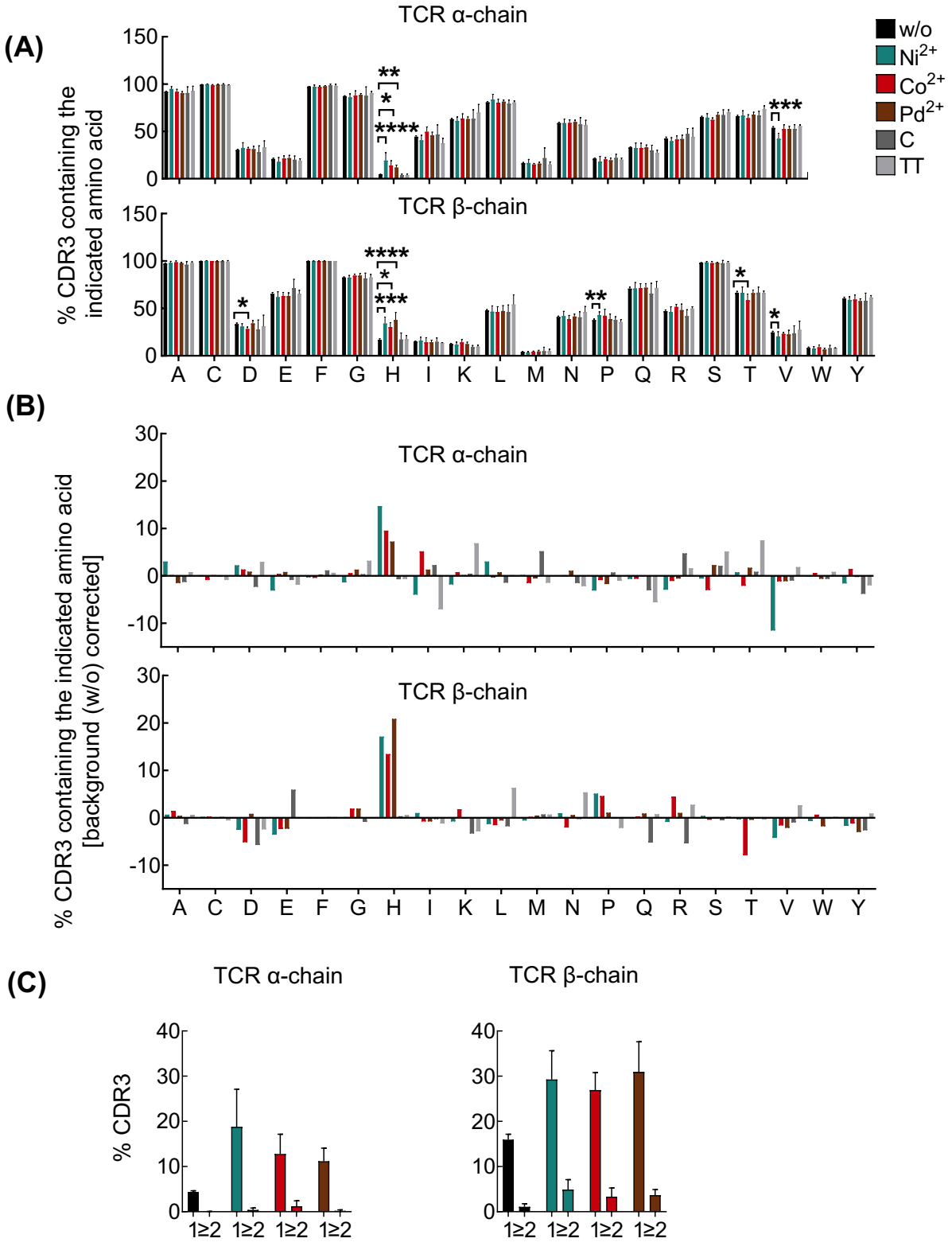

Figure S7**. CDR3 amino acid compositions of metal-specific TCR.** Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells (w/o) were sorted after 5 hours of antigen stimulation and their TCR were sequenced (**Table S4**). **(A) CDR3 amino acid composition.** Shown are mean percentages of CDR3 that contain the indicated amino acids (error bars indicate SD; diversity percentages). The first F and last C were included in the analysis. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal-Wallis) with Dunn's test as post-hoc test (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 vs. w/o). **(B) Background corrected amino acid use**. Mean values from random T cells were subtracted to highlight differences in CDR3 amino acid use among the indicated TCR repertoires. **(C)** Analysis of histidine number in α- and β-chain CDR3 that are specific for the indicated antigens (mean ± SD).

Figure S8

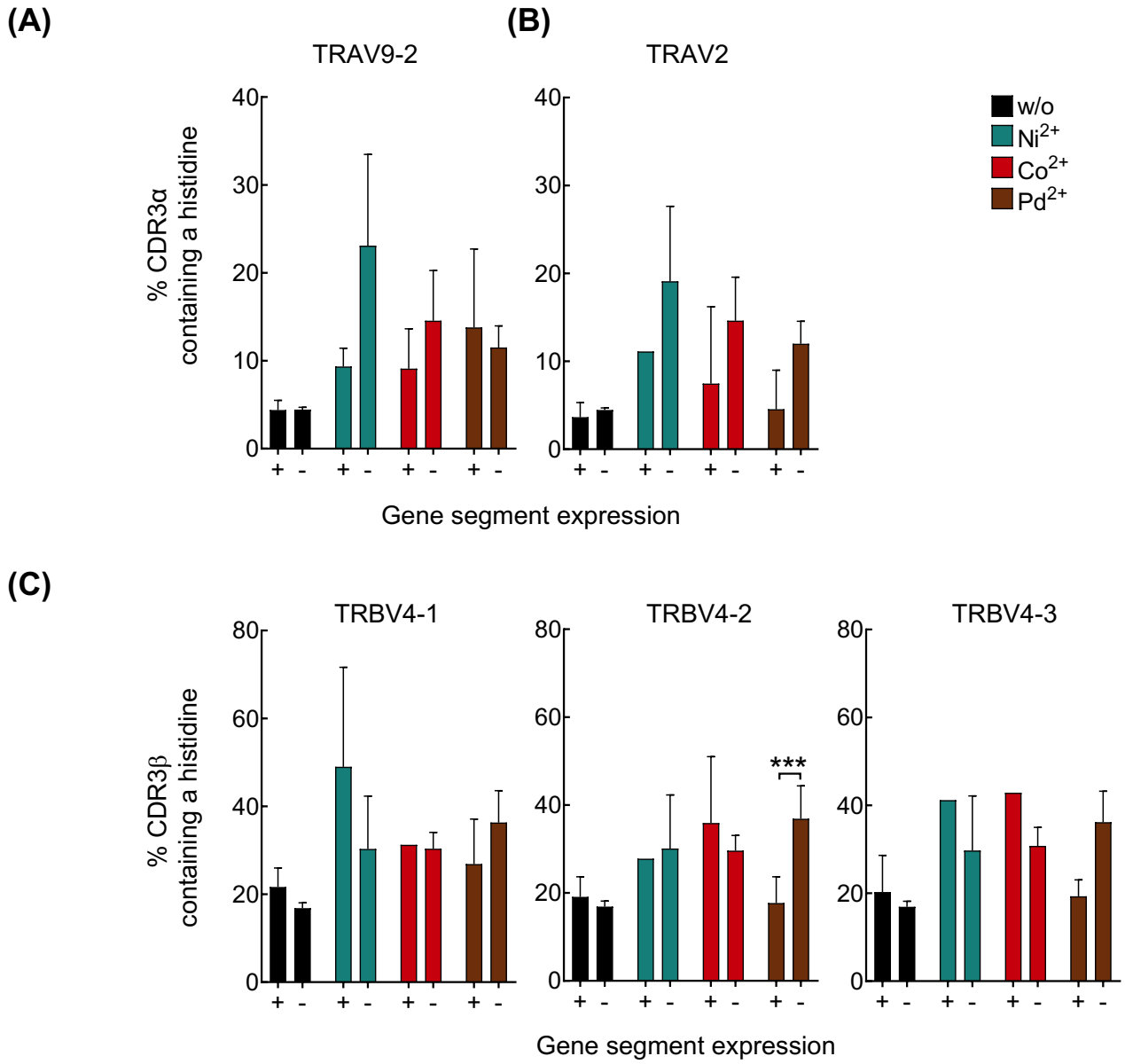

Figure S8**. CDR3 with histidine are less abundant among TCR with gene segment-associated metal ion recognition.** Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells (w/o) were sorted after 5 hours of antigen stimulation and their TCR were sequenced (**Table S4**). Shown are mean percentages of CDR3 containing a histidine among TRAV9-2+/- **(A)**, TRAV2+/- **(B)** and TRBV4-1, -2, -3+/- TCR **(C)**. Error bars indicate the SD. Statistical significances were determined by one-way non-parametric ANOVA analysis (Kruskal-Wallis) with Dunn's post-hoc test (***p <0.001 + vs. -).

Figure S9

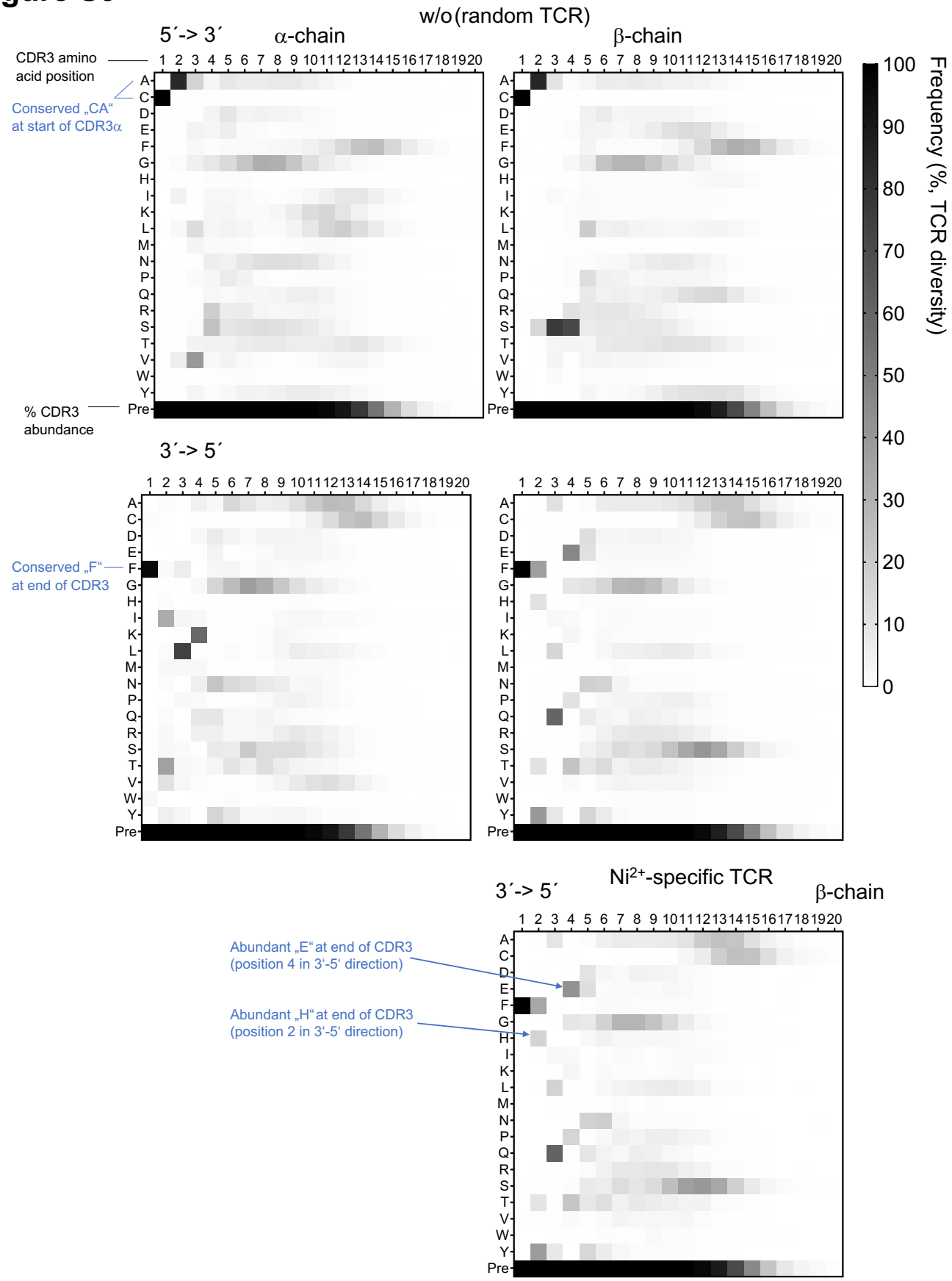

Figure S9. **Positional exact CDR3α and CDR3β amino acid compositions.** Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells (w/o) were sorted after 5 hours of antigen stimulation and their TCR were sequenced (**Table S4**). Shown are mean percentages of CDR3 that contain the indicated amino acid at the respective position. “Pre” – presence, i.e. the percentage of CDR3 that have sufficient length to reach the indicated position. The average length of a CDR3 is ~12-15 amino acids.

Figure S10

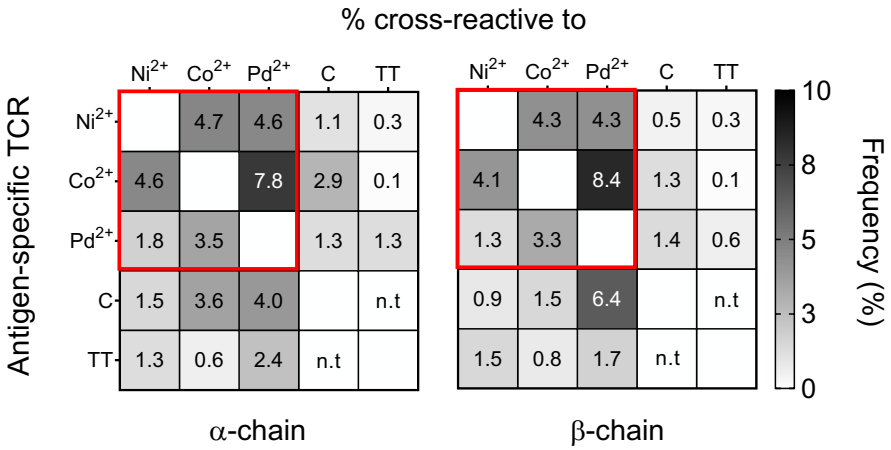

Figure S10. **Increased cross-reactivity among metal-specific TCR.** Antigen-specific CD154+CD4+ memory T cells and random CD4+ memory T cells (w/o) were sorted after 5 hours of antigen stimulation and their TCR were sequenced (**Table S4**). Shown are mean percentages of cross-reactive clonotypes (**Table S5**). For instance, 4.7% and 4.6% of Ni^{2+}-specific TCR were identified as cross-reactive to Co^{2+} and Pd^{2+}, respectively (1st row, left panel). "n.t." - not tested. The red box highlights metal-specific cross-reactive TCR.

