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

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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 $\alpha\beta$ 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 $\mu\text{mol/L}$ 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 specifically restimulated. Among nickel-specific CD4+ T cells of allergic and non

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.

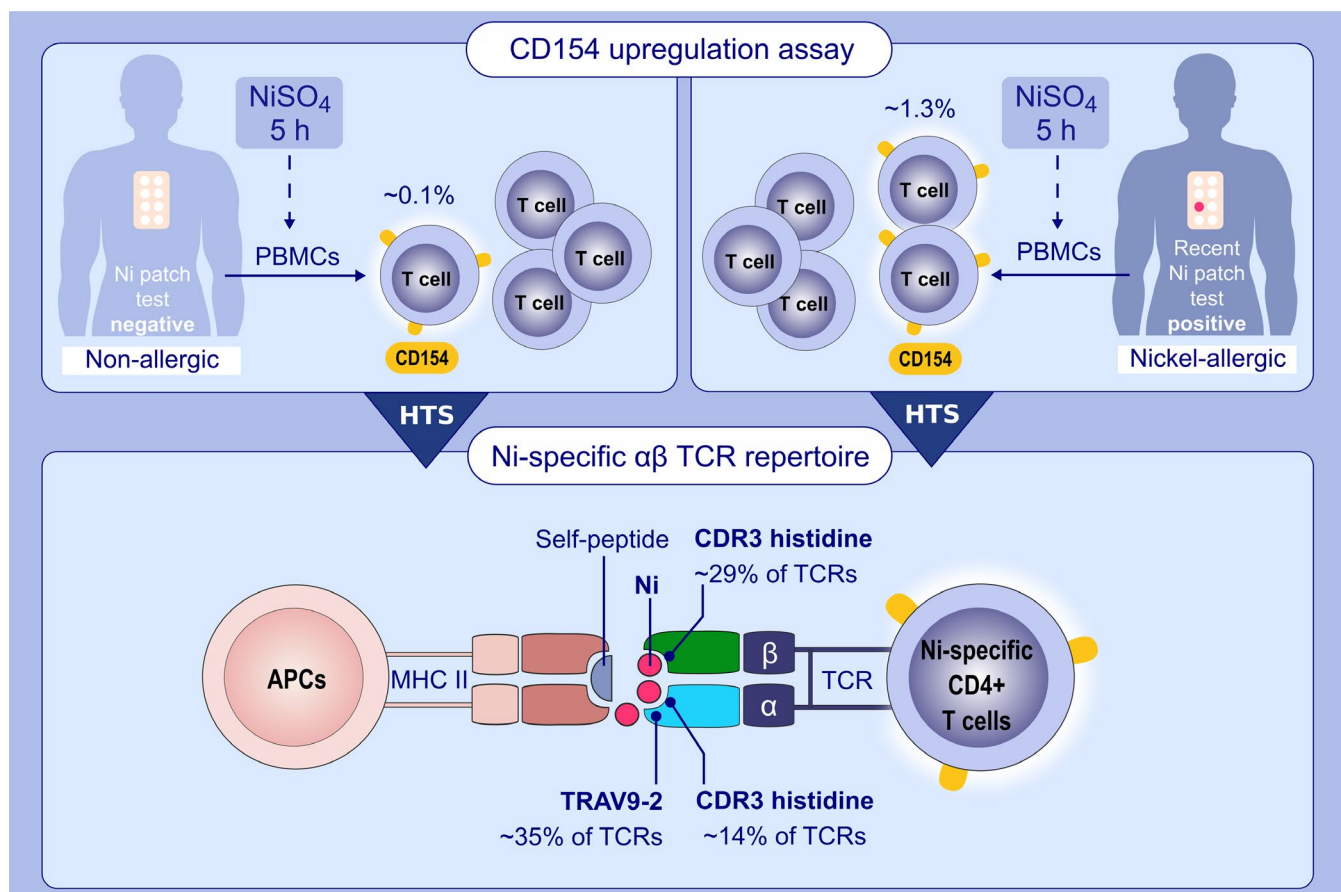
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allergic donors, TCRs expressing the α -chain segment TRAV9-2 or a histidine in their α - or β -chain complementarity determining region 3 (CDR3) were highly overrepresented. **Conclusions:** Induced CD154 expression represents a reliable method to 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 $\alpha\beta$ T cell receptors reveals an overrepresentation of α -chain segment TRAV9-2 and CDR3 histidines.

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 (V β 17 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 $\alpha\beta$ TCR repertoire more comprehensively, we here adopted induced CD154 (CD40L) expression²⁷⁻²⁹ and combined it with high-throughput sequencing of both TCR α - and β -chains³⁰. 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

Highlights

- Nickel-specific naïve and memory CD4+ T cells can be detected by induced CD154 (CD40L) expression.
- In some allergic donors with recent positive patch test, memory frequencies are increased and linked to markers of *in vivo* immune activation.
- In all donors, nickel activates mainly TCRs expressing α -chain segment TRAV9-2 or a histidine in their α - or β -chain CDR3.

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 μ mol/L NiSO₄ in the presence of CD40 blocking antibody (1 μ g/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 $\alpha\beta$ 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

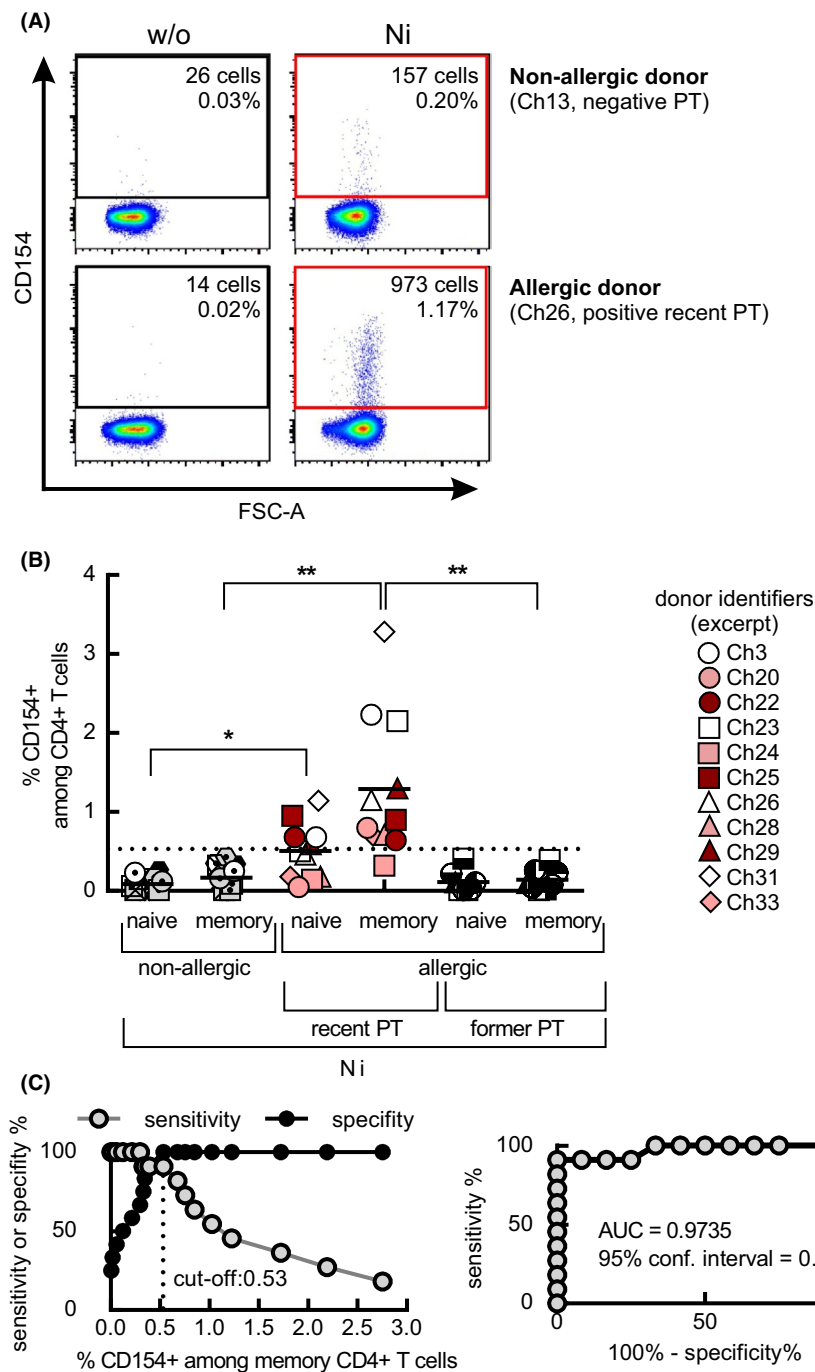


FIGURE 1 Detecting Ni-specific CD4+ T cells by CD154 upregulation assay. (A) Dot plots showing frequencies and cell numbers of CD154+ memory CD4+ T cells without antigen stimulation (w/o, left panels) and after 5 h of stimulation with 200 μ M NiSO₄ (Ni, right panels) on a 96-well plate. Data are from 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

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 μ M/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 μ M/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 non-allergic 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 (V β 17) but not the noninteracting β -chain TRBV6-5 (V β 13) 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). NiSO₄ titration showed increasing frequencies up to ~1 mmol/L (Figure S1G) while toxic effects, for example, decreasing monocyte numbers, started at ~400 μ mol/L. We then chose 200 μ mol/L NiSO₄ 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 CD154³² and because Ni-specific CD8⁺ T cells seem less frequent in general,⁹ they cannot be reliably detected on a 96-well plate format.

