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 NiSO₄. TCR α- and β-chains of sorted nickel-specific and control cells were studied by high-throughput sequencing.

Results: Stimulation of PBMCs with NiSO₄ 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; NiSO₄, 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 exposure²⁻³ 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.⁵⁻⁹ 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. However, Ni-reactive clones were often generated 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. Ni can bind to histidines on MHC II-presented peptides as has been shown for a single peptide. Also, peptide mimotopes have been identified that activate a Ni-specific TCR and likely replace an unknown Ni-loaded peptide. Alternatively, an interaction with TCR β-chain segment TRBV19 (Vβ17 in Arden nomenclature) has been discussed but mutation analysis of three TRBV19 clones rejected an involvement in Ni binding. 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.

To analyze the Ni-reactive αβ TCR repertoire more comprehensively, we here adopted induced CD154 (CD40L) expression and combined it with high-throughput sequencing of both TCR α- and β-chains. 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 NiSO₄ in the presence of CD40 blocking antibody (1 µg/mL, HB14, Miltenyi Biotec). 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. 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, 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 NiSO₄. 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 NiSO₄, 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
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, as observed in previous studies.

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). NiSO₄₄ 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 NiSO₄₄ 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\textsuperscript{32} and because Ni-specific CD8+ T cells seem less frequent in general,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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, was also more frequently expressed by Ni-specific T cells (Figure 2C). Nur77 and CD69, markers for TCR-mediated activation, 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.\textsuperscript{14-16} Many expressed INF\textsubscript{Y}, 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\textsubscript{h}17 or T\textsubscript{h}2 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 IFN\textsubscript{Y}.

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 repertoire differences 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.\textsuperscript{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,\textsuperscript{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\textsubscript{36} in the CDR1 of TRAV9-2.\textsuperscript{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
% CDR3 containing the indicated amino acid

% change in aa use among Ni-specific CDR3

% amino acid

% CDR3 containing histidines

TRA9-2+ TCR α-chains

TRA9-2- TCR α-chains

% CDR3 containing histidines

% CDR3 containing histidines
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 repertoires (Figure S5B). Since we observed and TCR diversity percentages were similar for the different repertoires of Ni-specific CD154+ CD4+ T cells in blood of allergic and nonallergic donors.

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

4 | DISCUSSION

The CD154 upregulation assay has several advantages. 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.

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 NiSO4 here vs 0.02% with ~40 µmol/L NiSO4). We used a high nontoxic concentration of NiSO4 to also capture low-affinity clones. The importance of low-affinity clones becomes increasingly recognized even in chronic T cell responses in contrast to antibody 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.

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.

The use of the TRAV9-2 segment has been previously described for only one Ni-specific clone termed “SE9”. Mutation studies of “SE9” showed that it recognizes Ni via tyrosine in the CDR1 of TRAV9-2 and via histidine 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. Given that MHC II histidine 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. Contrary, Beryllium disease, another metal allergy, has a HLA allele-associated mechanism where Be has no direct TCR contact. Additionally, drugs may cause type IV hypersensitivity by noncovalent binding to certain HLA alleles via "pharmacological interactions". 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 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).
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 in the α-chain CDR3 was mutated to histidine, suggesting a role in peptide but not Ni binding. Accordingly, some Ni-reactive clones are dependent on peptides presented by certain APCs, as reported here and elsewhere.

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 cells58 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.

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