Table S1. **Donor demographic and clinical characteristics.**

Donor	ID	Patch test results‡ (72 hours)			Time (days)§	Age	Sex	Atopic status¶	Smoking (cigarettes/ day)	CMV IgG titer [AE/ml]
		Ni^{2+}	Co^{2+}	Pd^{2+}						
VF4		++	-	n.d.	10	38	f	R,AA	-	224
VF6		++	-	-	3	64	f	AD,R,AA	-	870
VF8		+++	+	n.d.	3	81	f	R	-	226
VF9		++	-	n.d.	10	43	f	AD	5	523
VF10		+++	-	++	3	58	f	R	-	493
VF12		++	-	n.d.	7	33	f	neg.	-	1417
VF14		++	-	-	7	54	m	AD	-	n.d.
VF1		-	+	+	3	26	m	AD	1	neg.
VF3		-	+	-	7	36	f	AD	10	183
VF5		-	+	n.d.	12	32	f	R	5	neg.
VF7		-	+	n.d.	4	81	f	AD,R	-	1243
VF18		-	++	-	3	72	m	R	-	n.d.
VF19		-	+	n.d.	30	63	m	AD	-	196
VF20		-	+	-	14	62	f	AD	10	136
VF2		-	-	-	3	36	f	AD,R, AR	e-cig.	71
VF11		-	-	-	3	26	f	neg.	-	neg.
VF13		-	-	-	730	38	f	neg.	-	neg.
VF15		-	-	-	3	77	f	neg.	-	neg.
VF17		-	-	-	3	25	f	neg.	-	n.d.
VF21		-	-	-	3	29	f	neg.	-	neg.

Donors, n	20
Age (years), mean (SD)	49 (19)
Sex: female, n (%)	16 (80)
male, n (%)	4 (20)
Atopic, n (%)	14 (70)
Smoking, n (%)	6 (30)
CMV IgG+, n (%)	11 (55)

‡Patch testing was performed as suggested by the "Deutsche Kontaktallergiegruppe" (DKG, Germany, https://dkg.ivdk.org/testreihen.html; 05.02.2022) with 5% $NiSO_4$ x $6H_2O$, 1% $CoCl_2$ x $6H_2O$ and 1% $PdCl_2$ (all in petrolatum, Smart Practice) in Finn Chambers for 48 hours. The scoring system of the International Contact Dermatitis Research Group (ICDRG) was used. §Days between start of patch testing and blood draw. ¶Hints for atopic dermatitis (AD), allergic rhinoconjunctivitis (R) and/or allergic asthma (AA). Shaded rows indicate donors whose TCR have been sequenced. Bold and underlined donors indicate samples, where T cells have been sorted for *in vitro* expansion and re-stimulation experiments. n.d. – not determined, neg. – negative, ID - donor identifier (blue symbols - nickel (Ni^{2+}-) allergic, red symbols - cobalt (Co^{2+}-) allergic, white symbols - non-allergic).

Table S2. **Key frequencies of cellular surface marker expression in the CD154 upregulation assay.** Raw values for frequency percentages shown in **Figures 1, S3 and S4**. Empty values indicate that samples have not been analyzed, "-" indicates a value below the detection level of 20 cells for the analysis of CD69 or CLA co-expression by CD154+CD4+ memory T cells. Shaded areas indicate samples from which T cells were sorted for TCR HTS (**Table S4**). Bold and underlined donors indicate samples, where T cells have been sorted for *in vitro* expansion and restimulation experiments. Cell frequency values for allergic donors are indicated in bold, increased co-expression of CLA (+5%, rounded, compared to the respective total T cell pool) is shown in green. w/o – without antigen stimulation, Ni^{2+}, Co^{2+} and Pd^{2+} indicate stimulation with 400 µM $NiSO_4$, $CoCl_2$ and $PdCl_2$ in the CD154 upregulation assay.

Donor	**ID**	**CD4+ memory T cells**													
		% CD154+				**% CLA+**			**% CLA+ among CD154+**			**% CD69+ among CD154+**			
		w/o	**Ni^{2+}**	**Co^{2+}**	**Pd^{2+}**	**Ni^{2+}**	**Co^{2+}**	**Pd^{2+}**	**Ni^{2+}**	**Co^{2+}**	**Pd^{2+}**	**w/o**	**Ni^{2+}**	**Co^{2+}**	**Pd^{2+}**
VF4		0.01	**0.02**	0.01	1.81	**11.5**	12.0	13.5	**44.2**	-	11.9				
VF6		0.01	**0.25**	0.01	1.12	**9.50**	9.8	14.1	**8.60**	-	13.7				
VF8		0.02	**0.26**	**0.03**	2.08	**11.6**	10.8	17.0	**13.1**	-	19.5				
VF9		0.05	**0.24**	0.09	4.20	**15.4**	14.8	16.0	**20.1**	12.5	11.0				
VF10		0.01	**0.31**	0.02	**7.26**	**11.6**	13.5	15.8	**12.1**	47.5	**13.8**				
VF12		-	**0.69**	2.16	3.60	**7.03**	7.63	12.5	**5.75**	9.01	10.7				
VF14		0.01	**0.44**	2.17	5.01	**8.42**	8.84	12.8	**17.6**	6.37	7.19				
VF1		0.06	0.27	**0.60**	**6.93**	12.8	**11.4**	14.9	15.0	**13.6**	**10.9**				
VF3		0.01	0.11	**0.10**	0.38	8.00	**9.60**	10.8	8.30	**8.8**	-				
VF5		-	0.25	**0.32**	0.22	10.4	**10.4**	14.3	6.00	**5.40**	22.6				
VF7		-	0.17	**0.32**	4.01	8.20	**6.80**	9.80	11.4	**12.6**	7.30				
VF18		0.04	0.19	**0.45**	1.72	5.91	**6.59**	7.99	**-**	**8.72**	5.46	-	74.6	**77.9**	72.4
VF19		0.03	0.25	**2.31**	3.02	7.54	**5.99**	5.84	-	**10.8**	4.05	-	61.6	**81.3**	88.8
VF20		0.03	0.30	**0.04**	2.65	10.5	**9.20**	7.75	8.81	-	4.21	-	89.3	-	93.2
VF2		-	0.45		2.13	17.9		18.2	8.40		11.4				

Donor	ID	CD4+ memory T cells													
		% CD154+				% CLA+			% CLA+ among CD154+			% CD69+ among CD154+			
		w/o	Ni^{2+}	Co^{2+}	Pd^{2+}	Ni^{2+}	Co^{2+}	Pd^{2+}	Ni^{2+}	Co^{2+}	Pd^{2+}	w/o	Ni^{2+}	Co^{2+}	Pd^{2+}
VF11	○	0.02	0.75	0.64	5.90	17.4	15.2	22.0	15.5	17.6	18.3				
VF13	⬡	-	0.15	0.03		9.9	9.5		10.3	-		-	95.1	92.5	
VF15	▽	-	0.22		7.04							-	82.3		94.1
VF17	△	0.01	0.19	0.48	2.64	7.53	6.54	6.83	10.1	10.6	6.19				
VF21	⊠	0.04	0.27	0.01	3.74	10.6	9.69	10.3	6.24	-	7.75	-	86.8	82.6	81.7
Means		**0.03**	**0.29**	**0.54**	**3.44**	**10.6**	**9.90**	**12.8**	**13.1**	**13.6**	**10.9**		**81.6**	**83.6**	**86.0**
SD		0.02	0.17	0.77	2.11	3.26	2.65	4.16	8.75	10.7	5.22		10.9	5.43	8.11
Means (w/o corrected)			**0.30**	**0.51**	**3.42**	**10.7**	**8.6**	**15.4**	**17.4**	**9.99**	**12.4**				
Means – Allergic			**0.32**	**0.59**	**7.10**	**2.52**	**1.95**	**0.45**	**11.9**	**2.73**	**1.45**				
SD			0.19	0.72	0.17										
Means - Non-Allergic*			0.27	0.51	3.02	10.6	10.8	12.5	10.0	17.3	10.8				
SD			0.16	0.80	1.80	3.63	2.70	4.31	3.07	14.0	5.50				

*Non-allergic to the indicated metal allergen.

Table S3. **Ni^{2+}-, Co^{2+}- and Pd^{2+}-specific T cell clones.** Single metal-specific CD154+CD4+ memory T cells were sorted from CD154 upregulation assays and expanded in vitro. Restimulation experiments were done with the original and potentially cross-reactive metal antigen(s). "+" – positively tested, "-" – negatively tested. Donors are listed in Table S1, "B" within a donor name indicate buffy coat samples (no symbols assigned, additional numbers and letters (e.g., "ML") represent internal sample codes). Clones are listed according to the original antigen to which they reacted with increasing metal salt concentration. "T cells only" indicates restimulation experiments without additional CD3-depleted PBMC as antigen-presenting cells.

#	Donor	ID	Antigen specificity	Restimulation (original antigen)	Cross-reactivity to		T cells only (original antigen)
Ni^{2+}-specific CD4+ T cell clones							
1	MLB14		Ni50_1A	+	+	Pd^{2+}	-
2	MLB10		Ni200_4C	+	-		
3	MLB10		Ni200_4G	+	-		+
4	VF1	⊡	Ni200_1C	+	-		+
5	LMB4		Ni400_3D	-	-		
6	MLB33		Ni400_4C	+	-		+
7	MLB33		Ni400_4G	+	-		+
8	MLB33		Ni400_6E	+	+	Pd^{2+}	
9	MLB33		Ni400_7C	-	+	Pd^{2+}, Au^{3+}	-
10	MLB33		Ni400_6A	+	+	Pd^{2+}	+
11	SASB4		Ni400_7F	+	-		
12	SASB4		Ni400_5E	+	-		
13	SASB4		Ni400_6B	-	-		
14	VF21	⊠	Ni400_4E	+	-		+
15	VF21	⊠	Ni400_2F	+	-		-
16	MLB10		Ni800_7H	+	+	Co^{2+}, Pd^{2+}	-
17	MLB14		Ni800_3D	+	+	Pd^{2+}	-
18	VF1	⊡	Ni800_3C	+	+	Co^{2+}, Pd^{2+}, Pt^{2+}, Cu^{2+}	-
19	VF1	⊡	Ni800_4D	+	+	Pd^{2+}, Pt^{2+}	
Co^{2+}-specific CD4+ T cell clones							
1	VF1	⊡	Co200_1F	-	+	Pd^{2+}, Pt^{2+}	
2	VF1	⊡	Co200_5C	+	-		-
3	VF2	□	Co200_4D	+	+	Ni^{2+}	+

#	Donor	ID	Antigen specificity	Restimulation (original antigen)	Cross-reactivity to		T cells only (original antigen)
4	VF14	⊡	Co200_6A	-	-		
5	LMB9		Co400_9E	+	+	Ni^{2+}, Pd^{2+}	+
6	MLB33		Co400_9F	+	-		-
7	MLB33		Co400_12C	+	-	Pd^{2+}	-
8	MLB33		Co400_12D	+	+		-
9	MLB33		Co400_9A	-	-		
10	SASB4		Co400_10C	+	+	Ni^{2+}	
11	VF21	⊠	Co400_12A	-	-		
12	VF21	⊠	Co400_12B	-	-		
13	MLB10		Co800_3H	+	+	Ni^{2+}, Pd^{2+}	-
14	VF1	⊡	Co800_7B	+	+	Pd^{2+}, Pt^{2+}	-
Pd^{2+}-specific CD4+ T cell clones							
1	VF1	⊡	Pd50_8F	+	-		+
2	MLB10		Pd200_8H	+	+	Ni^{2+}, Co^{2+}, Pt^{2+}	-
3	VF1	⊡	Pd200_9A	+	+	Pt^{2+}	
4	VF14	⊡	Pd200_9E	+	-		+
5	LMB4		Pd400_2F	+	-		+
6	LMB4		Pd400_5B	+	-		+
7	LMB9		Pd400_4G	+	-		+
8	LMB9		Pd400_2C	+	-		+
9	MLB33		Pd400_2H	-	+	Ni^{2+}	+
10	MLB33		Pd400_3D	+	-		+
11	MLB33		Pd400_3A	-	-		
12	MLB33		Pd400_3H	-	-		
13	MLB34		Pd400_7E	+	-		-
14	MLB34		Pd400_6E	+	-		-
15	MLB14		Pd800_9B	+	+	Co^{2+}	-
16	MLB14		Pd800_9A	+	-		+
17	VF1	⊡	Pd800_9F	+	-		+
18	VF1	⊡	Pd800_8A	+	+	Pt^{2+}	+

Table S4. **Overview of TCR sequencing data.** This table lists the number of sorted T cells, obtained αβ TCR sequence counts (each count derives from one unique UMI) and TCR diversity (number of different clonotypes) from the indicated donors and samples. w/o – without antigen stimulation (random CD4+ memory T cells were sorted). Antigen-specific CD154+CD4+ memory T cells were sorted from samples stimulated with 400 µM Ni^{2+} ($NiSO_4$), 400 µM Co^{2+} ($CoCl_2$), 400 µM Pd^{2+} ($PdCl_2$), CMV and TT. *Replicate samples, mean values were calculated for TCR repertoire feature analysis.