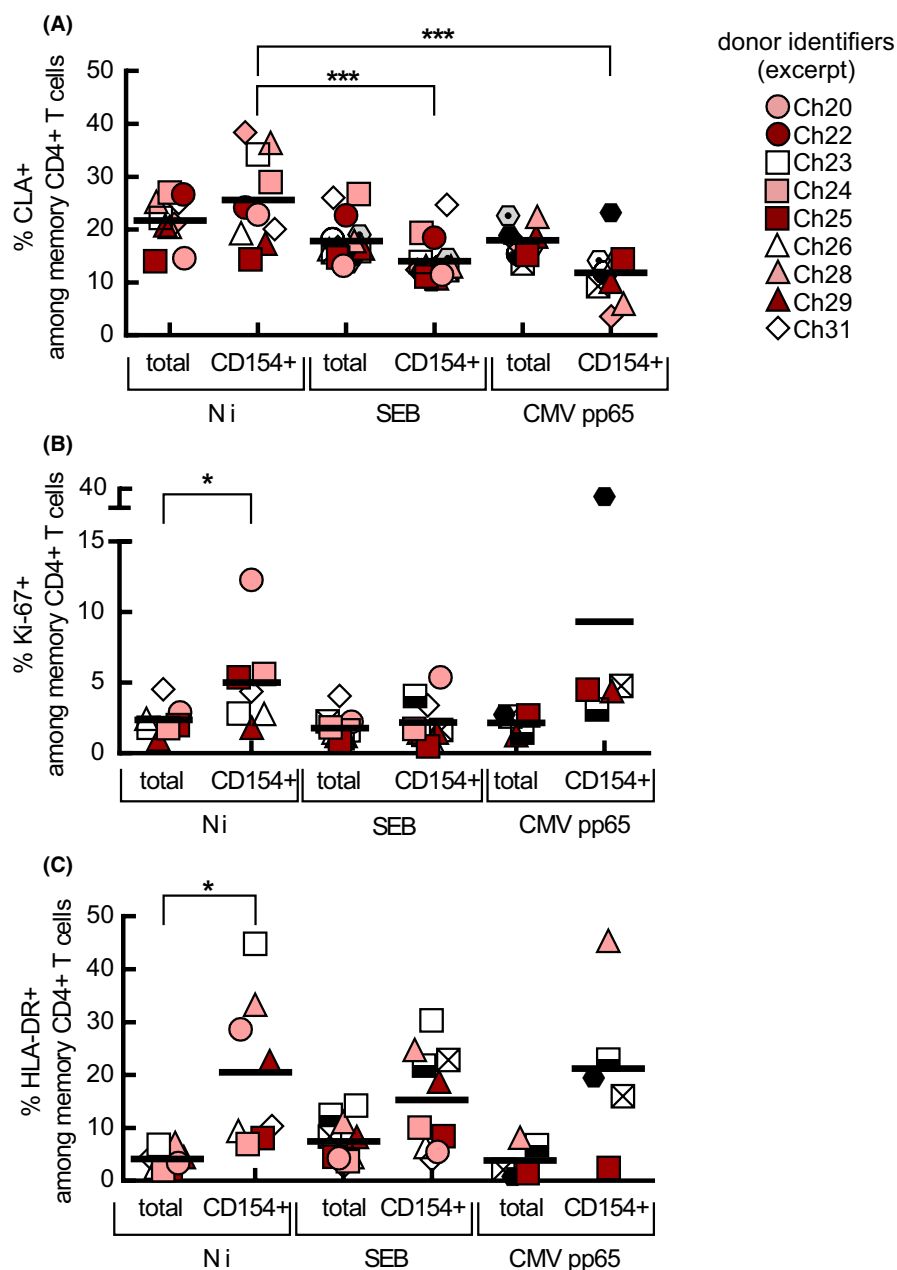


FIGURE 2 In vivo activation of Ni-specific T cells in allergic donors. Expression of (A) CLA, (B) Ki-67, and (C) HLA-DR by total or antigen-specific CD154⁺ CD4⁺ memory T cells in donors with recent positive patch test after 5 h of in vitro stimulation. Only populations with ≥ 20 cells are shown. Lines 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 (* $P < .05$, *** $P < .001$)

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⁺

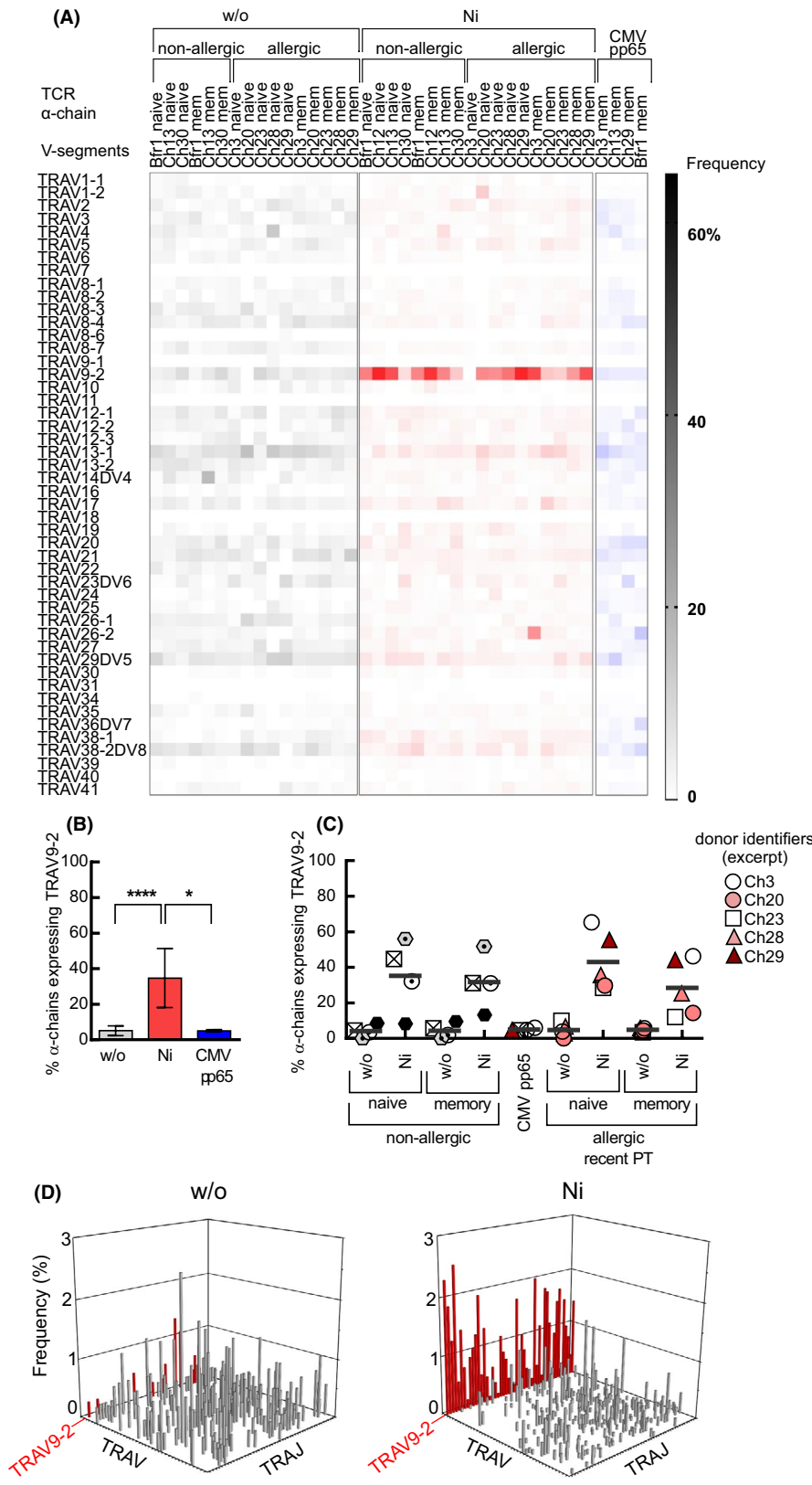


FIGURE 3 TCR α-chain segment TRAV9-2 is overrepresented among Ni-specific TCRs. (A) Heat plot depicting TCR α-chain V-segment use among randomly sorted (without antigen stimulation, w/o, grey), Ni-specific (red), and CMV pp65-specific (blue) CD4⁺ T cells (frequencies). Cells were sorted from CD154 upregulation 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)

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 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 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 INF γ (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 IFN γ .