Donor	ID	Antigen	Sorted cells	TCR chain	Counts (UMI)	diversity	Library number
VF8	◆	w/o	100,000	α	76,528	28,753	1
				β	57,787	24,458	2
		Ni^{2+}	846	α	532	209	3
				β	525	193	4
		Pd^{2+}	2,432	α	1,511	749	5
				β	2,344	992	6
		CMV	943	α	628	160	7
				β	748	143	8
VF9	◭	w/o	100,000	α	21,359	10,065	9
				β	16,821	8,466	10
		Ni^{2+}	1,078	α	72	51	11
				β	75	52	12
		Pd^{2+}*	4,239	α	324	246	13
				β	492	360	14
			1,660	α	124	93	15
				β	183	138	16
			2,922	α	229	169	17
				β	443	303	18
		CMV	1312	α	89	43	19
				β	105	46	20
VF10	◈	w/o	100,000	α	6,985	3,759	21
				β	10,620	5,240	22
		Ni^{2+}	1,610	α	163	80	23
				β	158	87	24
		Pd^{2+}*	1,383	α	100	67	25
				β	186	134	26
			1,253	α	69	56	27
				β	132	108	28
			1,334	α	92	74	29
				β	143	110	30
		CMV*	1967	α	98	32	31
				β	147	49	32
			2814	α	150	44	33
				β	195	47	34
			2604	α	155	47	35

Donor	ID	Antigen	Sorted cells	TCR chain	Counts (UMI)	diversity	Library number
				β	180	59	36
VF12		w/o	100,000	α	3,159	2,008	37
				β	4,321	2,627	38
		Ni^{2+}	1,348	α	182	108	39
				β	236	145	40
		Co^{2+}	4,574	α	405	286	41
				β	522	328	42
		Pd^{2+}	2,750	α	282	194	43
				β	427	280	44
VF1		w/o	100,000	α	14,696	10,017	45
				β	25,752	15,061	46
		Ni^{2+}	1,058	α	403	222	47
				β	497	254	48
		Pd^{2+*}	1,000	α	333	233	49
				β	462	329	50
			14,910	α	3,935	2,589	51
				β	7,298	3,822	52
VF5		w/o	100,000	α	37,211	18,022	53
				β	51,181	21,692	54
		Ni^{2+}	3,103	α	1,715	760	55
				β	2,198	827	56
		Co^{2+}	2,238	α	1,977	779	57
				β	2,133	797	58
		TT	826	α	606	158	59
				β	504	136	60
VF7		w/o	100,000	α	13,559	6,175	61
				β	10,537	5,071	62
		Co^{2+}	654	α	212	38	63
				β	211	41	64
		Pd^{2+}	1,667	α	361	163	65
				β	504	217	66
		CMV	1,570	α	387	87	67
				β	458	79	68
VF18		w/o	100,000	α	29,271	17,260	69
				β	26,566	15,152	70
		Ni^{2+}	407	α	36	29	71
				β	45	37	72
		Pd^{2+*}	758	α	158	128	73
				β	230	167	74
			1,240	α	282	243	75
				β	362	286	76
			2,021	α	353	306	77

Donor	ID	Antigen	Sorted cells	TCR chain	Counts (UMI)	diversity	Library number
				β	523	407	78
		TT	1,069	α	96	85	79
				β	148	127	80
VF19	◆	w/o	100,000	α	42,452	18,139	81
				β	43,849	16,678	82
		Ni^{2+}	725	α	71	55	83
				β	112	79	84
		Co^{2+}*	1,950	α	514	376	85
				β	557	369	86
			1,719	α	300	220	87
				β	329	237	88
			2,965	α	498	352	89
				β	483	353	90
		Pd^{2+}*	2,247	α	498	352	91
				β	483	353	92
			1,672	α	337	239	93
				β	542	362	94
			1,457	α	215	158	95
				β	389	275	96
		CMV	414	α	108	41	97
				β	99	45	98
VF20	▼	w/o	100,000	α	32,113	15,798	99
				β	32,836	14,795	100
		Ni^{2+}	2,701	α	500	303	101
				β	603	342	102
		Co^{2+}	848	α	141	99	103
				β	133	100	104
		Pd^{2+}*	5,439	α	620	417	105
				β	888	575	106
			3,077	α	319	209	107
				β	609	381	108
			6,311	α	240	84	109
				β	451	103	110
		CMV*	4,252	α	389	127	111
				β	494	126	112
			4,023	α	189	101	113
				β	292	98	114
			3,372	α	707	484	115
				β	1259	750	116
VF2	□	w/o	23,670	α	41,52	2425	117
				β	3,263	1995	118
		Ni^{2+}	1,078	α	368	185	119

Donor	ID	Antigen	Sorted cells	TCR chain	Counts (UMI)	diversity	Library number
				β	341	192	120
		Pd^{2+}	2,819	α	574	380	121
				β	759	504	122
		CMV	1,596	α	193	38	123
				β	188	28	124
VF11	◯	w/o	100,000	α	9,921	8,657	125
				β	10,003	9,562	126
		Ni^{2+}	1,399	α	107	79	127
				β	108	88	128
		Co^{2+}	1,165	α	84	72	129
				β	70	63	130
		Pd^{2+}	2,636	α	290	230	131
				β	460	336	132
		TT	1,295	α	86	66	133
				β	120	87	134

Total number of sorted cells	1,246,173
Sequence counts α-chains	314,315
Sequence counts β-chains	325,636
Total sequence count	639,951
TCR diversity α–chain	153,921
TCR diversity β-chain	157,420
Total TCR diversity	311,341
Counts/cell α-chain	0.21
Counts/cell β–chain	0.26
Overall counts/cell	0.23 ± 0.20

Table S5. **Cross-reactive TCR.** This table lists TCR commonly identified in the indicated sample pairs. w/o – without antigen stimulation (for sort of random CD4+ memory T cells). Antigen-specific CD154+CD4+ memory T cells were sorted from samples stimulated with 400 μM $NiSO_4$ (Ni^{2+}), $CoCl_2$ (Co^{2+}), $PdCl_2$ (Pd^{2+}) and CMV or TT. Div. – diversity (number of TCR clonotypes). "-" – no shared clonotypes identified.

Donor	ID	TCR chain	Antigen 1 – 2	div.	% div. 1	% div. 2	Antigen 1 – 2	div.	% div. 1	% div. 2
VF8	◆	α	w/o – Ni^{2+}	70	0.2	33.5	Ni^{2+} - Pd^{2+}	16	7.7	2.1
		β		50	0.2	25.9		19	9.8	1.9
		α	w/o – Pd^{2+}	294	1.0	39.3	Ni^{2+} - CMV	2	1.0	1.3
		β		344	1.4	34.7		1	0.5	0.6
		α					Pd^{2+} - CMV	3	0.4	1.9
		β						6	0.6	3.5
VF9	◭	α	w/o – Ni^{2+}	8	0.1	15.7	Ni^{2+} - Pd^{2+}	2 2 -	3.9 3.9 -	2.2 1.2 -
		β		11	0.1	21.2		1 1 -	1.9 1.9 -	0.7 0.3 -
		α	w/o – Pd^{2+}	21 64 35	0.2 0.6 0.4	22.6 26.0 20.7	Ni^{2+} - CMV	1	2.0	2.3
		β		24 56 74	0.3 0.7 0.9	17.4 18.5 20.6		-	-	-
		α					Pd^{2+} - CMV	1 1 1	1.1 0.4 0.6	2.3 2.3 2.3
		β						1 - -	0.3 - -	2.2 - -
VF10	◈	α	w/o – Ni^{2+}	12	0.3	15.0	Ni^{2+} - Pd^{2}	- - -	- - -	- - -
		β		15	0.3	17.2		3 2 1	3.5 2.3 1.2	2.2 1.9 0.9
		α	w/o – Pd^{2+}	9 11 12	0.2 0.3 0.3	16.1 16.4 16.2	Ni^{2+} - CMV	- - -	- - -	- - -
		β		14 17 20	0.3 0.3 0.4	13.0 15.5 14.9		- - -	- - -	- - -
		α					Pd^{2+} - CMV	1 1 1 1 1 1 2 1	1.8 1.5 1.4 1.8 1.8 1.5 2.7 1.5	3.1 3.1 3.1 2.1 2.3 2.1 4.3 2.3

Donor	ID	TCR chain	Antigen 1 – 2	div.	% div. 1	% div. 2	Antigen 1 – 2	div.	% div. 1	% div. 2
								2	2.7	4.6
		β						2 1 1 2 1 1 2 1 1	1.9 0.9 0.8 1.9 0.9 0.8 1.9 0.9 0.8	3.4 1.7 1.7 4.1 2.0 2.0 4.3 2.1 2.1
VF12		α	w/o – Ni^{2+}	13	0.7	12.0	Ni^{2+} - Co^{2+}	7	6.5	2.5
		β		20	0.8	13.8		11	7.6	3.4
		α	w/o – Co^{2+}	31	1.5	10.8	Ni^{2+} - Pd^{2+}	5	4.6	2.6
		β		35	1.3	10.7		5	3.5	1.8
		α	w/o – Pd^{2+}	15	0.8	7.7	Co^{2+} - Pd^{2+}	11	3.9	5.7
		β		37	1.4	13.2		18	5.5	6.4
VF1		α	w/o – Ni^{2+}	45	0.5	20.3	Ni^{2+} - Pd^{2+}	33 10	14.9 4.5	1.3 4.3
		β		73	0.5	28.7		10 43	3.9 16.9	3.0 1.1
		α	w/o – Pd^{2+}	621 83	6.2 0.8	24.0 35.6				
		β		1068 133	7.1 0.9	27.9 40.4				
VF5		α	w/o – Ni^{2+}	103	0.6	13.2	Ni^{2+} - Co^{2}	37	4.8	4.9
		β		78	0.4	9.8		42	5.3	5.1
		α	w/o – Co^{2+}	106	0.6	14.0	Ni^{2+} - TT	2	0.3	1.3
		β		129	0.6	15.6		2	0.3	1.5
		α					Co^{2+} - TT	1	0.1	0.6
		β						1	0.1	0.8
VF7		α	w/o – Co^{2+}	18	0.3	47.4	Co^{2+} - Pd^{2}	7	18.4	4.3
		β		19	0.4	46.3		8	19.5	3.7
		α	w/o – Pd^{2+}	38	0.6	23.3	Co^{2+} - CMV	2	5.3	2.3
		β		54	1.1	24.9		1	2.4	1.3
		α					Pd^{2+} - CMV	2	1.2	2.3
		β						6	2.8	7.6
VF18		α	w/o – Ni^{2+}	5	0.0	17.2	Ni^{2+} - Pd^{2}	- - -	- - -	- - -
		β		7	0.1	18.9		1 1 -	2.7 2.7 -	0.4 0.3 -
		α	w/o – Pd^{2+}	51 30 62	0.3 0.2 0.4	21.0 23.4 20.3	Ni^{2+} - TT	-	-	-
		β		48 76 29	0.3 0.5 0.2	16.8 18.7 17.4		-	-	-

Donor	ID	TCR chain	Antigen 1 – 2	div.	% div. 1	% div. 2	Antigen 1 – 2	div.	% div. 1	% div. 2
		α					Pd^{2+} - TT	3 1 -	2.3 0.3 -	3.5 1.2 -
		β						2 1 1	0.7 0.3 0.6	1.6 0.8 0.8
VF19	◆	α	w/o – Ni^{2+}	16	0.1	29.1	Ni^{2+} - Co^{2}	1 2 4	1.8 7.3 3.6	0.3 1.1 0.9
		β		10	0.1	12.7		6 3 3	7.6 3.8 3.8	1.6 0.9 1.3
		α	w/o – Co^{2+}	102 113 73	0.6 0.6 0.4	27.1 32.1 33.2	Ni^{2+} - Pd^{2+}	1 1	1.8 1.8	0.4 0.6
		β		106 94 67	0.6 0.6 0.4	30.0 25.5 28.3		3 1	3.8 1.3	0.8 0.4
		α	w/o – Pd^{2+}	74 52	0.4 0.3	31.0 32.9	Ni^{2+} - CMV	-	-	-
		β		101 76	0.6 0.5	27.9 27.6		-	-	-
		α					Co^{2+} - Pd^{2+}	5 8 7 4 8 8	1.3 2.1 3.2 1.8 2.3 2.3	2.1 5.1 2.9 2.5 3.4 5.1
		β						13 6 15 14 15 12	3.7 1.7 4.1 3.8 6.3 5.1	3.6 2.2 4.1 5.1 4.1 4.4
		α					Co^{2+} - CMV	1 3	0.3 0.9	2.4 7.3
		β						1 1	0.3 0.4	2.2 2.2
		α					Pd^{2+} - CMV	2 2	0.8 1.3	4.9 4.9
		β						5 -	1.4 -	11.1 -
VF20	▼	α	w/o – Ni^{2+}	75	0.5	24.8	Ni^{2+} - Co^{2}	9	3.0	9.1
		β		80	0.5	23.4		9	2.6	9.0
		α	w/o – Co^{2+}	41	0.3	41.4	Ni^{2+} - Pd^{2+}	8 5 12	2.6 1.7 4.0	1.9 2.4 2.5
		β		37	0.3	37.0		14 11 14	4.1 3.2 4.1	1.9 2.9 2.4

Donor	ID	TCR chain	Antigen 1 – 2	div.	% div. 1	% div. 2	Antigen 1 – 2	div.	% div. 1	% div. 2
		α	w/o – Pd^{2+}	82 145 171	0.5 0.9 1.1	39.2 34.8 35.3	Ni^{2+} - CMV	1 1 -	0.3 0.3 -	1.0 0.8 -
		β		127 205 185	0.9 1.4 1.3	33.3 27.3 32.2		1 1 2	0.3 0.3 0.6	1.0 1.0 1.6
		α					Co^{2+} - Pd^{2+}	7 4 11	7.1 4.0 11.1	1.7 1.9 2.3
		β						7 7 6	7.0 7.0 6.0	1.8 1.2 0.8
		α					Co^{2+} - CMV	- - -	- - -	- - -
		β						1 1 -	1.0 1.0 -	1.0 0.8 -
		α					Pd^{2+} - CMV	6 9 9 13 7 17 9 6 14	2.9 2.2 1.9 3.1 3.4 3.5 2.2 2.9 2.9	7.1 10.7 10.7 10.2 5.5 13.4 8.9 5.9 13.9
		β						12 14 11 9 12 8 14 19 14	3.2 1.9 1.9 2.4 1.6 1.4 3.7 2.5 2.4	12.2 14.3 11.2 8.7 11.7 7.8 11.1 15.1 11.1
VF2	□	α	w/o – Ni^{2+}	15	0.6	8.1	Ni^{2+} - Pd^{2+}	5	2.7	1.3
		β		11	0.6	5.7		4	2.1	0.8
		α	w/o – Pd^{2+}	37	1.5	9.7	Ni^{2+} - CMV	-	-	-
		β		41	2.1	8.1		-	-	-
		α					Pd^{2+} - CMV	-	-	-
		β						-	-	-
VF11	○	α	w/o – Ni^{2+}	19	0.2	24.1	Ni^{2+} - Co^{2}	4	5.1	5.6
		β		18	0.2	20.5		1	1.1	1.6
		α	w/o – Co^{2+}	19	0.2	26.4	Ni^{2+} - Pd^{2+}	3	3.8	1.3
		β		17	0.2	27.0		3	3.4	0.9
		α	w/o – Pd^{2+}	56	0.7	24.4	Ni^{2+} - TT	-	-	-
		β		89	0.9	26.5		-	-	-
		α					Co^{2+} - Pd^{2+}	5	6.9	2.2