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 $\alpha\beta$ 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 $\pm$ 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 $\alpha\beta$ 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 $43\% \pm 15\%$ (mean $\pm$ SD) and $28\% \pm 14\%$ of T cells, respectively, in allergic individuals and by $35\% \pm 18\%$ and $32\% \pm 14\%$ 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 tyrosine₃₆ 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

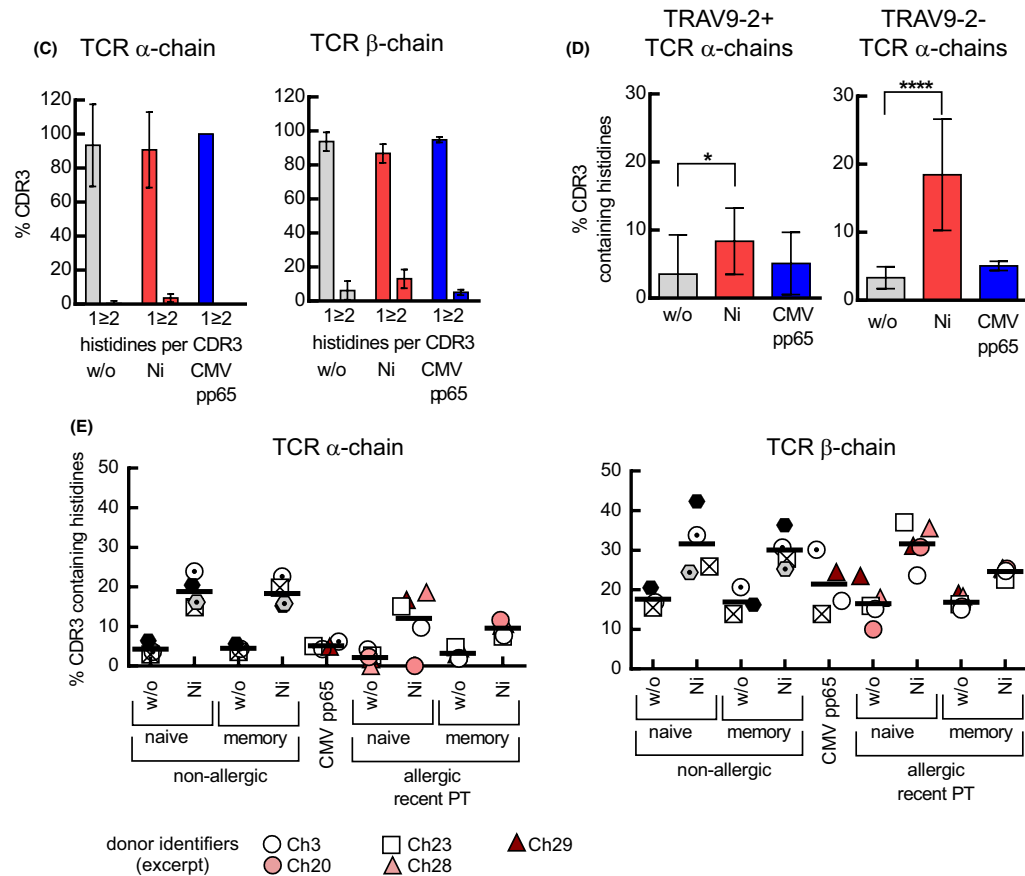
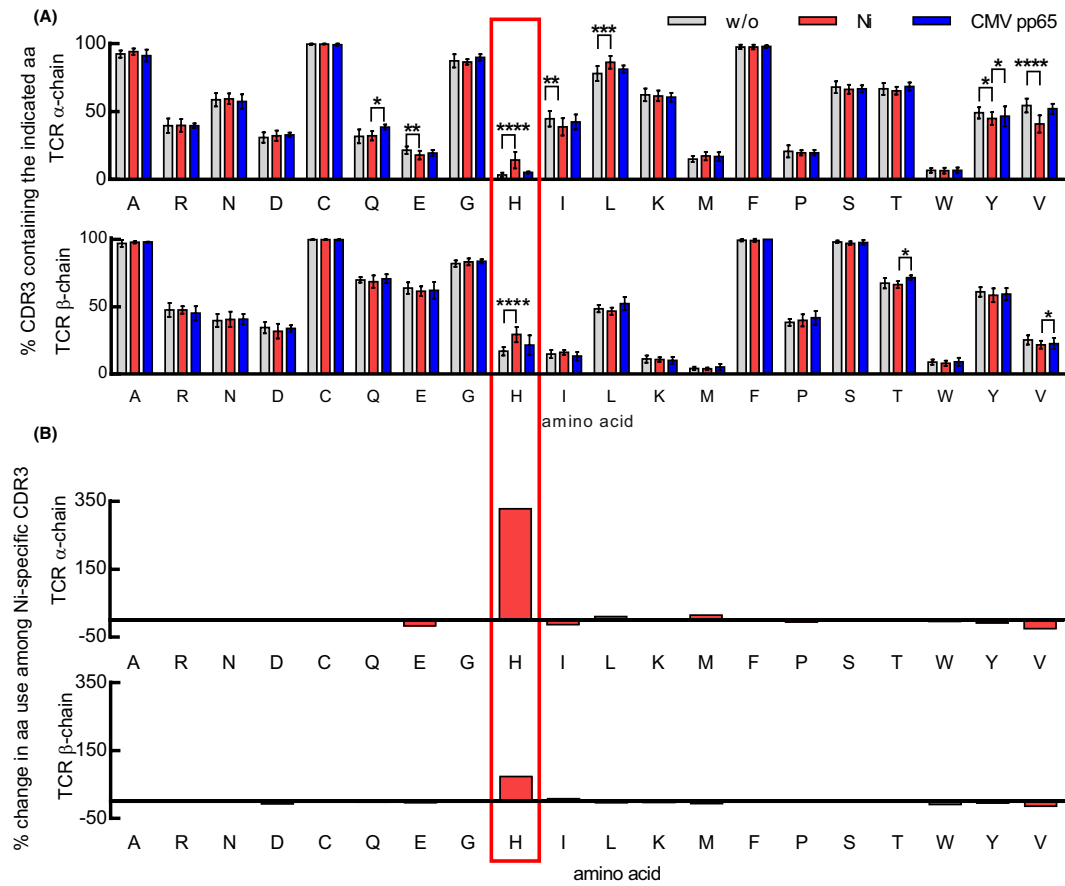


FIGURE 4 Ni-specific TCR α - and β -chains frequently contain a histidine in their CDR3. (A) Percentage of α - and β -chain CDR3 that contain the indicated amino acids among randomly sorted (without antigen stimulation, w/o, grey), Ni-specific (red), and CMV pp65-specific (blue) CD4⁺ T cells (diversity). 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 $\pm$ 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 $\pm$ SD). (D) Percentage of CDR3 containing a histidine among TRAV9-2⁺ and TRAV9-2⁻ TCRs for the indicated antigens (means $\pm$ 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)

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 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 S5C). 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 $\alpha\beta$ 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 μ mol/L NiSO₄ (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 $\alpha\beta$ 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 μ mol/L NiSO₄ here vs 0.02% with ~40 μ mol/L NiSO₄⁹). 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,⁴³ 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.¹¹

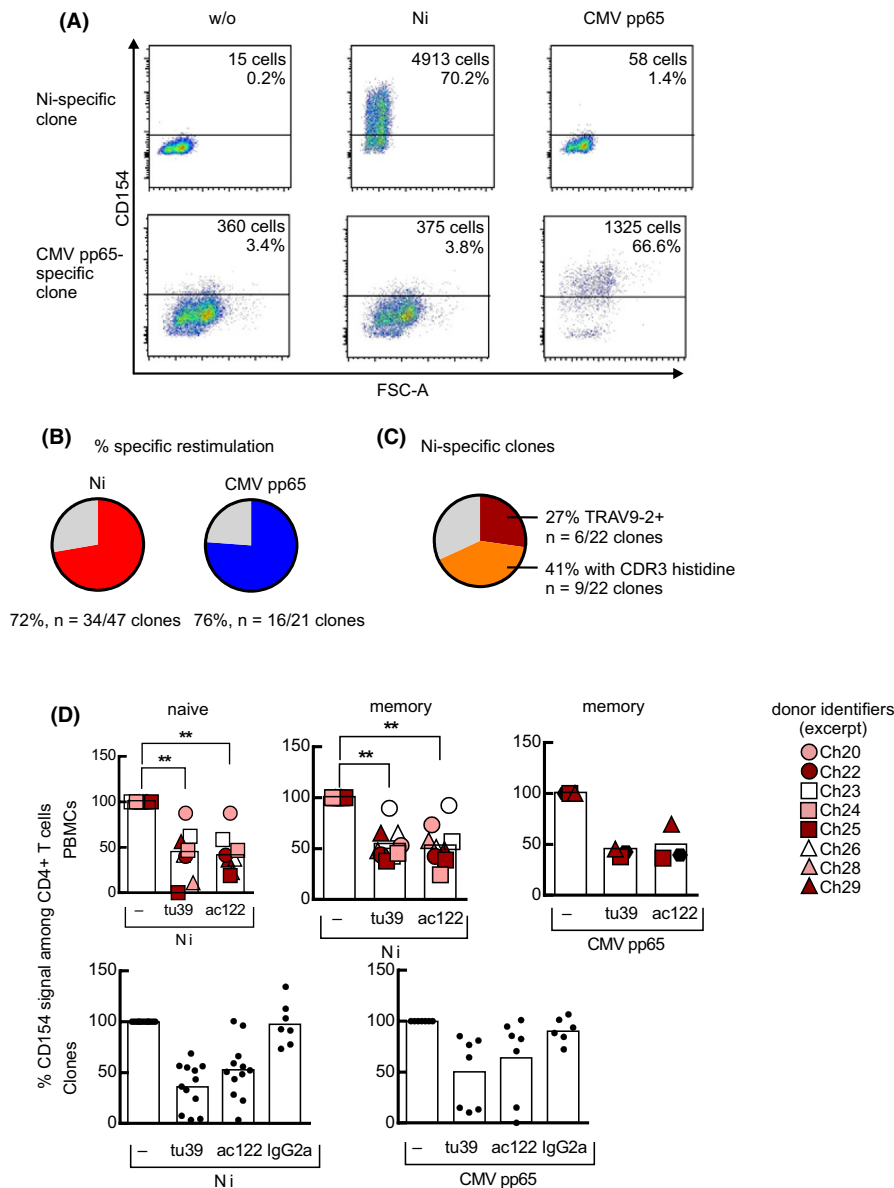


FIGURE 5 Ni-induced CD154 expression is TCR-mediated. (A) Representative dot plots depicting CD154 expression after 5 h of restimulation of a Ni- and a CMV pp65-specific T cell clone with the different antigens. Clones were derived from single CD154⁺ CD4⁺ memory T cells that were sorted from a CD154 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)

Analyzing thousands of Ni-specific $\alpha\beta$ 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 tyrosine₃₆ in the CDR1 of TRAV9-2 and via histidine₈₁ 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

histidine₈₁ 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 Be²⁺ 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.

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 tyrosine₁₀₈ 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 (IFN γ -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 cells²⁸ 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

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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.

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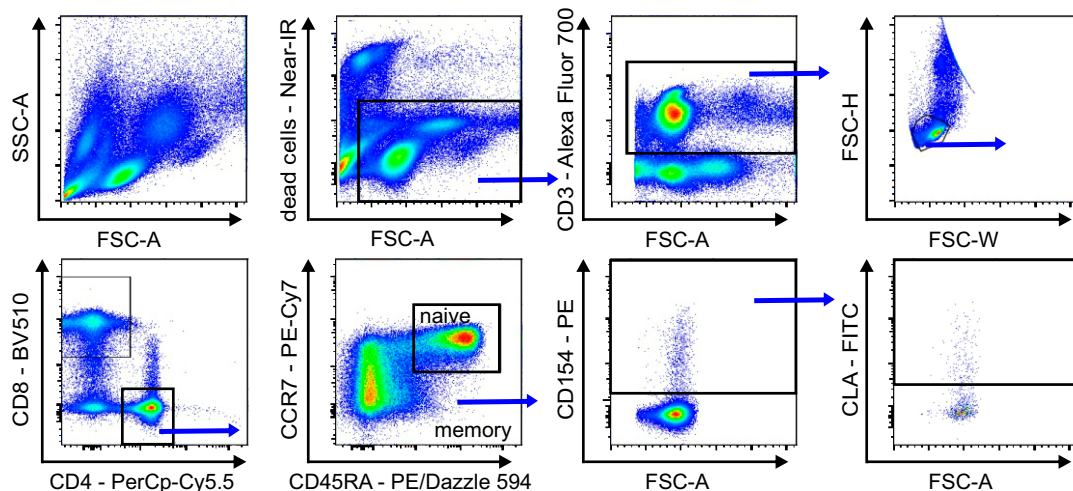
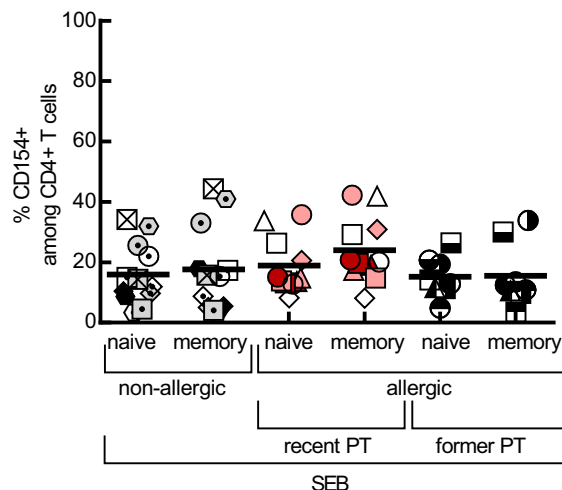
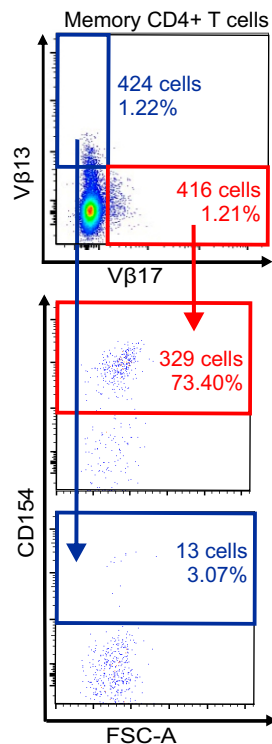
Fig. S1**A****B****C**

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+ naïve 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 (V β 17) or the non-SEB-interacting β -chain TRBV6-5 (V β 13) (one representative experiment out of three).

Fig. S1, cont. 1

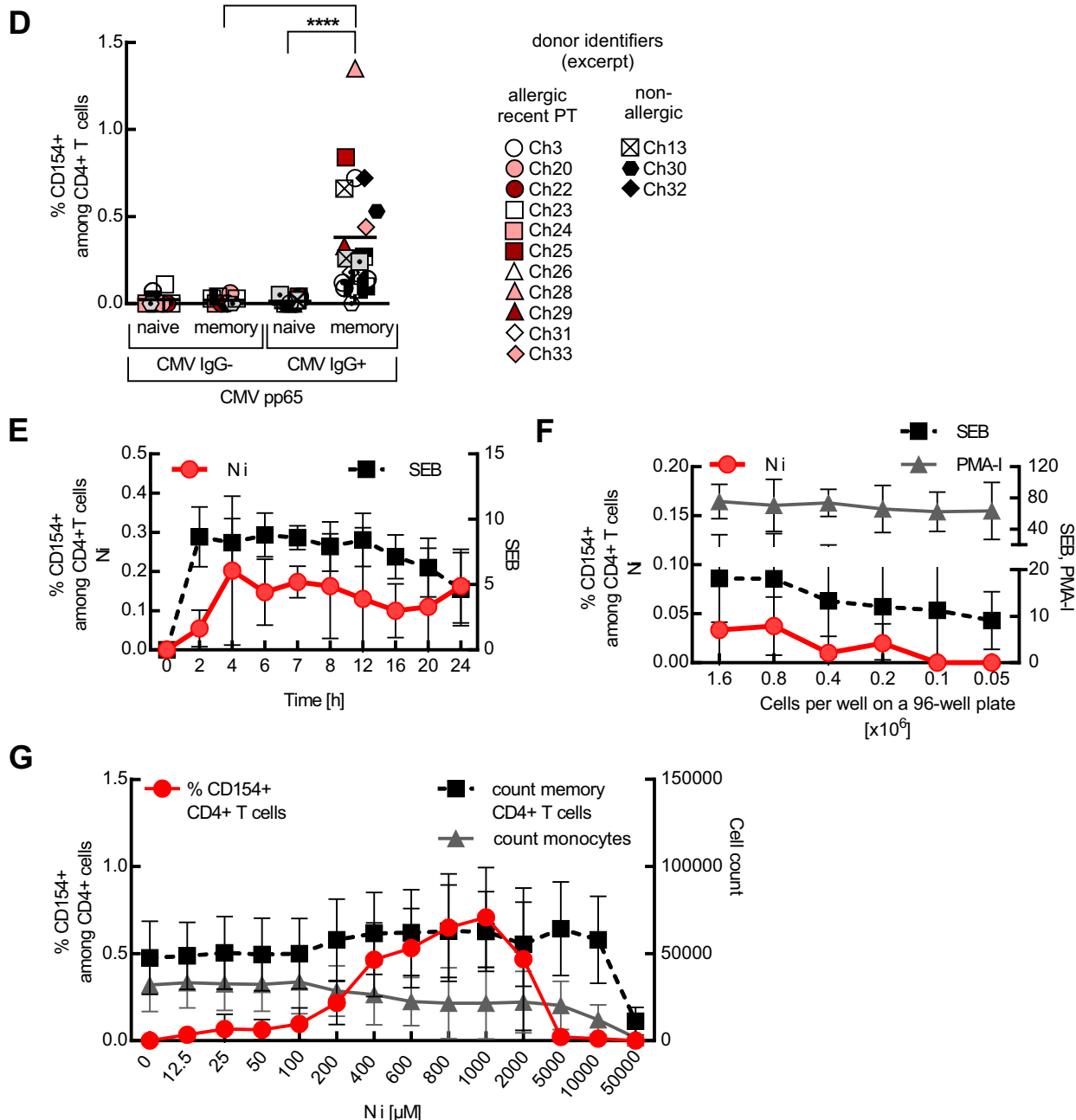


Figure S1 cont. 1: **(D) CMV pp65 stimulation.** Frequencies of CD154+ naive and memory CD4+ T cells after stimulation with CMV pp65 for 5 h for donors with negative (CMV IgG-, n = 9) and positive (CMV IgG+, n = 19) CMV titer (Table S1, also lists all donor identifiers). Lines indicate the means. One-way 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 NiSO₄ (n ≥ 3 donors). (E) - (G) Means $\pm$ SD, frequencies of CD154+ T cells without antigen stimulation were subtracted.