Annex III: Supplementary Material

Research paper: Frequencies and TCR Repertoires of Human 2,4,6-Trinitrobenzenesulfonic Acid-specific T Cells

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Published online on 22 February 2022 in:
Frontiers in Toxicology. 4:827109
Link: https://doi.org/10.3389/ftox.2022.827109

Figure S1

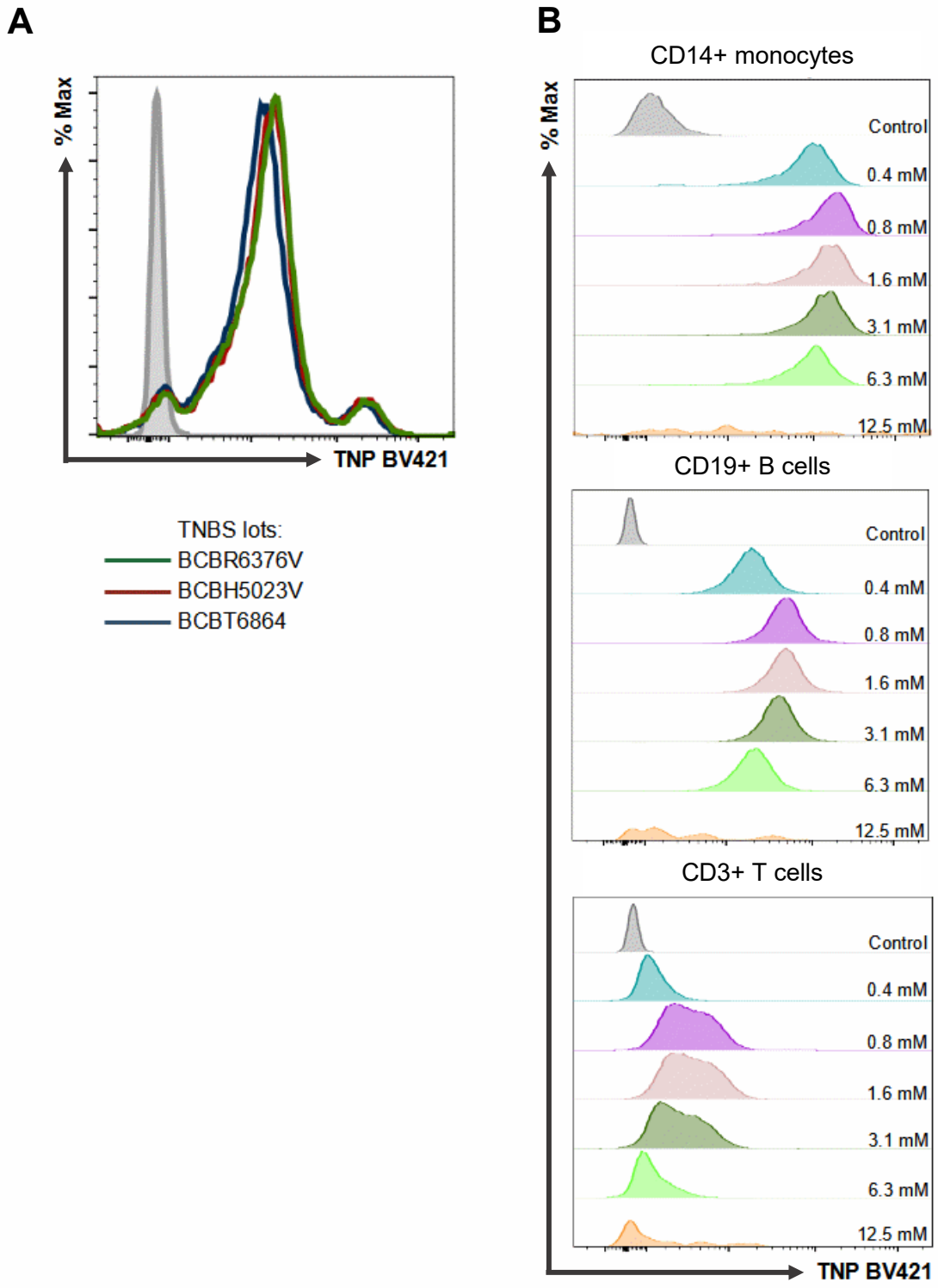


Figure S1. TNBS modification of PBMC. (**A**) Reproducibility of TNBS-modifications. PBMC were treated with 3 mM TNBS from different lots, stained with anti-TNP antibody and analyzed by flow cytometry. Gated on live cells. (**B**) TNBS modification of cell subtypes. PBMC were treated with the indicated TNBS concentrations in a two-fold dilution series starting from 12.5 mM TNBS. TNBS-modification of cell subpopulations were analyzed according to the indicated surface markers after live cell gating. For both graphs, one representative experiment is shown from n = 2 – 3 experiments.

Figure S2

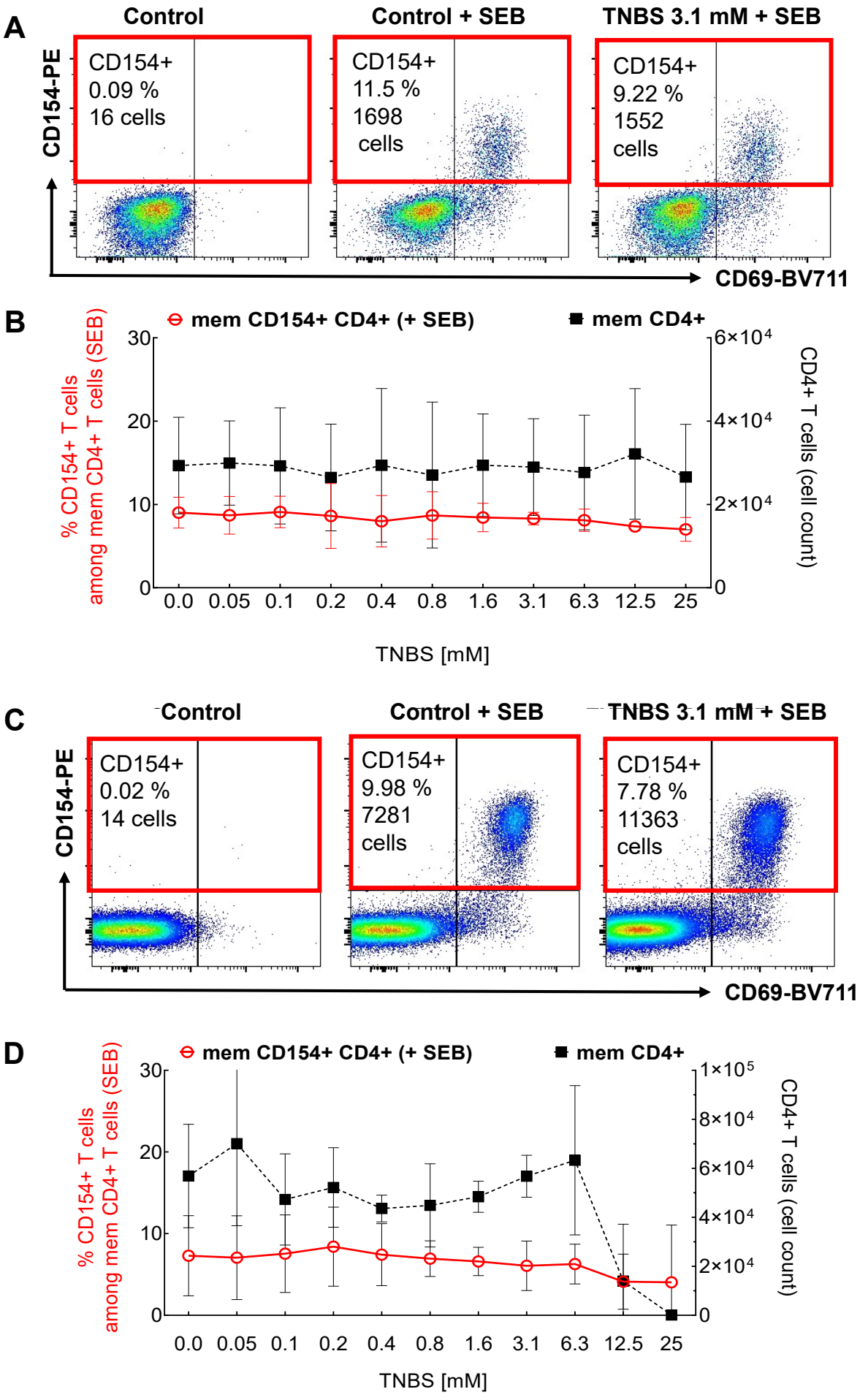

A
Control
Control + SEB
TNBS 3.1 mM + SEB
CD154-PE
CD154+
0.09 %
16 cells
CD154+
11.5 %
1698
cells
CD154+
9.22 %
1552
cells
CD69-BV711
B
mem CD154+ CD4+ (+ SEB)
mem CD4+
% CD154+ T cells
among mem CD4+ T cells (SEB)
CD4+ T cells (cell count)
TNBS [mM]
C
Control
Control + SEB
TNBS 3.1 mM + SEB
CD154-PE
CD154+
0.02 %
14 cells
CD154+
9.98 %
7281
cells
CD154+
7.78 %
11363
cells
CD69-BV711
D
mem CD154+ CD4+ (+ SEB)
mem CD4+
% CD154+ T cells
among mem CD4+ T cells (SEB)
CD4+ T cells (cell count)
TNBS [mM]

Figure S2. Effects of TNBS on viability and T cell CD154 upregulation. **(A, B)** Analysis of "responder" CD4+ memory T cells in a CD154 upregulation assay (5 h). PBMC were CFSE labeled and modified with PBS (control) or TNBS (0.05 – 25 mM, two-fold dilution series) and mixed as APCs in a 1:1 ratio with unmodified "responder" PBMC. In addition, some samples were stimulated with SEB, a superantigen activating a large fraction of T cells. **(A)** Representative dot plots showing CD154 expression upon different stimulation conditions (red boxes, MLB19). **(B)** Graph summarizing data from $n \geq 3$ experiments with different buffy coats showing frequencies of SEB-stimulated CD154+CD4+ memory T cells (left y-axis) and total CD4+ memory T cell numbers (right y-axis) in the presence of APC treated with different TNBS concentrations. **(C, D)** Analysis of T cells from TNBS-treated PBMC (APC, without CFSE label and "responder" cells, thus with double input cell number to maintain optimal cell surface densities). **(C)** Representative dot plots (as in (A), MLB16). **(D)** Graph summarizing data from $n \geq 3$ experiments, as in (B). All data were gated on live, CD3+, single, CD4+ memory T cells (see **Figure S4**) and have been performed on a 96-well plate. Lines represent mean values, error bars the standard deviation.

Figure S3

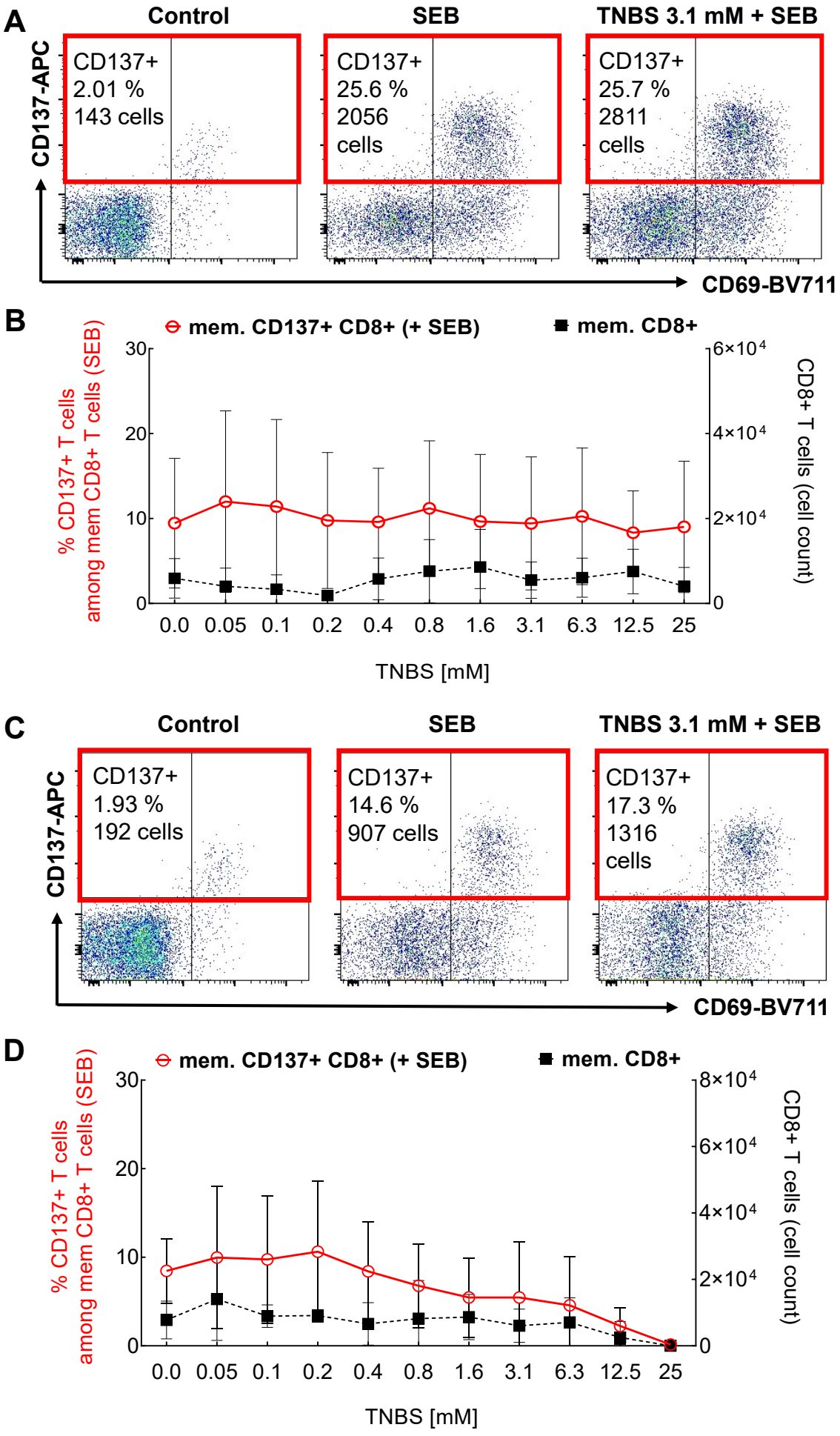

A
Control
SEB
TNBS 3.1 mM + SEB
CD137+
2.01 %
143 cells
CD137+
25.6 %
2056
cells
CD137+
25.7 %
2811
cells
CD137-APC
CD69-BV711
B
mem. CD137+ CD8+ (+ SEB)
mem. CD8+
% CD137+ T cells
among mem CD8+ T cells (SEB)
CD8+ T cells (cell count)
TNBS [mM]
C
Control
SEB
TNBS 3.1 mM + SEB
CD137+
1.93 %
192 cells
CD137+
14.6 %
907 cells
CD137+
17.3 %
1316
cells
CD137-APC
CD69-BV711
D
mem. CD137+ CD8+ (+ SEB)
mem. CD8+
% CD137+ T cells
among mem CD8+ T cells (SEB)
CD8+ T cells (cell count)
TNBS [mM]