Fig. S1, cont. 2

H

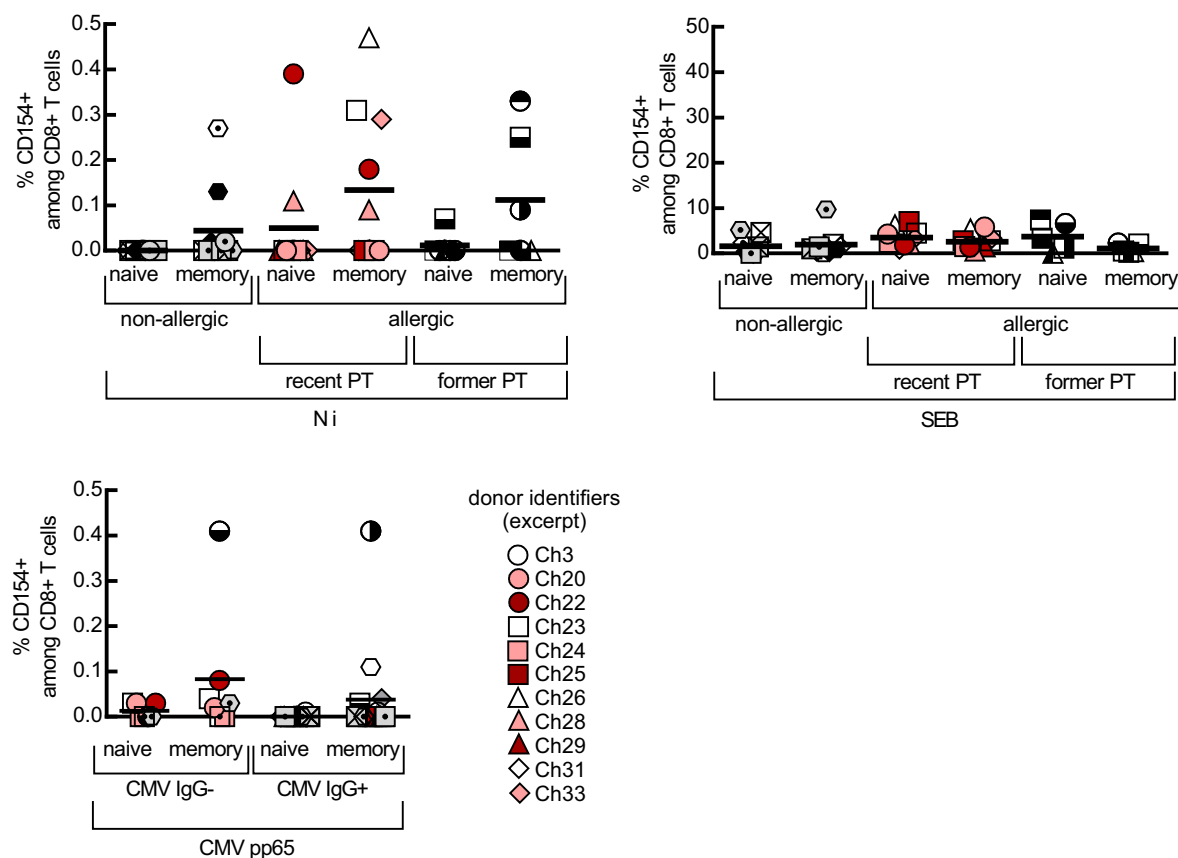


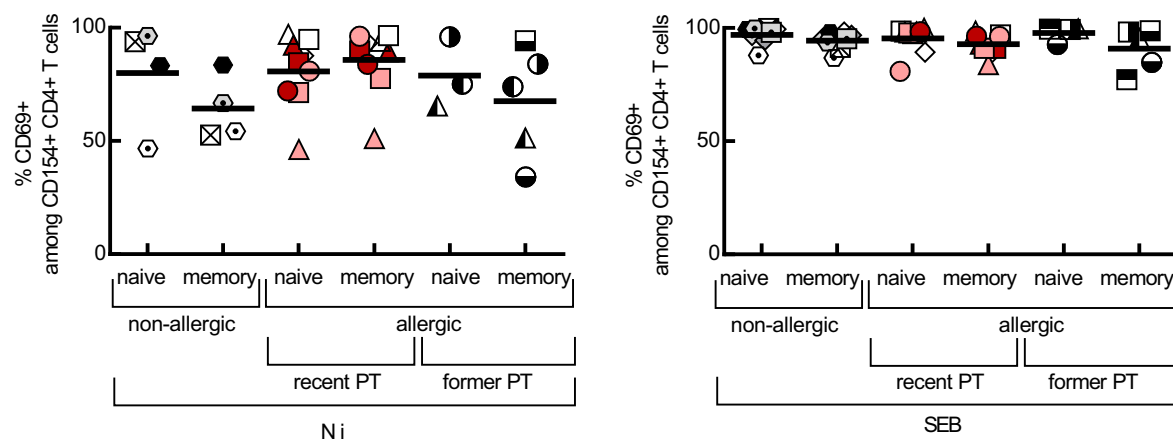
Figure S1 cont. 2: **(H) Ni-specific CD154+ CD8+ T cells.** Frequencies of CD154+ CD8+ T cells after stimulation of PBMCs with the indicated antigens for 5 h. Frequency values from samples without antigen stimulation were subtracted. Lines indicate the means. One-way nonparametric ANOVA analysis (Kruskal-Wallis) 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

A



B



C

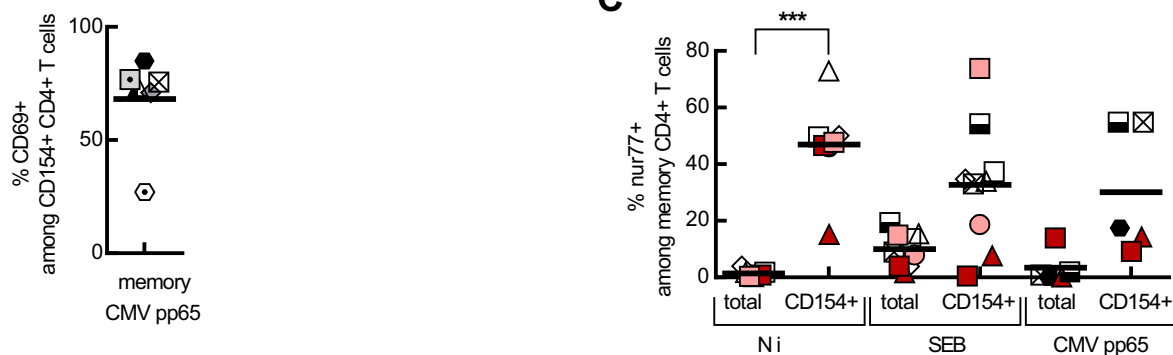


Figure S2: **Co-expression of activation marker by Ni-specific CD4+ T cells.** PBMCs were incubated with 200 μ M NiSO₄ or with the indicated antigens for 5 h and the expression of (A) CCR9, (B) CD69, and (C) nur77 was analyzed for total or CD154+ T cells by flow cytometry. Only populations with ≥ 20 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

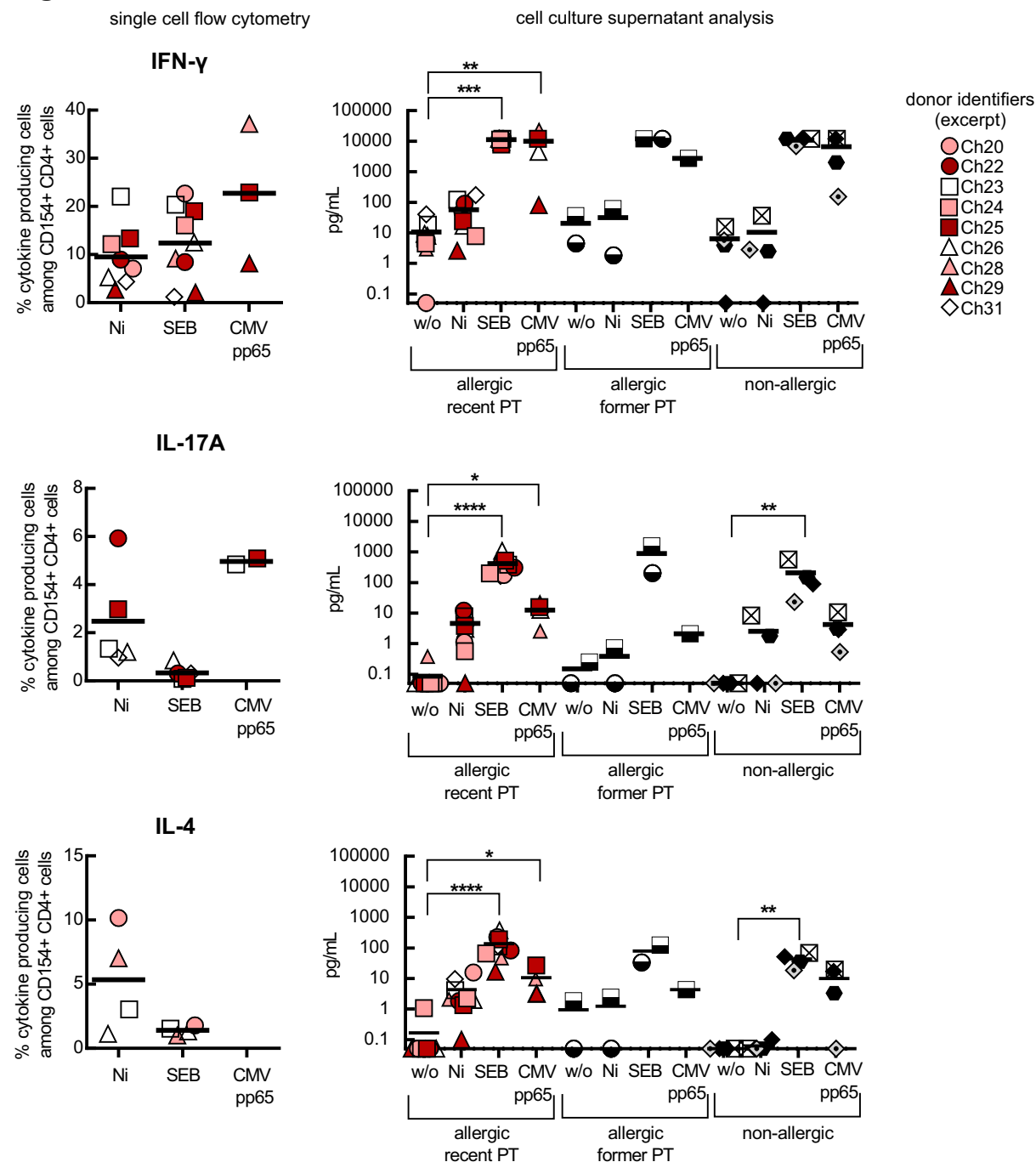


Figure S3: **Cytokine expression by Ni-specific T cells.** Left panels: Cytokine expression of CD154+ CD4+ T cells was analyzed by flow cytometry. PBMCs were stimulated with the indicated antigens in CD154 upregulation assays. Only populations with ≥ 20 cells are shown. For Ni-specific cells, this detection 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