Figure S3. Effects of TNBS on viability and T cell CD137 upregulation. **(A, B)** Analysis of "responder" CD8+ memory T cells in a CD137 upregulation assay (16 h). PBMC were CFSE labeled and modified with PBS (control) or TNBS (0.05 – 25 mM, two-fold dilution series) and mixed as APCs in a 1:1 ratio with unmodified "responder" PBMC. In addition, some samples were stimulated with SEB. **(A)** Representative dot plots showing CD137 expression upon different stimulation conditions (red boxes, MASB7). **(B)** Graph summarizing data from n ≥ 3 experiments with different buffy coats and TNBS concentrations for APC modification showing frequencies of SEB-stimulated CD137+CD8+ memory T cells (left y-axis) and total CD8+ memory T cell numbers (right y-axis). **(C, D)** Analysis of T cells from TNBS-treated PBMC (APC). **(C)** Representative dot plots (as in (A), MASB7). **(D)** Graph summarizing data from n ≥ 2-3 experiments, as in (B). All data were gated on live, CD3+, single, CD8+ memory T cells (see **Figure S4**) and have been performed on a 96-well plate. Lines represent mean values, error bars the standard deviation.

Figure S4

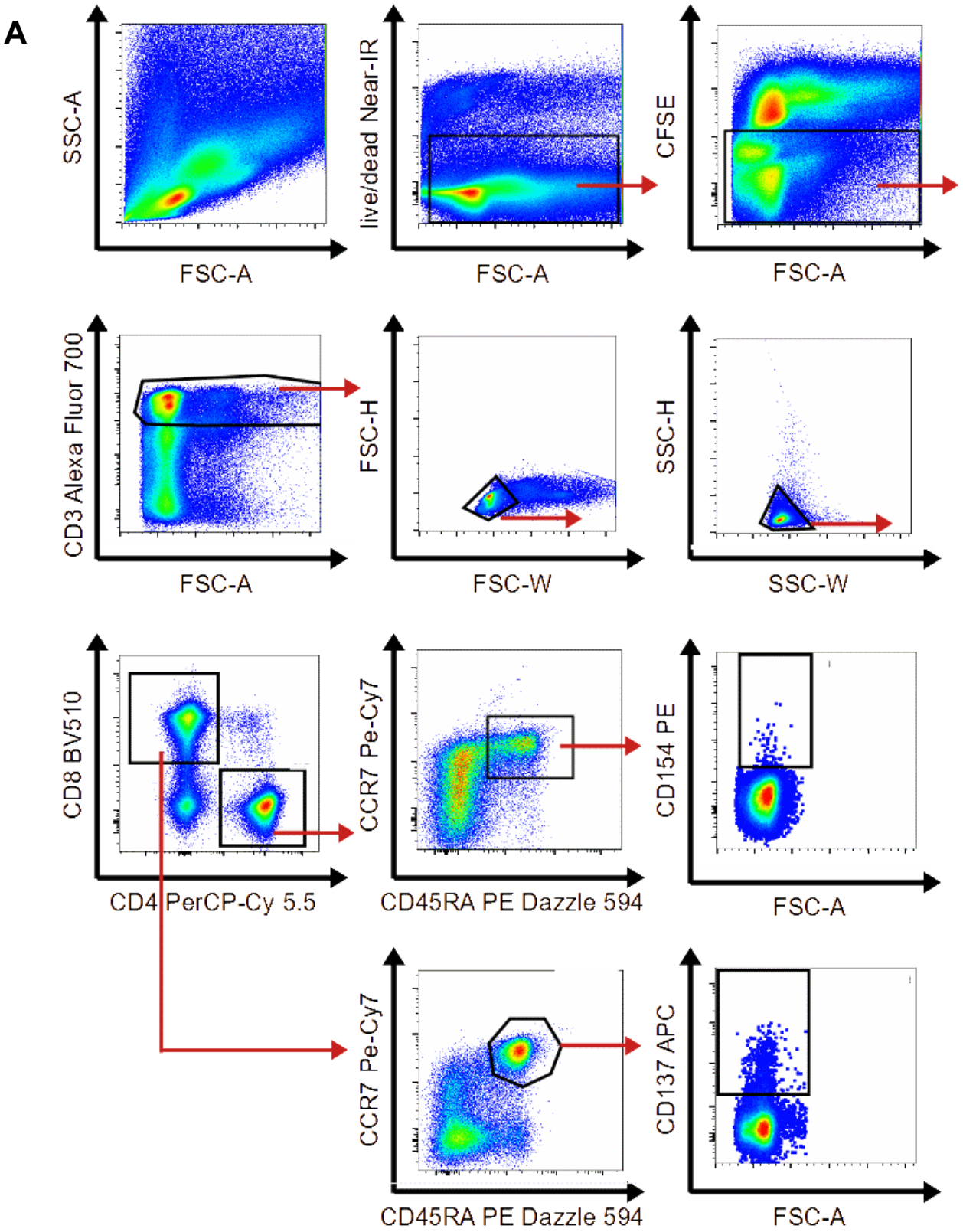


Figure S4. Gating strategy. Responder (non-modified) PBMC were gated on live, CFSE- (excluding CFSE-high population), CD3+, single, CD4+ or CD8+, naïve (CCR7+, CD45RA+) or memory (non-naïve) T cells to analyze TNBS-induced CD154 (5 h) and CD137 (16 h) expression (example from donor MLB20). Expression of further activation markers, e.g. CD69, was assessed among total and antigen-specific CD154+ and CD137+ T cells.

Figure S5

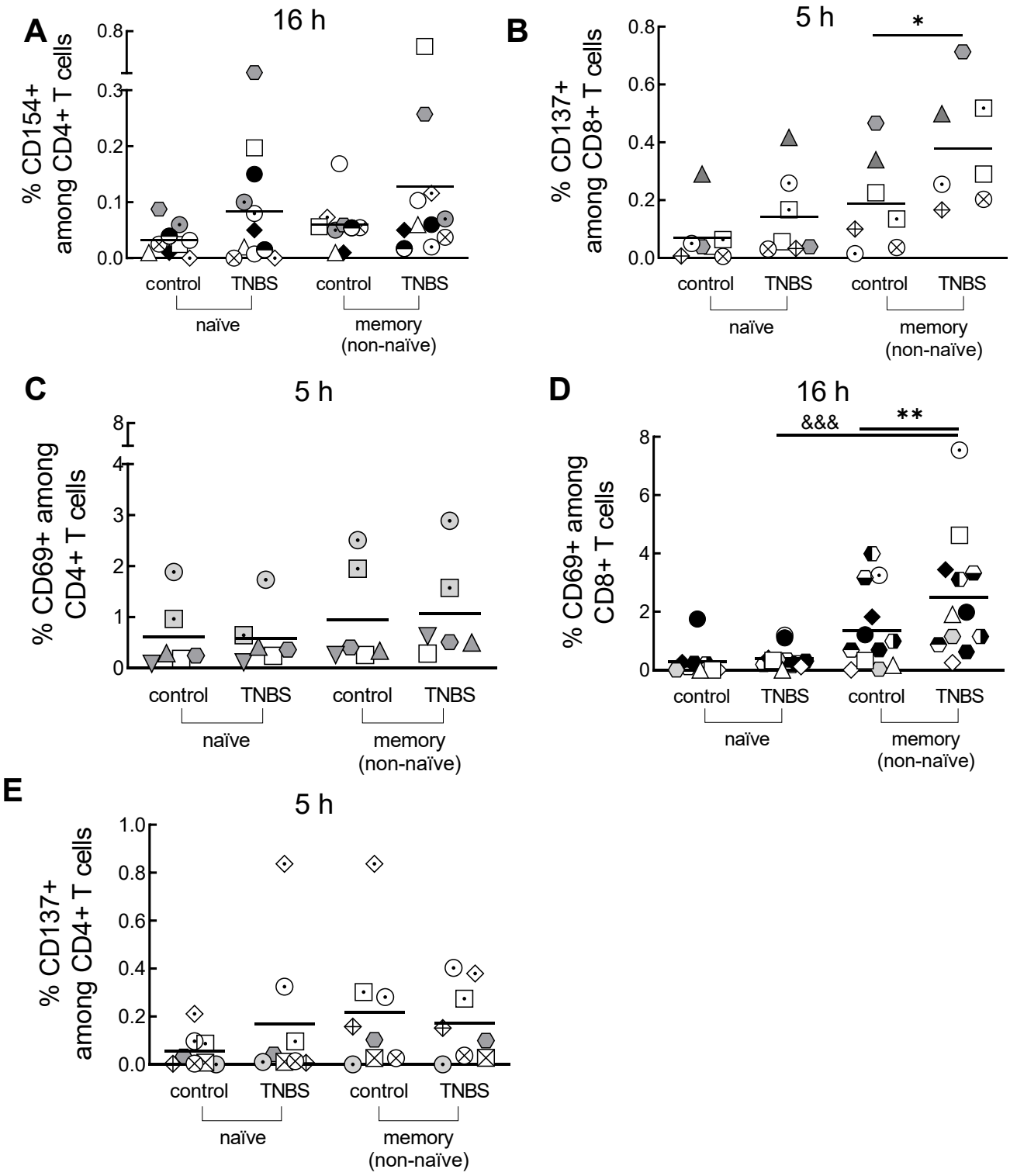


Figure S5. Additional activation marker expression in CD154/CD137 upregulation assays. PBMC were CFSE-labeled and modified with PBS (control) or TNBS as APC and incubated in a 1:1 ratio with unmodified "responder" PBMC for the indicated assay times. **(A)** CD154 upregulation assay (n = 11 buffy coats). **(B)** CD137 upregulation assay (n = 7 buffy coats). **(C, D)** CD69 upregulation by CD4+ **(C)** or CD8+ **(D)** T cells. **(E)** CD137 upregulation by CD4+ T cells. Symbols represent buffy coat identifiers (**Table S1**). Horizontal lines indicate the mean values. Statistical significances were determined by non-parametric Mann-Whitney t-test (P<.*0.5, **0.01 vs memory control; &&& P<.001 vs naïve TNBS).

□ LMB1, ⬡ ML_B31

Figure S6

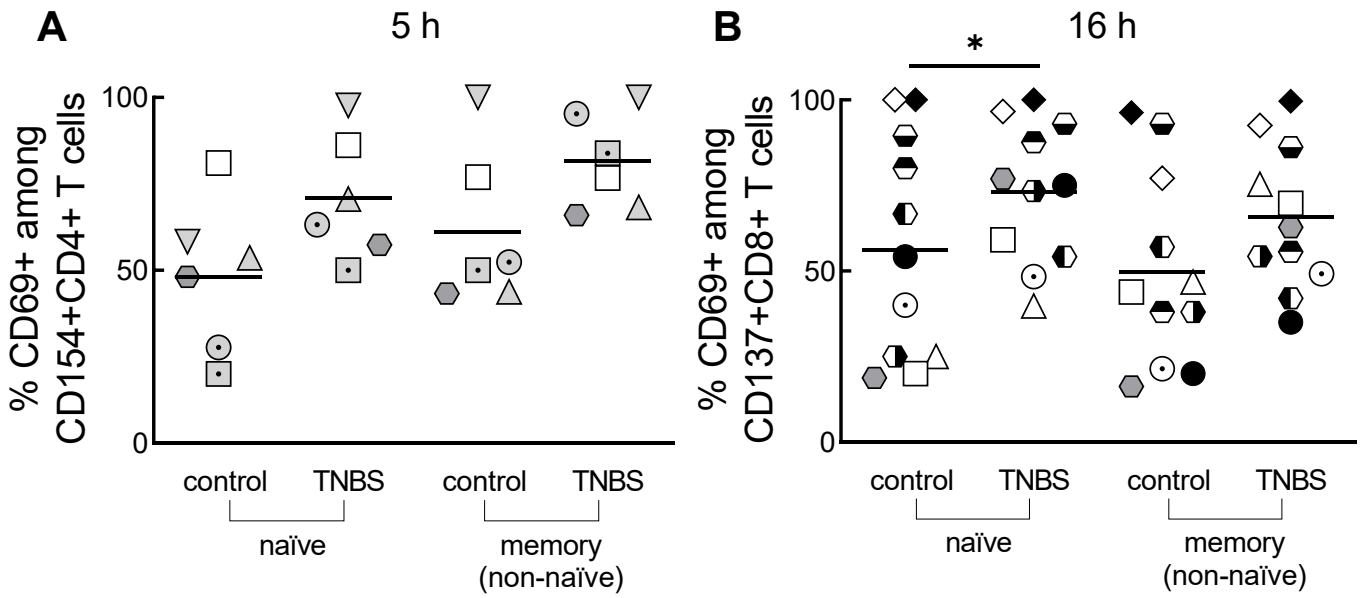


Figure S6. CD69 co-expression analysis. PBMC were CFSE-labeled and modified with PBS (control) or TNBS as APC and incubated in a 1:1 ratio with unmodified "responder" PBMC for the indicated times. **(A)** CD69 co-expression by CD154+CD4+ T cells. **(B)** CD69 co-expression by CD137+CD8+ T cells. Symbols represent buffy coat identifiers (**Table S1**). Horizontal lines indicate the mean values. Statistical significances were determined by non-parametric Mann-Whitney t-test (P<.*0.5 vs memory control).