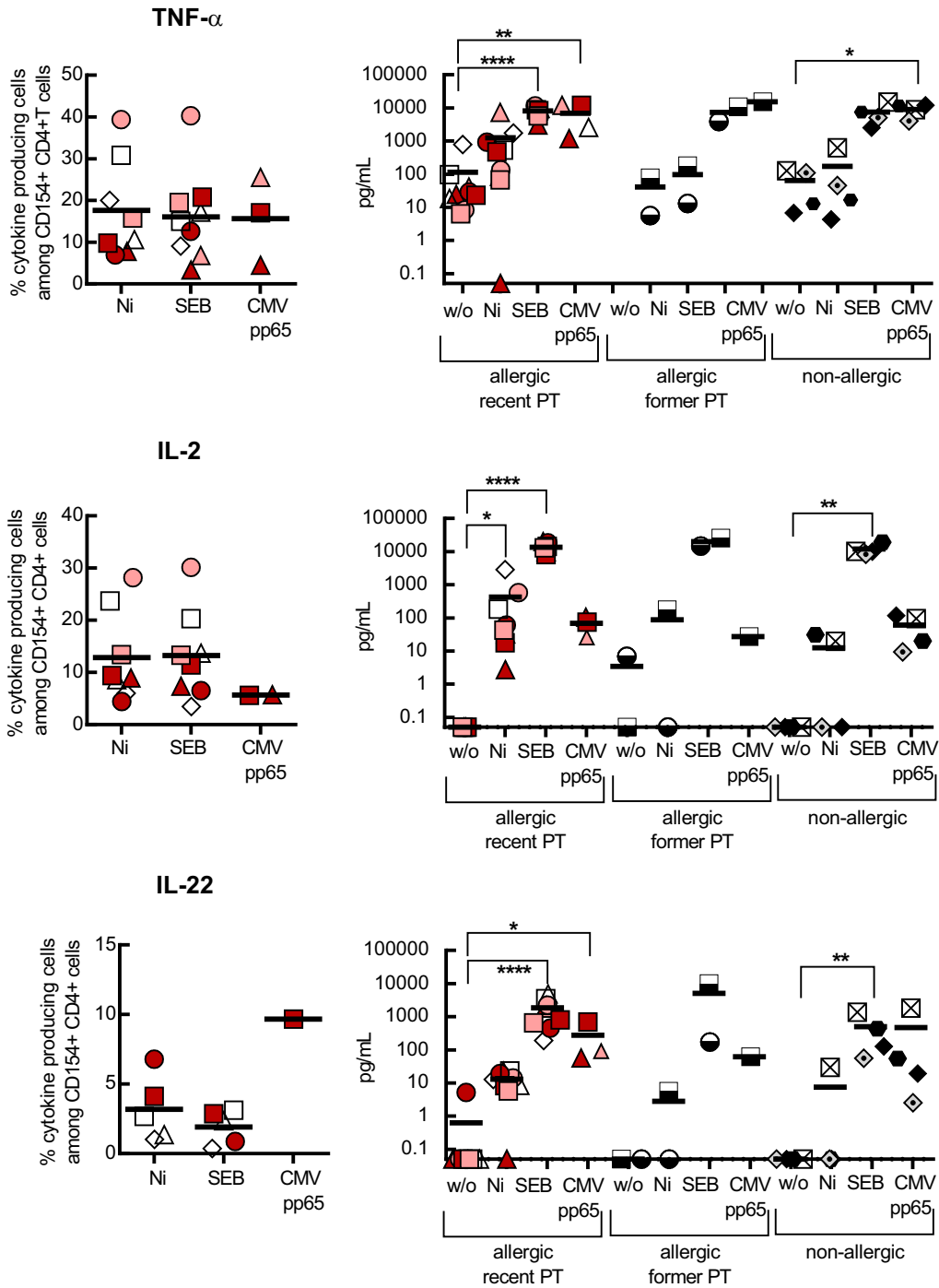


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Fig. S3 cont. 2

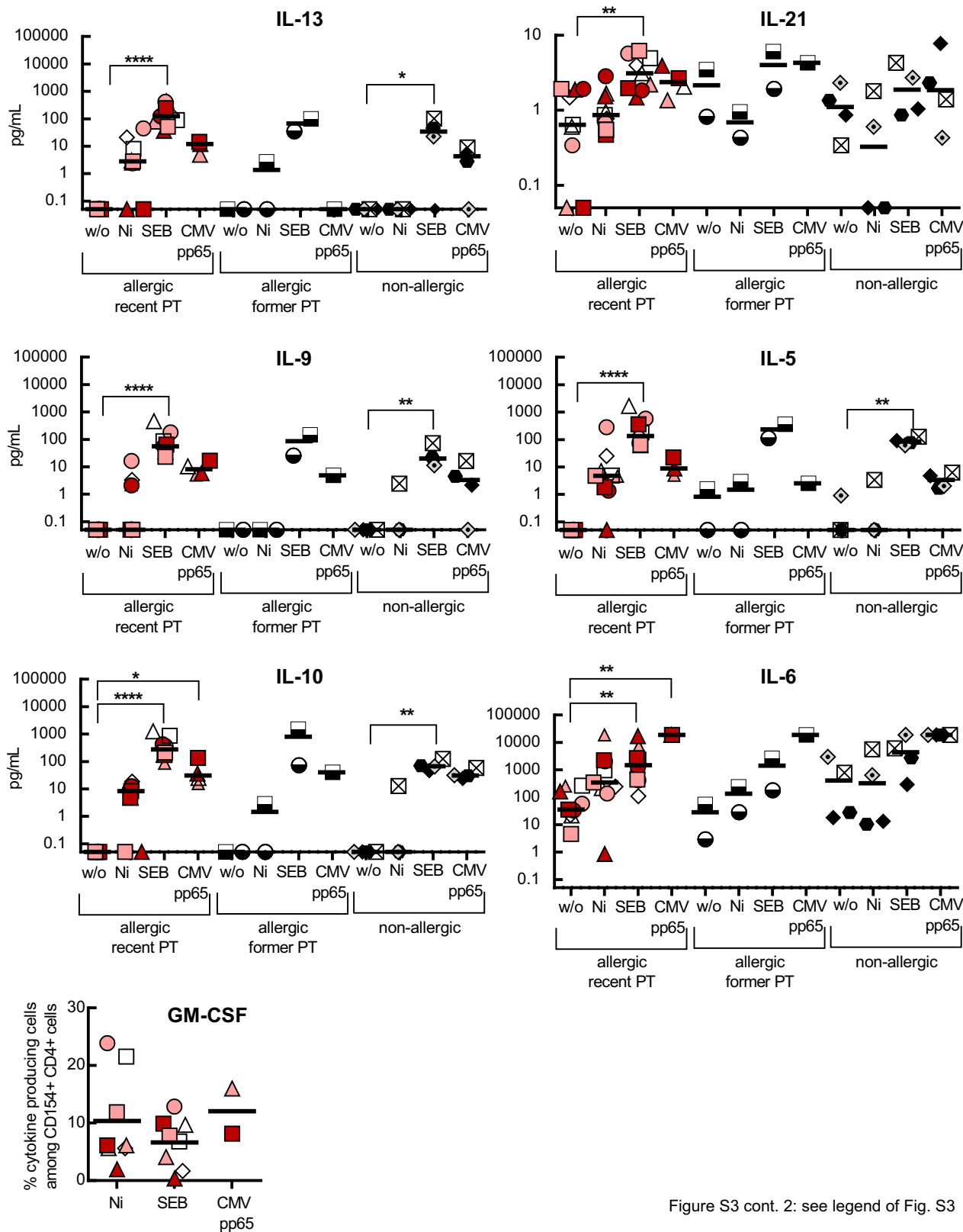


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Fig. S4

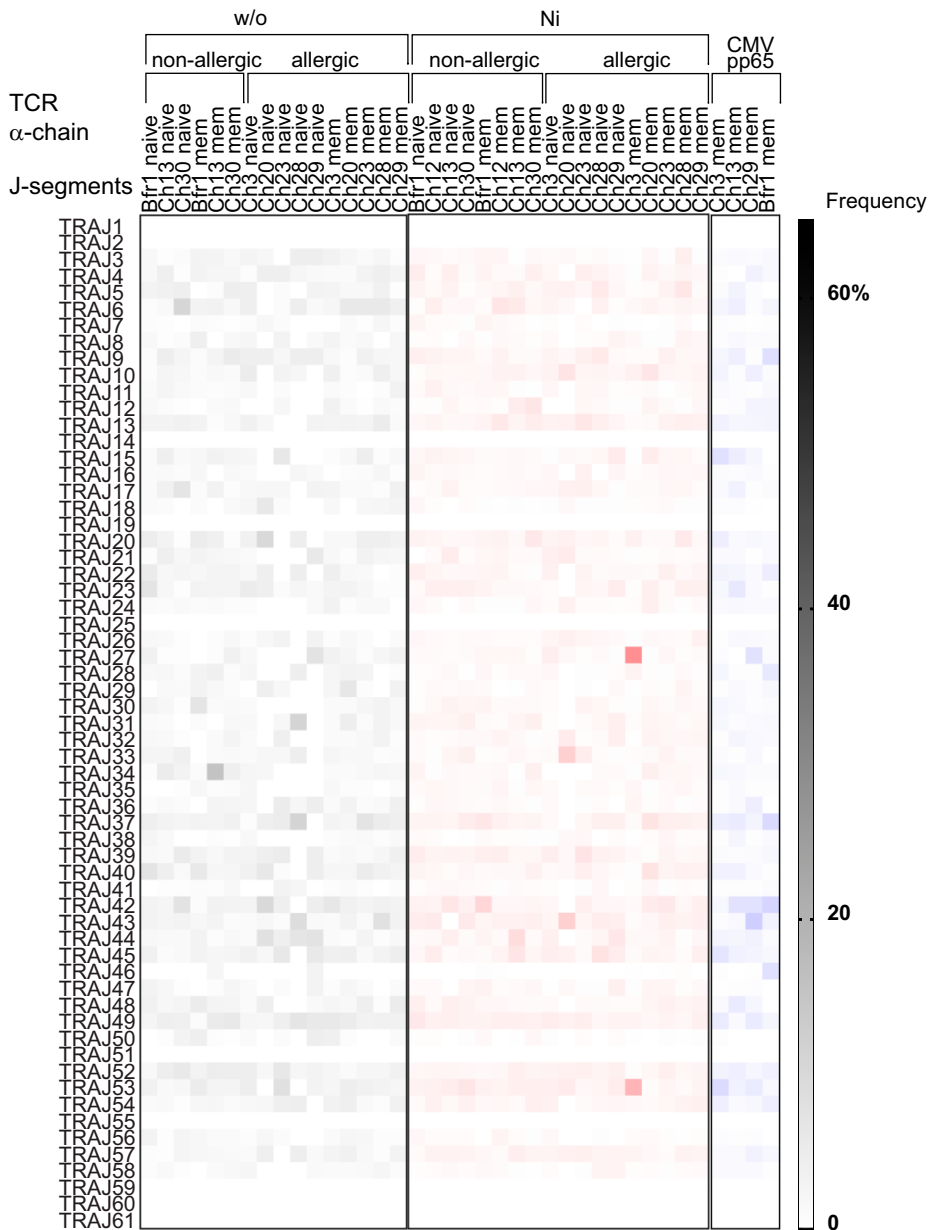


Figure S4: **TRAJ, TRBV, and TRBJ segment use.** Heat plot depicting segment use among randomly sorted CD4+ T cells (black), Ni-specific CD154+ CD4+ T cells (red), and CMV pp65-specific CD154+ CD4+ T cells (blue, frequencies). Cells were sorted from CD154 upregulation assays (Table S2) and their $\alpha\beta$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