Figure S7

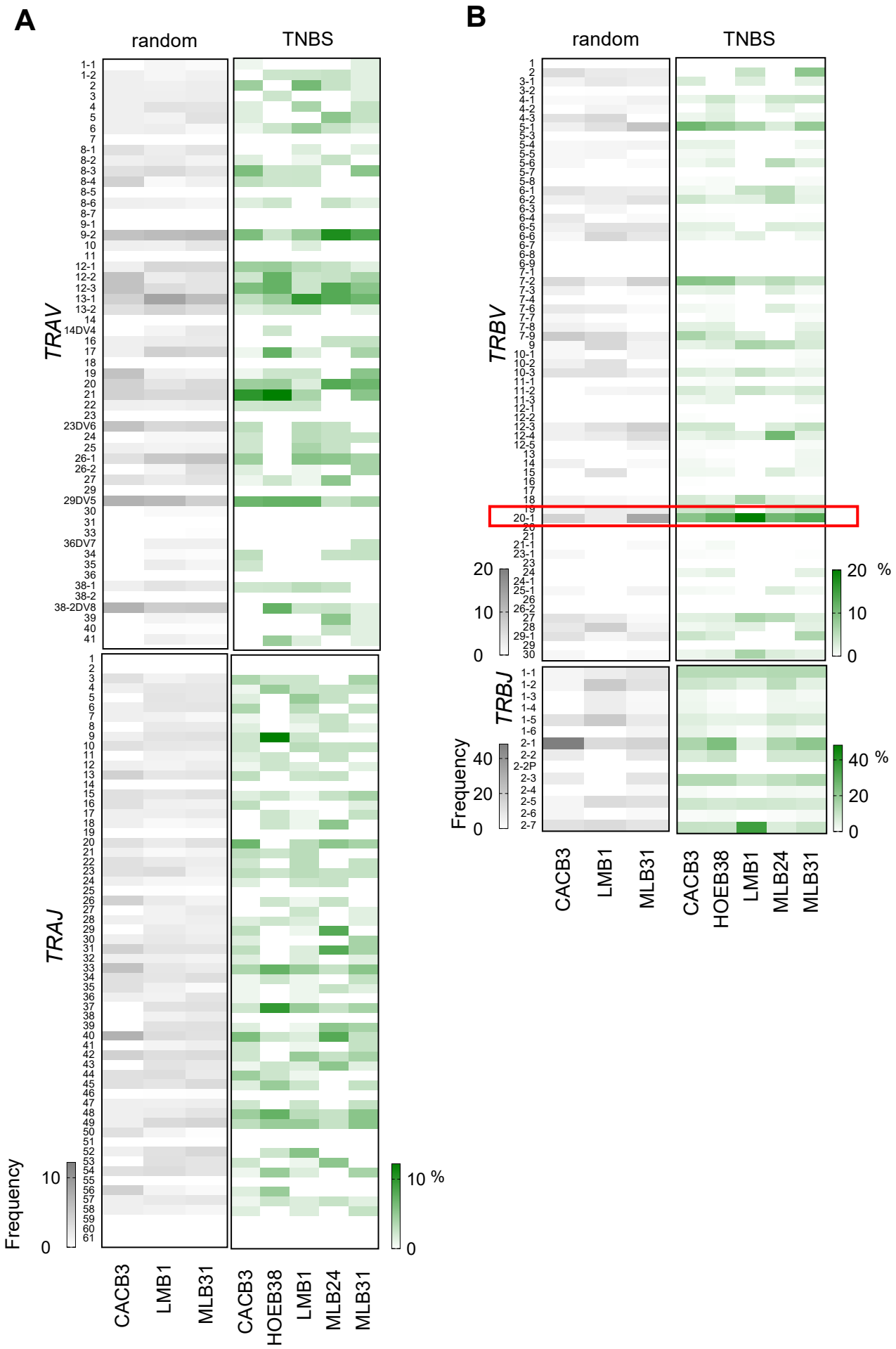
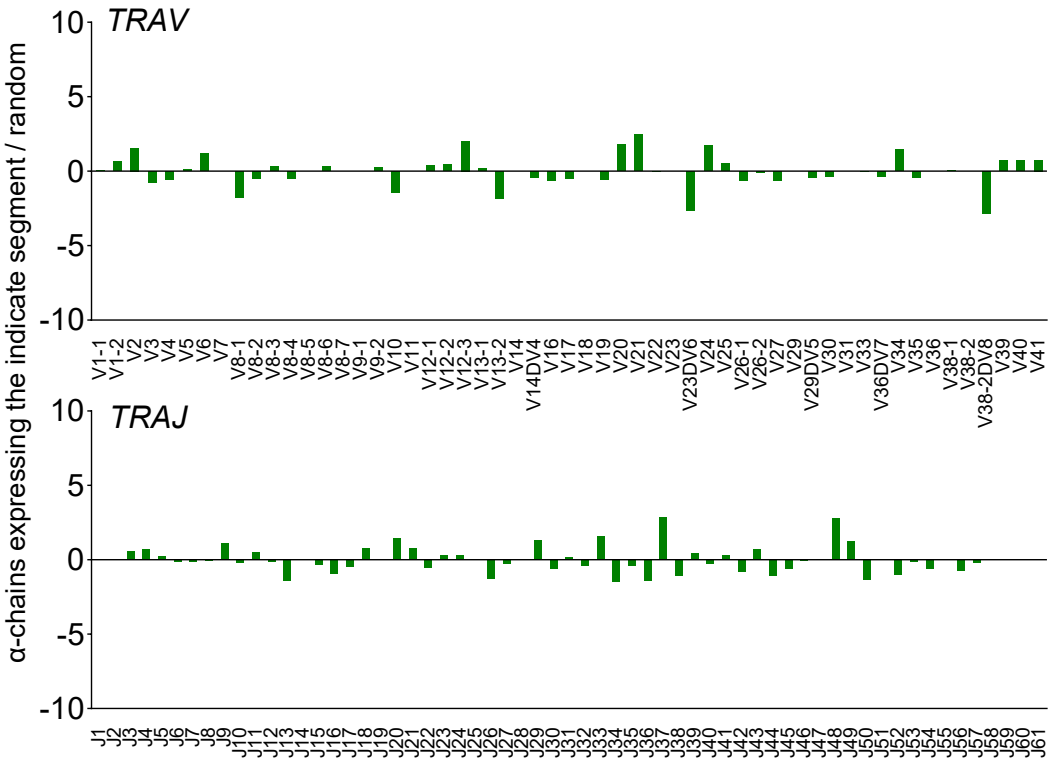

A
random
TNBS
TRAV
TRAJ
Frequency
10
0
10 %
0
CACB3
LMB1
MLB31
CACB3
HOEB38
LMB1
MLB24
MLB31
B
random
TNBS
TRBV
TRBJ
Frequency
20
10
0
40
20
0
20 %
10
0
40 %
20
0
CACB3
LMB1
MLB31
CACB3
HOEB38
LMB1
MLB24
MLB31

Figure S7, cont.

C



D

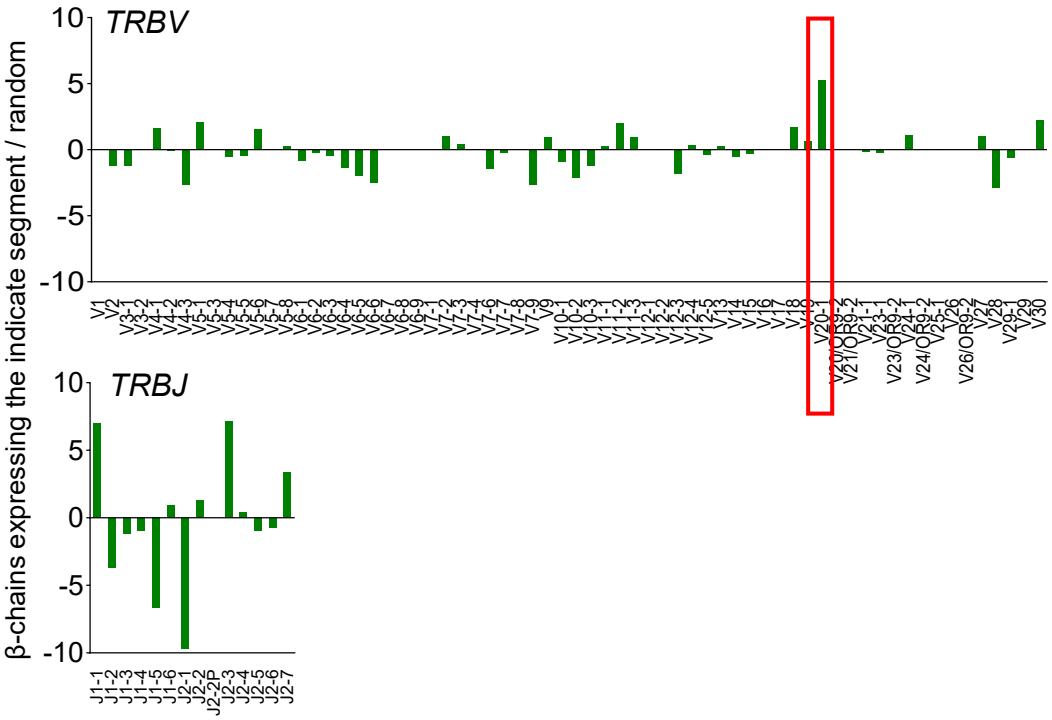


Figure S7, cont.

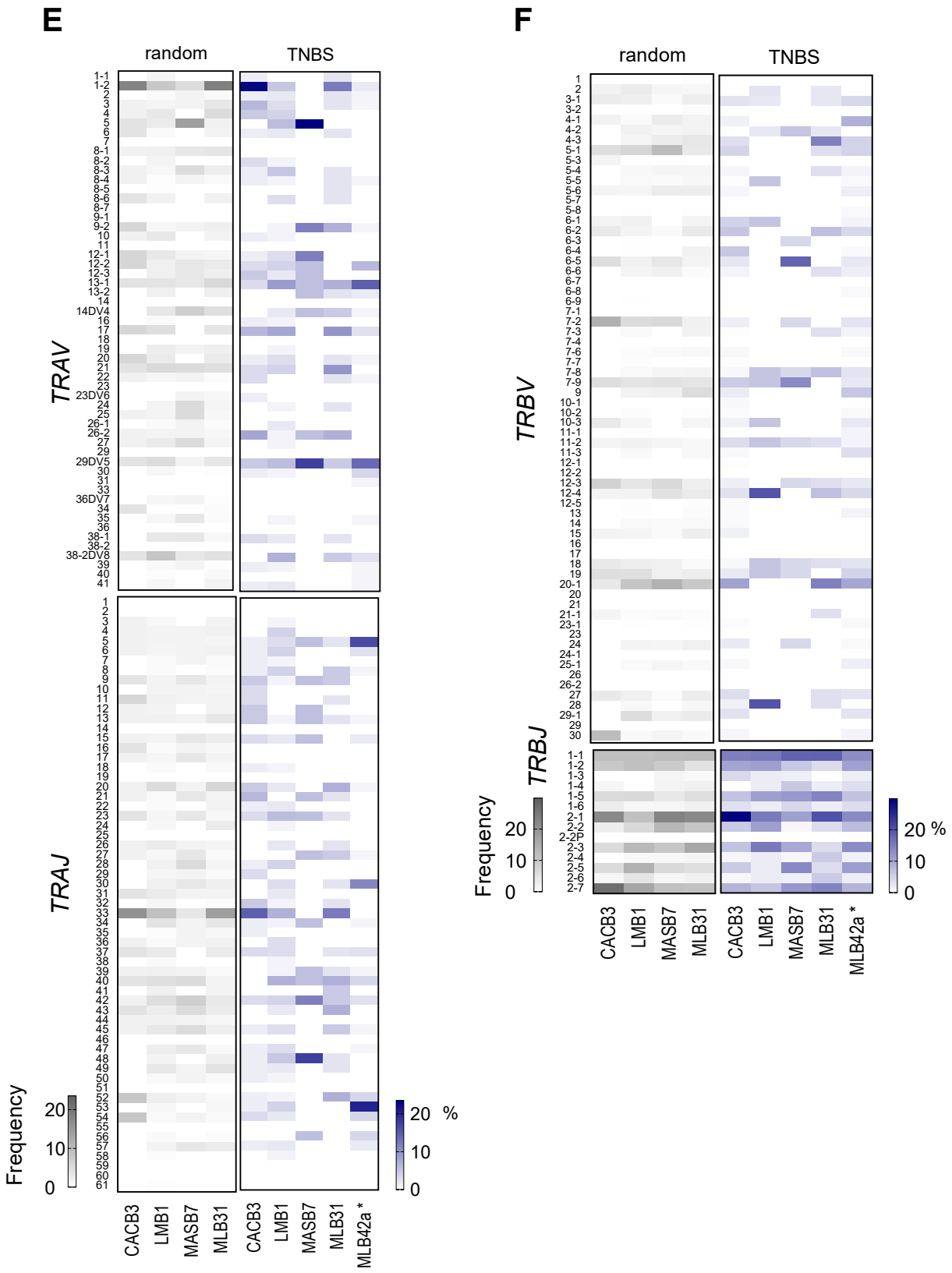


Figure S7, cont.