Fig. S4 cont.

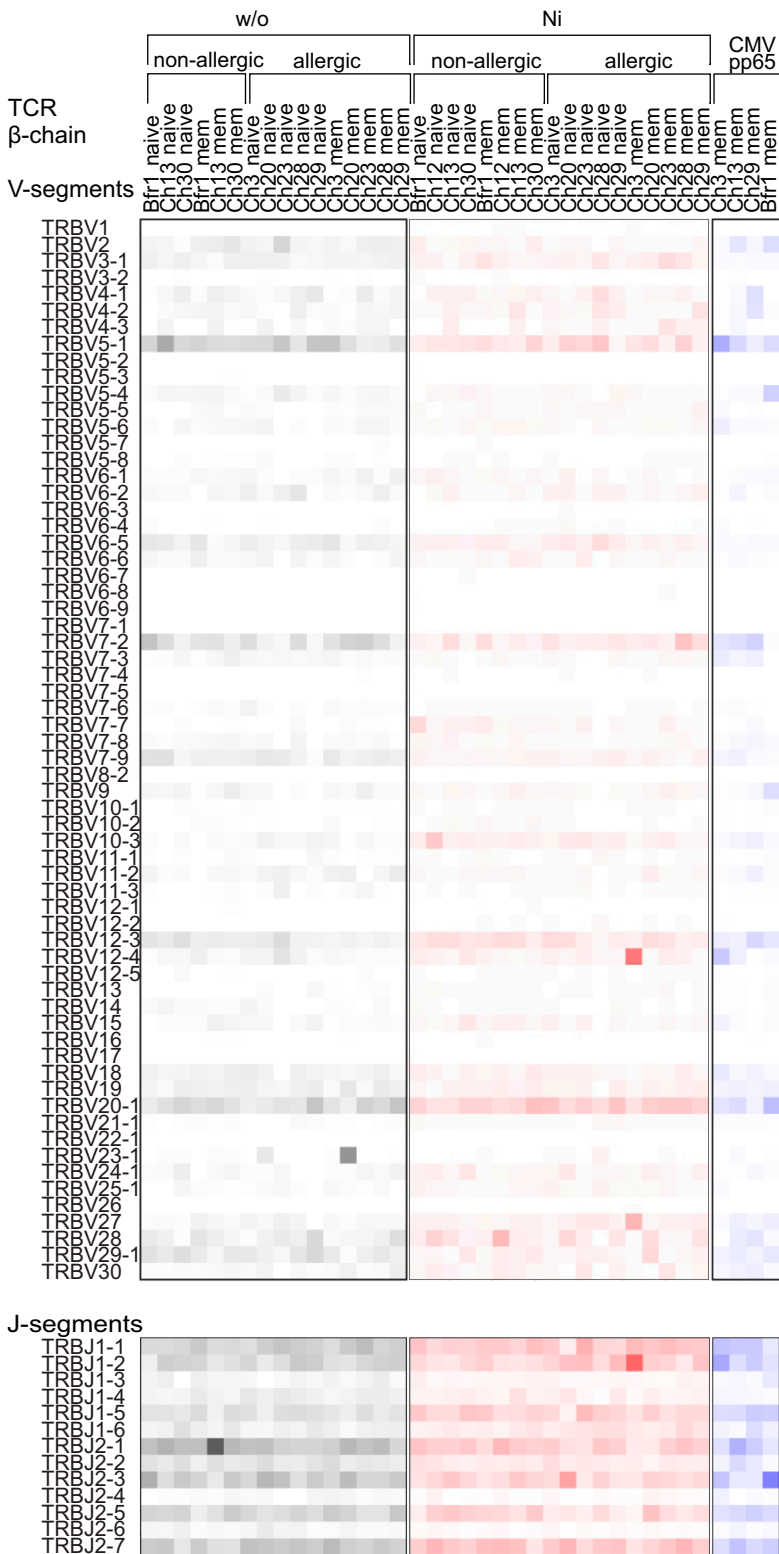
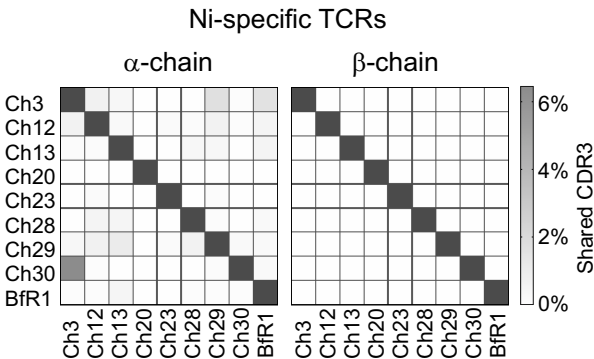


Figure S4 cont.: see legend of Fig. S4

Fig. S5

A



B

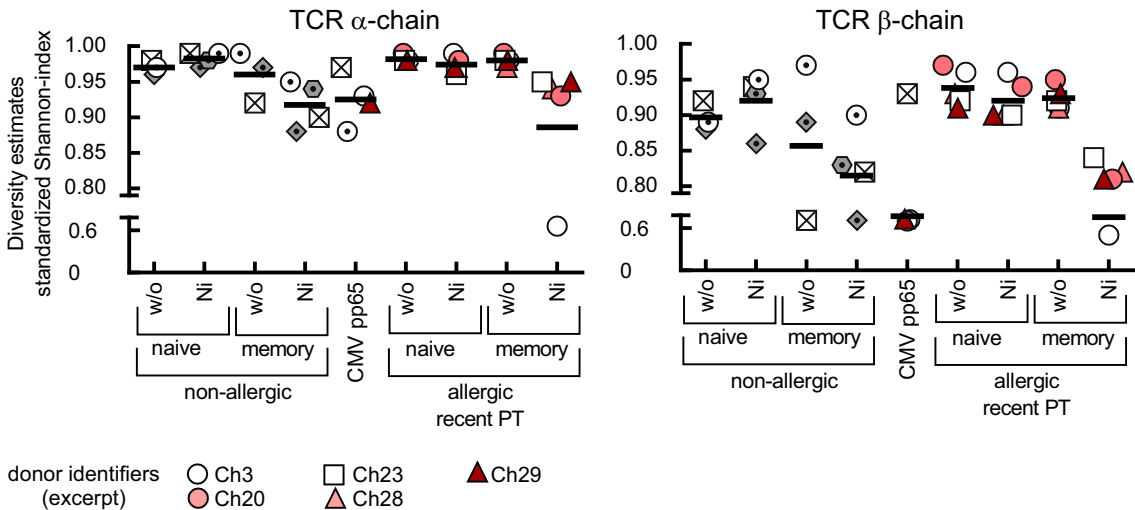


Figure S5: **(A) Clonal congruence of Ni-specific TCR.** The heat plots depict overlap of TCR α - and β -chains from different donors (frequencies, nucleotide level). Ni-specific CD154⁺ CD4⁺ memory T cells were identified by CD154 upregulation assay and their TCRs were analysed by high throughput sequencing (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.