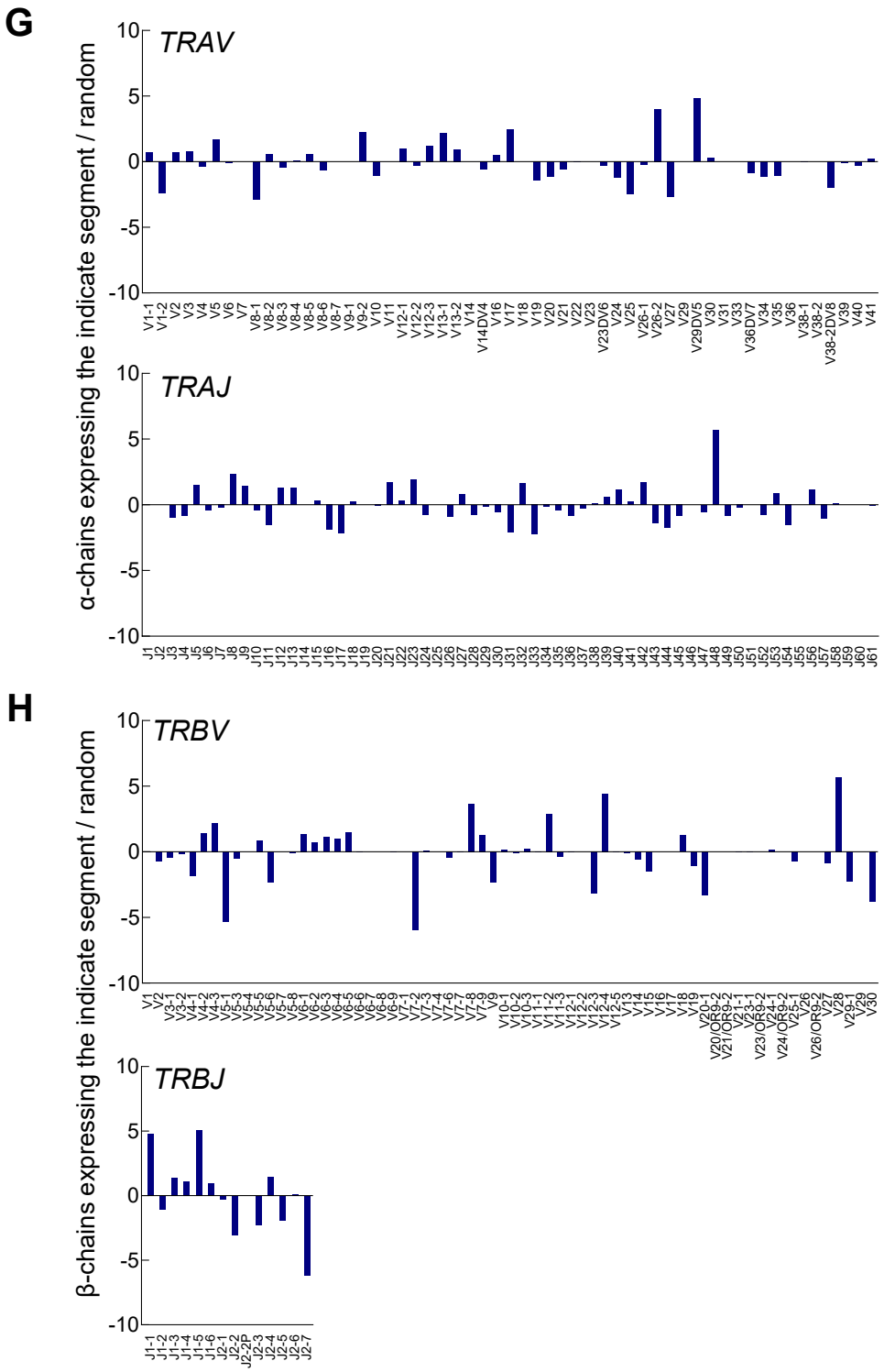


Figure S7. Gene segment use by TNBS-specific T cells. TNBS-specific CD154+CD4+ and CD137+CD8+ memory T cells were sorted from 5 h and 16 h experiments, respectively, and their TCR were sequenced (**Table S3**). Random CD4+ and CD8+ memory T cells from the same buffy coats served as control. Heatmaps and bar charts depict TCR α- **(A, C, E, G)** and β-chain **(B, D, F, H)** V- and J-segment gene use expressed as TCR diversity frequencies. Random TCR are shown in grey, TNBS-specific TCR in green (CD4+ T cells) and blue (CD8+ T cells). Highlighted in red **(B, D)** is the TRBV20-1 segment. Statistical significance was determined by multiple t-test with Sidak-Holm correction for multiple comparison (n = 3 – 5, adj.-P-value = 0.004).

Figure S8

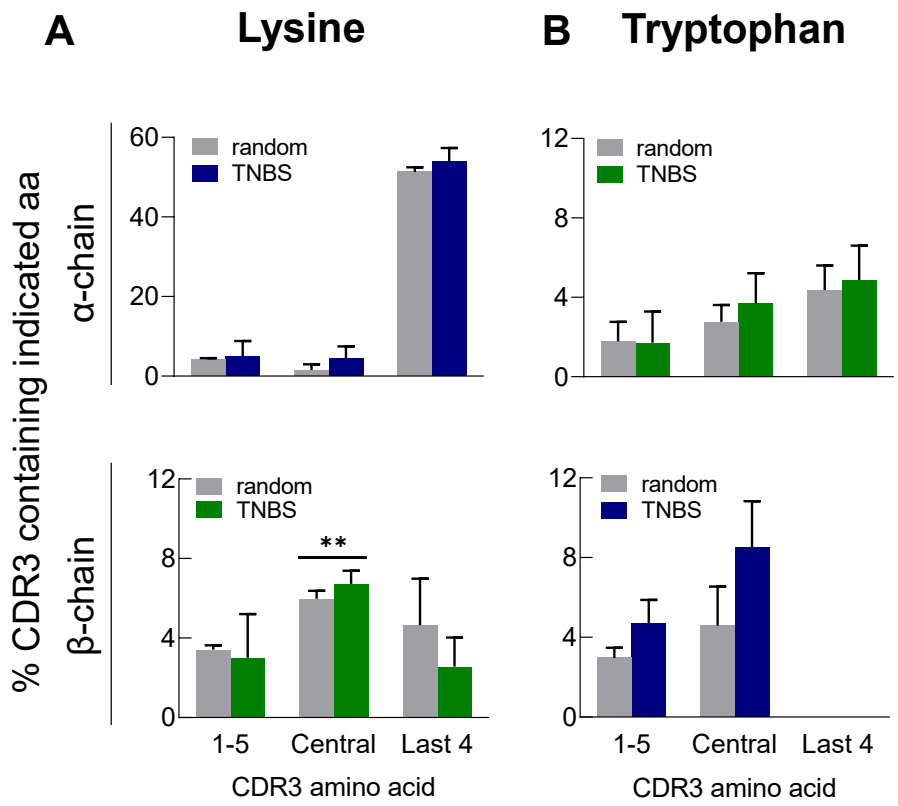


Figure S8. Lysine and tryptophan location in the CDR3. Random and TNBS-specific CD4+ and CD8+ memory T cells were sorted and their TCR sequenced (**Table S3**). (**A, B**) The graphs depict lysine (**A**) and tryptophan (**B**) occurrence according to their locations in the CDR3 of TCR α- (upper panels) and β-chains (lower panels) among random (grey) and TNBS-specific CD4+ (green) and CD8+ (blue) T cells (see also **Figure 3G**). Statistical significance was determined by t-test and corrected for multiple comparisons with the Holm-Sidak's method (n = 3 – 5, **P<.01).

Figure S9

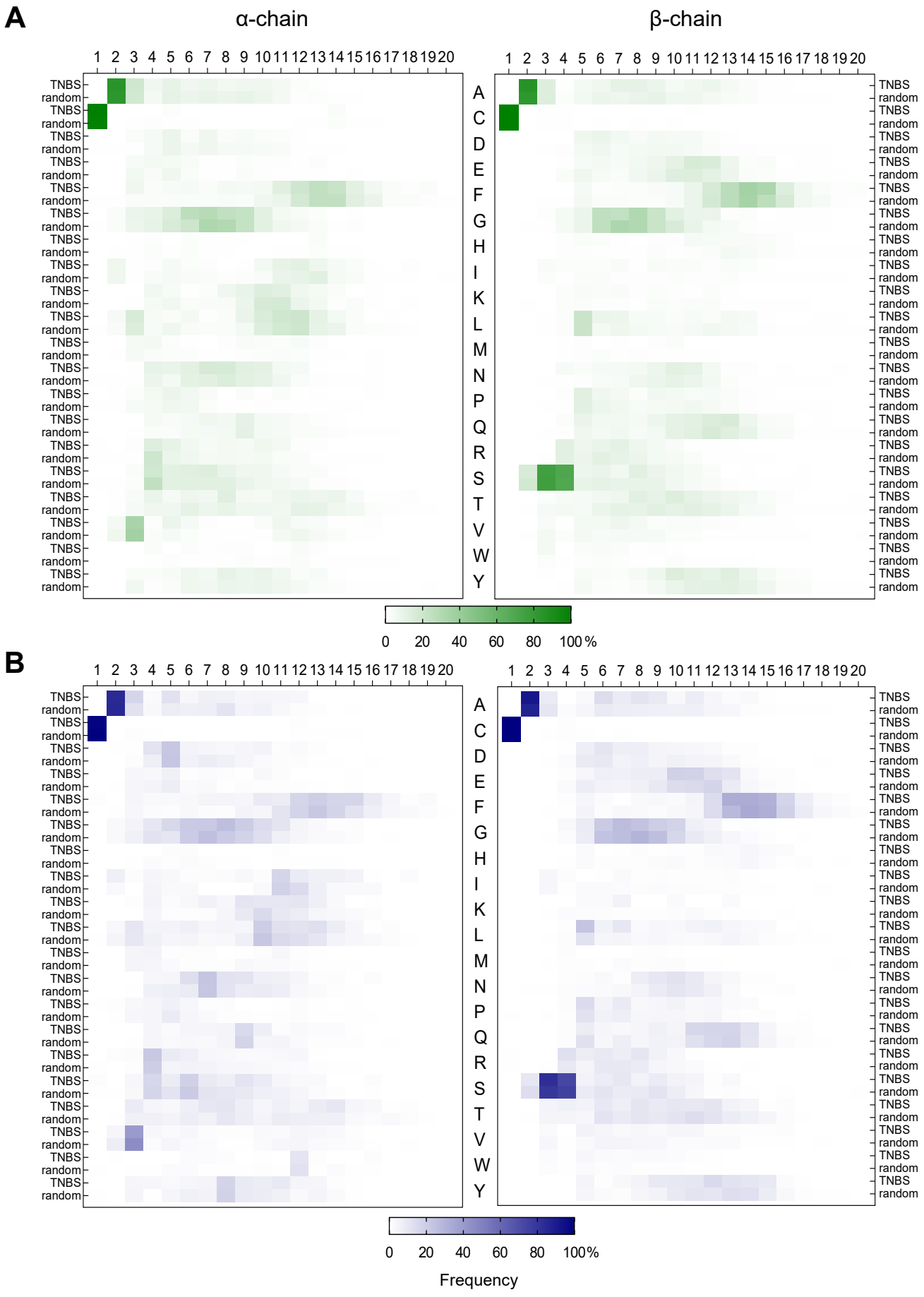

A
α-chain
β-chain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
TNBS
random
A C D E F G H I K L M N P Q R S T V W Y
0 20 40 60 80 100 %
B
0 20 40 60 80 100%
Frequency

Figure S9, cont.

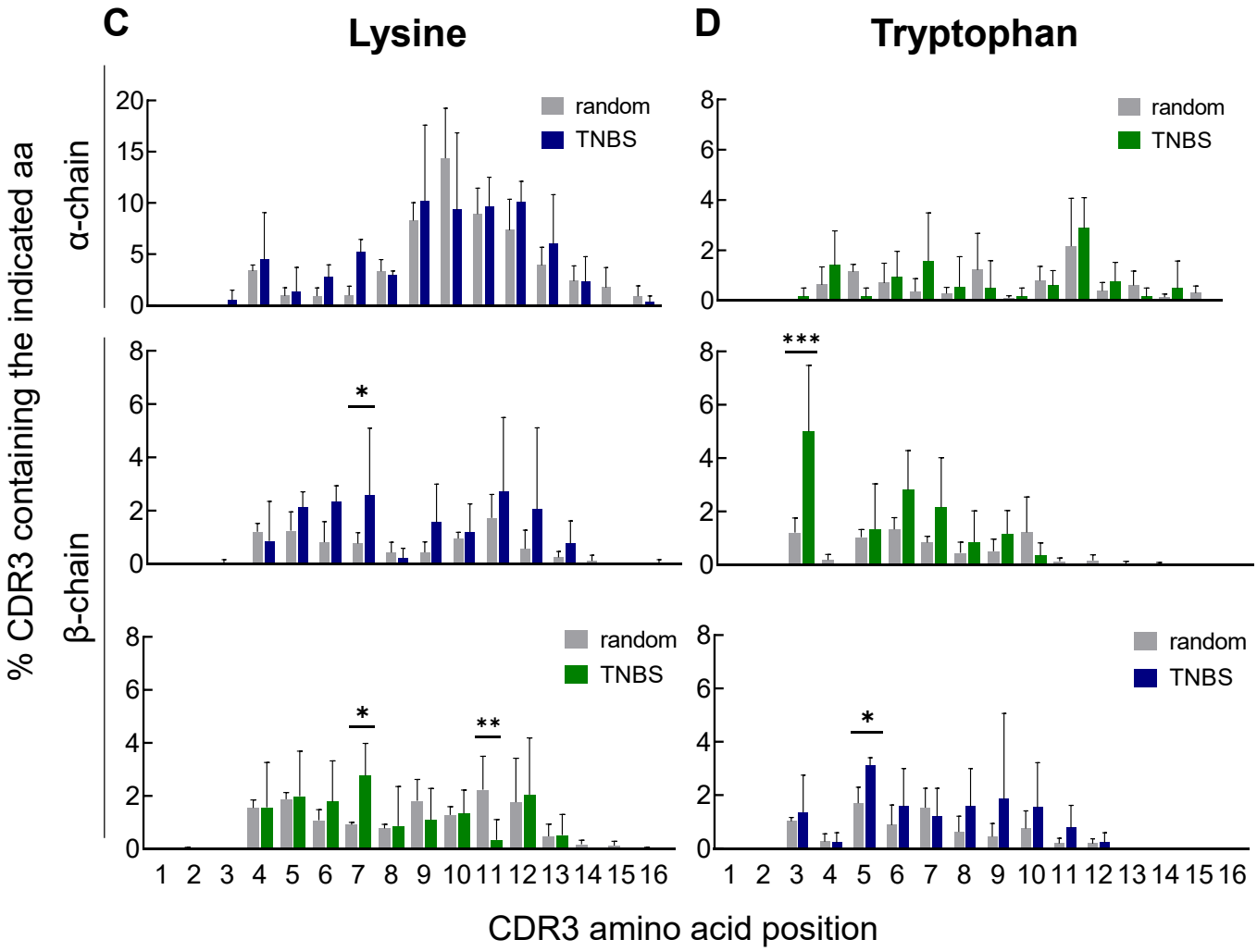


Figure S9. Positional exact analysis of CDR3 amino acids. Random and TNBS-specific CD4+ and CD8+ memory T cells were sorted and their TCR sequenced (**Table S3**). (**A, B**) Heatmaps depicting the occurrence of all amino acids in the CDR3 according to their positions (positions 1 to 16 cover most of the CDR3 length) for TCR α- and β-chains of CD4+ (green, **A**) and CD8+ (blue, **B**) T cells (mean values, TCR diversity, n = 3-5). (**C, D**) Bar charts depict lysine (**C**) and tryptophan (**D**) occurrence according to their CDR3 position in TCR α- (upper panels) and *β*-chains (middle and lower panels) for TCR from random (grey) and TNBS-specific CD4+ (green) and CD8+ (blue) T cells. Statistical significances were determined by multiple t-test and corrected for multiple comparison with the Sidak-Holm's method (n = 3 – 5, P<*0.05,**0.01, ***0.001).

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Supplementary Material

Table S1. **Sample overview.** This table lists buffy coats, frequency percentages of activated T cells and CD69 co-expression (raw values from **Figures 1, S6**) and unique buffy coat identifiers (donor symbols). Shaded areas indicate samples from which T cells were sorted for TCR HTS. Empty values indicate that samples have not been analyzed. Bold and underlined values indicate samples, where T cells have been sorted for *in vitro* expansion and restimulation experiments.

Buffy coat	**CD4+ memory T cells** (5 hour assays)					**CD8+ memory T cells** (16 hour assays)					**Symbol**
	Well size	**Control**		**TNBS**		**Well size**	**Control**		**TNBS**		
		% CD154+	**% CD69 co-exp.****	**% CD154+**	**% CD69 co-exp.****		**% CD137+**	**% CD69 co-exp.****	**% CD137+**	**% CD69 co-exp.****	
ABAB3						6-well	0.11	78.38	0.27	81.58	
CACB2	6-well*&	0.01		0.04							
CACB3	6-well	0.07		**0.11**							
HOEB38	12-well*&	0.02		0.07							
IWB1	12-well	0.01	43.8	0.02	68.2						
IWB2	12-well	0.01	100.0	0.02	100.0						
LMB1	12-well	0.04	76.9	0.09	76.7	12-well	1.19	43.88	**5.25**	69.8	
MASB7						12-well	0.37	46.79	**0.84**	75.4	
MLB20	12-well	0.10	52.4	**0.25**	95.3	12-well	0.49	73.3	0.62	76.3	
MLB21	12-well	0.05	50.0	0.08	83.9						
MLB23	6-well*&	0.17		0.18							

Buffy coat	**CD4+ memory T cells** (5 hour assays)					**CD8+ memory T cells** (16 hour assays)					**Symbol**
	Well size	**Control**		**TNBS**		**Well size**	**Control**		**TNBS**		
		% CD154+	**% CD69 co-exp.**[**]	**% CD154+**	**% CD69 co-exp.**[**]		**% CD137+**	**% CD69 co-exp.**[**]	**% CD137+**	**% CD69 co-exp.**[**]	
MLB24	6-well	0.00		0.03		96-well	0.07	25.0	0.90	28.3	
MLB27						12-well[*]	0.18	39.24	1.76	59.33	
MLB31	12-well	0.04	43.33	0.06	65.95	12-well	0.47	16.3	1.23	62.8	
MLB35						12-well	0.37		0.89		
MLB36						12-well	0.03	77.1	**0.13**	95.4	
MLB37						12-well	0.08	96.3	0.42	99.6	
MLB38						12-well	0.12	21.4	1.27	49.6	
MLB39						12-well	0.49	20.5	1.13	34.2	
MLB40						12-well	0.26		0.19		
MLB42a						12-well	0.07	38.46	**0.11**	55.3	
MLB43						12-well	0.29	56.2	0.12	41.2	
MLB44						12-well	0.10	38.5	0.10	54.3	
MLB45						12-well	0.18	93.3	0.22	86.2	
Means ± SD		0.05 ± 0.05	61.1 ± 20.7	0.09 ± 0.07	81.7 ± 12.8		0.29 ± 0.27	58.9 ± 22.8	0.91 ± 1.19	67.7 ± 19.0	
Background (control) corrected[#]				**0.04 ± 0.02**	**20.6 ± 7.90**				**0.62 ± 0.92**	**8.80 ± 3.80**	

[*]control samples analyzed on 24-well plates. [&]TNBS samples analyzed on 6-well plates. [**]percentage of CD154+ or CD137+ T cells that co-express CD69. [#]Values from the respective controls were subtracted.

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Supplementary Material

Table S2. Overview of TNBS-specific T cell clones and lines. This table lists sorted T cells from CD154/CD137 upregulation assays. For lines, the initially sorted cell number is given in the line designation in bold. After *in vitro* expansion, cells were tested for TNBS specificity in restimulation assays. "+" indicates a positive result, "-" indicates a negative result. In addition, some clones and lines were tested for MHC restriction.

Buffy coat	**T cell clone/line designation**	**Restimulation result**	**Tested for MHC restriction**
CACB3	CD4_5T	+	yes
CACB3	CD4_19T	+	yes
CACB3	CD4_15T	-	no
CACB3	CD4_1T	+	yes
CACB3	CD4_2T	+	yes
CACB3	CD4_14T	+	yes
CACB3	CD4_16T	+	yes
CACB3	CD4_20T	+	yes
MLB20	CD4_T3-**50**	+	no
MLB20	CD4_8D	-	no
MLB20	CD4_11E	+	no
MLB20	CD4_10D	+	no
MLB20	CD4_9B	-	no
LMB1	CD8_F5	+	yes
LMB1	CD8_F6	+	yes
LMB1	CD8_B3-**100**	-	no
MASB7	CD8_12D2	+	yes
MASB7	CD8_9C1	-	no
MASB7	CD8_4A2	-	no
MLB42a	CD8_1A-**50**	+	no
MLB42a	CD8_1B-**50**	-	no
MLB42a	CD8_1C-**50***	+	no
MLB42a	CD8_1D-**50**	+	yes
MLB42a	CD8_4D	+	yes
MLB42a	CD8_4E	+	yes
MLB42a	CD8_4F	+	yes
MLB36	CD8_B7	+	no
MLB36	CD8_8E	+	no

*analyzed by TCR-HTS (see Table S3)

Supplementary Material

Table S3. **Overview of TCR sequencing data.** This table lists the number of sorted cells from the respective buffy coats, obtained TCR sequences (counts, each count is represented by one UMI) and TCR diversity (clonotypes).

Buffy coat	**CD4+ memory T cells***						**CD8+ memory T cells****					
	Control (random T cells)			TNBS-specific (CD154+)			Control (random T cells)			TNBS-specific (CD137+)		
	Sorted cells	Counts / diversity		Sorted cells	Counts / diversity		Sorted cells	Counts / diversity		Sorted cells	Counts / diversity	
		α	β		α	β		α	β		α	β
CACB3	500	91 69	144 109	393	296 129	197 116	500	79 50	169 85	450	100 61	122 60
HOEB38				524	71 41	77 49						
LMB1	10 000	1524 1159	1765 1396	600	208 122	178 114	10 000	820 453	1345 552	2 556	157 98	242 155
MLB24				503	66 37	88 49						
MLB31	10 000	1015 708	1425 976	369	111 72	62 45	10 000	607 345	867 518	727	73 41	59 36
MLB42a §										5 000	32471 108	13225 22

*Sorted from 5 hour assays, **sorted from 16 h assays; §$CD137^{+}CD8^{+}$ T cell line (MLB42a_CD8_1C-50, consisting of initially 50 sorted cells that had been expanded *in vitro*, see also **Table S2**)

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Supplementary Material

Supplementary Methods

Blood samples and isolation of PBMC

T cell media (TCM) consisted of RPMI 1640 medium supplemented with 5% (v/v) human AB serum (both PAN Biotech), 2 mM glutamine (GlutaMAX), 55 µM β-mercaptoethanol (both Thermo Fisher Scientific), 1x non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (all PAN Biotech), 1x Penicillin/Streptomycin (Capricorn Scientific) and 1.5 mM $CaCl_2$ (Sigma Aldrich) for optimal T cell responses (Zimmermann et al., 2015)

Antigen presenting cells (APC) preparation

CFSE modification was done for 15 min at 37°C with $20x10^6$ cells/ml in a water bath. Cells were washed once by addition of a 4-fold volume of MACS buffer (0.5% fetal calf serum (FCS, Biochrome), 2 mM EDTA in PBS). TNBS modification (Sigma Aldrich, Cat# 92822-1ML) was done afterwards with $20x10^6$ cells/ml and cells were washed three times in a 4-fold volume of MACS buffer.

T cell antigen stimulation assay

Antibodies for T cell antigen stimulation assays and major histocompatibility complex *(*MHC) blocking experiments:

Antigen	Clone	Concentration	Company	Catalog number and/or identifier
CD40	HB14	1 µg/ml	Miltenyi Biotec	Cat# 130-094-133; RRID:AB_10839704
HLA-DR, -DP, -DQ	Tu39	10 µg/ml	BD Biosciences	Cat# 555556; RRID:AB_395938
HLA-DR	Ac122	10 µg/ml	Miltenyi Biotec	Cat# 130-108-056; RRID:AB_2661330
HLA-DR	L243	10 µg/ml	BioLegend	Cat# 307602; RRID:AB_314680
HLA-ABC	W6/32	10 µg/ml	BioLegend	Cat# 311428; RRID:AB_2561492
-(IgG2a isotype control)	MOPC-173	10 µg/ml	BioLegend	Cat# 400224; RRID:AB_2861018

Supplementary Material

Antibody staining and flow cytometry analysis

Antibodies for flow cytometry were titrated to determine optimal dilutions and staining volumes.

Antigen	Clone	Fluorochrome conjugate	Company	Catalog number and/or identifier
CD3	UCHT1	AlexFluor® 700	BioLegend	Cat# 300424; RRID:AB_493741
CD4	SK3	BB700	BD Biosciences	Cat# 566392; RRID:AB_2744421
CD8	SK1	BV510	BioLegend	Cat# 344732; RRID:AB_2564624
CD45RA	HI100	PE/Dazzle™ 594	BioLegend	Cat# 304146; RRID:AB_2564079
CCR7	G043H7	PE-Cy7	BioLegend	Cat# 353226; RRID:AB_11126145
CD154	5C8	PE	Miltenyi Biotec	Cat# 130-113-607; RRID:AB_2751142
CD137	4B4-1	APC	BioLegend	Cat# 309810; RRID:AB_830672
CD69	FN50	BV711	BioLegend	Cat# 310944; RRID:AB_2566466
CD14	M5E2	BV421	BioLegend	Cat# 301830; RRID:AB_10959324
CD19	HIB19	APC	BD Biosciences	Cat# 555415, RRID:AB_398597
TNP	A19-3	BV421	BD Biosciences	Cat# 562601; RRID:AB_11153675
dead cells	-	*	Thermo Fisher Scientific	Cat# L10119

*LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit. Dead cell stain was added immediately before use to the antibody mix to stain cells simultaneously for surface markers and dead cells.

Stainings were performed in FACS buffer (PBS, 2 mM EDTA, 0.5% FCS). Our BD FACSAria III is equipped with three lasers (405 nm, 488 nm, 633 nm) and mirror/filter combinations for the simultaneous detection of 11 colors. For samples from 96- and 24-well plates, all events were acquired. For samples from 12- and 6-well plates, we collected 1 million total events and afterwards stored for remaining cells all events contained in the SSC single cell gate (see gating strategy, **Figure S4**).

Single cell sort, expansion and restimulation of T cell clones

Additional functional antibody for single clone expansion and restimulation assays.

antigen	clone	concentration	company	catalog number and/or identifier
CD3	OKT3	30 ng/ml (in feeder medium) 1 µg/ml (plate coating)	Miltenyi Biotec	Cat# 130-093-387; RRID:AB_1036144

CD28	15E8	1 µg/ml	Miltenyi Biotec	Cat# 130-093-375; RRID:AB_1036134

Feeder cells were prepared by incubation of $50x10^6$ PBMC/ml with 50 µg/ml mitomycin C (Abcam) in PBS and 10 % FCS (Biochrome) for 30 minutes at 37°C with occasional mixing and extensive washing afterwards. Cells were kept in liquid nitrogen until use.

Positive specific clone restimulation was assumed if the following conditions were fulfilled: (a) the frequency of CD154+CD4+ or CD137+CD8+ cells was 3-times higher in antigen-stimulated samples compared to samples without antigen, (b) the frequency of CD154+CD4+ or CD137+CD8+ T cells after Phorbol-12-myristat-13-acetat (PMA)-Ionomycine stimulation was >30% in T cells without APC and (c) the count of CD154+CD4+ or CD137+CD8+ cells was >20 cells.

Supplementary References

Zimmermann, J., Radbruch, A., and Chang, H.D. (2015). A Ca(2+) concentration of 1.5 mM, as present in IMDM but not in RPMI, is critical for maximal response of Th cells to PMA/ionomycin. *Eur J Immunol* 45**,** 1270-1273.