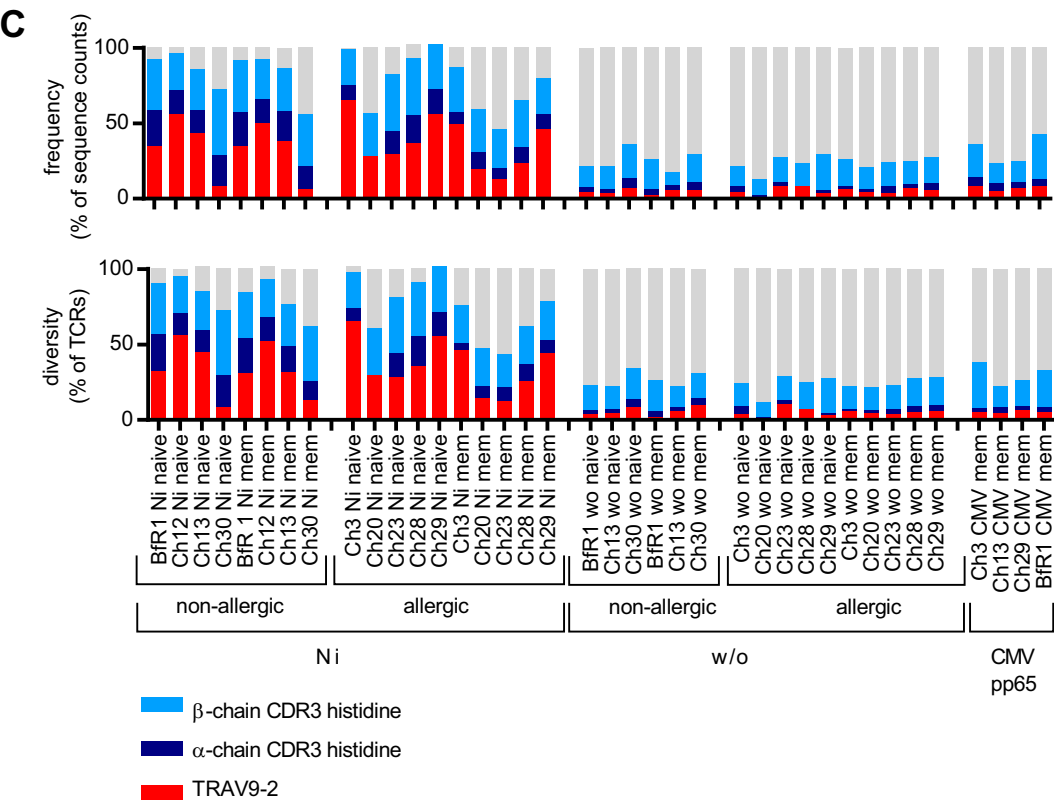


Figure S5 cont: **(C) Frequency and diversity of TCR repertoires features.** Frequency (percentages of sequence counts, upper panel) and diversity (percentages of TCR clonotypes, lower panel) of TCRs expressing TRAV9-2 (red), α -chain CDR3 histidine (dark blue) or β -chain CDR3 histidine (light blue)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

[illegible]

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 (cigarettes/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 and 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% NiSO₄ 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 ~ TCR sequencing data

Summary of sorted CD4⁺ T cells for $\alpha\beta$ 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+ ^s) 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

		naïve	809	β	1175	505
				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 μ l AcR St4/bc1R (2 μ M each, final dilution 0.2 μ M) 3 μ l Smart Adapter (2 μ M, final dilution 0.2 μ M) 9 μ l RNA 6 μ l 5X First-Strand Buffer 3 μ l dNTP (10 mM, final dilution, 1 mM) 3 μ l DTT (20 mM, final dilution 2 mM) 3 μ l SMARTScribe RT (Takara, final dilution 10 U/ μ l) 1.5 μ l 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 μ l 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)	10.5 μ l H ₂ O 10 μ l Q5® Reaction Buffer 1 μ l dNTP (final dilution 0.2 mM) 2 μ l ac2R/bc2R-mix (final dilution 0.4 μ M) 2 μ l Na-SBX-M1_V2 (final dilution 0.4 μ M) 0.5 μ l Q5® High-Fidelity DNA Polymerase (NEB) 10 μ l purified cDNA
Temperature program	95°C 2 min 18 cycles o 95°C 10 s o 61°C 20 s o 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 NNNCGAGCGTGACGACGACAG XXXXXX GGGTCAGGGTTCTGGATAT XXXXXX ACACSTTKTTCAGGTCCTC
Reaction mixture (setup according to ¹⁷ ; separate for TCR α - and β - chain)	30.5 μ l H ₂ O 10 μ l Q5® Reaction Buffer 1 μ l dNTP (final dilution 0.2 mM) 2 μ l NNa-mix (final dilution 0.4 μ M) 2 μ l ac3R-SBX or bc3R-SBX (final dilution 0.4 μ M) 0.5 μ l Q5® High-Fidelity DNA Polymerase (NEB) 4 μ l purified product of 1 st PCR
Temperature program	95°C 2 min ~19 cycles (optimal cycle number determined for each sample): o 95°C 10 s o 61°C 20 s o 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 ~ 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	CASSLVLTTPPGYEQYF
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	CASSYRGAENSPLHF
14	Ch33Ni6A	α	TRAV8-1	TRAJ39	CAVNEADAGNMLTL
		β	TRBV10-3	TRBJ2-3	CATSSGGVTDQTQYF
15	Ch33Ni6E	α	TRAV20	TRAJ5	SAVQVNQFYF
		β	TRBV12-5	TRBJ2-3	CASGHDDSTTDQTQYF
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	CAISPGGGRGTDQTQYF
21	Ch33Ni11A	α	TRAV30	TRAJ28	CGTEIRSGAGSYQLTS
		β	TRBV6-6	TRBJ1-5	CASSYRGSNQPQHF
22	Ch33Ni11B	α	TRAV9-2	TRAJ57	CALRTQGGSEKLVL
		β	TRBV27	TRBJ1-1	CASSLFGGTEAFF

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 2.5×10^6 cells/cm² in flat bottom tissue culture plates. For antigen stimulation assays 200 μ M NiSO₄·6H₂O (Sigma-Aldrich, 40 mM stock dissolved in H₂O) or control antigens CMV pp65 (1:100, Miltenyi Biotec), staphylococcal enterotoxin B (SEB, 1 μ g/mL), PMA-Ionomycin (PMA-I, 10 ng/ml and 1 μ g/ml, all Sigma-Aldrich) were added to the PBMC cultures in the presence of CD40 blocking antibody to prevent contact-dependent downregulation of CD154¹. For MHC block experiments, anti-HLA-DR, DP, DQ (10 μ g/ml, tu39, AB_395938), anti-HLA-DR (10 μ g/ml, ac122, AB_2661330) or an isotype control antibody (10 μ g/ml, eBM2a, eBioscience) were added 30 min prior to stimulation. In case of intracellular cytokine staining, Brefeldin A (1 μ g/ml, Merck) and BD GolgiStop™ (1:1000, BD Biosciences) were added for an additional 2 h of incubation. We used 200 μ M NiSO₄ 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 10×10^6 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 columns³ (Miltenyi Biotec; 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: TNF α -BV421 (MAb11), IL-2-BV711 (MQ1-17H12), GM-CSF-APC (BVD2-2 1C11, all Biolegend), IL-22-FITC (22URTI, eBiosciences), IFN γ -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 μ l cloning medium (complete medium without extra CaCl₂, 15% human AB serum, 200 U/ml IL-2 [IS grade, Miltenyi Biotec], 30 ng/ml α -CD3 [OKT-3, pure, Miltenyi Biotec] 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 μ l medium under the same conditions as used for bulk culture antigen stimulation assays plus CD28 antibody (1 μ g/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 $\alpha\beta$ chains was performed as described⁴ (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 UMI⁵). TCRs were extracted with MIXCR (v3.0.2; library repseqio.v1.5⁶) 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 clonotypes⁷), 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 v1.2.9⁸) is used throughout the manuscript (Arden nomenclature in brackets⁹). $\alpha\beta$ TCRs of clones were determined with a simplified protocol using 50 000 cells directly lysed in 25 μ l distilled water supplemented with 1 mM DTT and 12.5 U recombinant RNase inhibitor (Takara, Kusatsu, Japan). After a freeze thaw cycle, 3 μ l 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-Index¹⁰ 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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