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Possible involvement of regulatory T cell abnormalities and variational usage of TCR repertoire in children with autoimmune neutropenia

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Abbreviations:

AIN, autoimmune neutropenia; anti-HNA Abs, antibodies against human neutrophil antigens; Cnt, control; FSC, forward scatter; PBMCs, peripheral blood mononuclear cells; SD, standard deviations; SLE, systemic lupus erythematosus; SSC, side scatter; Tcons, conventional T cells; TCR, T cell receptor; TCR-Vβ, TCR beta chain variable region; TRBV, TCR beta chain variable gene; Tregs, regulatory T cells;

Abstract

Autoimmune neutropenia (AIN) in childhood is characterized by chronic neutropenia and positivity for antineutrophil antibodies, resulting in the excessive destruction of neutrophils. In this study, we investigated the involvement of regulatory T cells (Tregs) in the pathogenesis of AIN in childhood. Tregs have been classified into three subpopulations based on the expressions of CD45RA and FOXP3: resting Tregs, activated Tregs, and non-suppressive Tregs. The frequency of activated Tregs ($CD4^+CD25^+FOXP3^{high}CD45RA^-$ T cells) as well as that of total Tregs ($CD4^+CD25^+FOXP3^+$ T cells) in peripheral blood was significantly decreased in patients with AIN. Analysis of the T cell receptor (TCR)-Vβ repertoire of $CD4^+$ T cells revealed skewed usages in patients with AIN compared with that observed in age-matched control subjects. Regarding T cell subsets, the use of four of 24 TCR-Vβ families in Tregs and one in conventional T cells were increased in patients with AIN. The number of patients with AIN who showed skewed usages of TCR-Vβ family in conventional and Tregs was significantly higher than that reported in control subjects. When the preference between Tregs and conventional T cells in each TCR-Vβ family was individually compared, different use was prominently observed in the TCR-Vβ 9 family in patients with AIN. These results suggest that the quantitative abnormalities of Tregs and the skew of the TCR-Vβ repertoire in $CD4^+$ T cells, including Tregs and conventional T cells, may be related to autoantibody production through a human neutrophil antigen-reactive T cell clone.

Introduction

Autoimmune neutropenia (AIN) in children is characterized by a low absolute neutrophil count caused by the excessive destruction of neutrophils through antibodies against human neutrophil antigens (anti-HNA Abs) (1). The median patient age at diagnosis of AIN is 7–9 months (2, 3). During the period of neutropenia, many patients tend to present bacterial, but not severe infections, because their bone marrow is intact and immediately produces neutrophils after receiving an infectious signal. Some patients receive prophylactic medication, such as sulfamethoxazole-trimethoprim combination to avoid recurrent infections (4). Unlike other autoimmune diseases with autoantibodies, AIN is a self-limited disease which does not require any special therapies (e.g., steroid medications). Because anti-HNA Abs disappear gradually in many cases, almost all patients recover without treatment in 2–3 years (3, 5).

Although AIN is not a rare neutropenia of childhood, its etiopathogenesis remains unclear. Several researchers have attributed causes of this disease to the modification of antigens after exposure to drugs, molecular mimicry of microbial antigens, post-infectious autoantibodies, and differences in human leukocyte antigen types (5–8). Previously, we reported a deficiency of regulatory T cells (Tregs; $CD4^+CD25^+$ $FOXP3^+$ T cells) in children with AIN as another cause of this disease (9). Tregs play a key role in suppressing the immune response based on the control of autoimmunity in peripheral tissue (10). In fact, the deficiency of Tregs has been shown in several autoimmune diseases (11). Furthermore, Tregs could separate subpopulations such as resting Tregs, activated Tregs, and non-suppressive Tregs according to the extent of expression of CD45RA and FOXP3. Activated Tregs, defined as $CD4^+CD25^+FOXP^{high}CD45RA^-$ T cells, have the most suppressive function in these subpopulations (12), and the fluctuation of the aforementioned three subpopulations is associated with autoimmune diseases (13, 14).

T cells, in combination with B cells, play an important role in antibody production. For the recognition of various antigens, each T cell has a specific T cell receptor (TCR) on its surface (a heterodimer comprised of α- and β-chains). TCR is formed by random re-combinations of TCR gene elements termed V, D, J-segments

(15). The random re-combination causes diversity of the TCR repertoire. The complementarity determining region on the β-chain made from the V-region is the most important region for diversity (16). Recently, associations with repertoire of TCR-Vβ and autoimmune diseases, such as systemic lupus erythematosus (SLE), type 1 diabetes mellitus, autoimmune thyroiditis, and idiopathic thrombocytopenic purpura, have been reported in numerous research studies (17–20). The results have shown several different usages or expansions of the TCR-Vβ family in each disease and indicated an association between disease and an unusual repertoire of the TCR-Vβ family. However, there has been no study analyzing the repertoire of the TCR-Vβ family in patients with AIN. Although the high throughput sequencing is often used for TCR-Vβ repertoire analysis nowadays, it requires much cost. The analysis using flow cytometry could give us less information than high throughput sequencing, but this method is easy and useful to get overview of the repertoire of the TCR-Vβ family in each T cell subset.

In this study, we analyzed the frequency of total Tregs and activated Tregs in $CD4^+$ T cells. Subsequently, we investigated the repertoire of the TCR-Vβ family in patients with AIN using flow cytometry to obtain information on the usages of the TCR-Vβ family in T cells.

Patients and Methods

Subjects

The diagnosis of AIN in children was based on the presence of chronic neutropenia and positivity for antineutrophil antibodies in sera according to the criteria published in the Nelson Textbook (21). Antineutrophil antibody was detected using an indirect granulocyte immunofluorescence test, as previously described (4). **Table I** presents the characteristics of 25 patients with AIN enrolled in this study: 13 patients for the analysis of the frequency of total Tregs and activated Tregs (median age: 17 months, range: 7–69 months) and 17 patients for the analysis of the TCR-Vβ repertoire (median age: 20 months, range: 11–57 months). Five patients were included in both analyses. AIN in childhood shows spontaneous resolution within a few years without the need for treatment. Hence, these TCR repertoire assays were performed in the middle of the neutropenic period. Parts of the assays in some patients were performed at the end of the spontaneous recovery from neutropenia. Treg and TCR repertoire assays were longitudinally performed in three patients with AIN. The results of the analyses in these patients did not show any fluctuations. Therefore, the TCR repertoire assay was compared between patients with AIN and age-matched control subjects. Eighteen (median age: 17.5 months, range: 4–78 months) and 22 (median age: 20 months, range: 8–49 months) heathy children without neutropenia were also examined as age-matched control subjects for respective analyses.

Informed consent was obtained from the guardians of patients and control subjects, and approval for these studies was obtained from the institutional review board.

Isolation of peripheral blood mononuclear cells (PBMCs)

Heparinized peripheral blood (approximately 3 mL) was obtained from patients and healthy children. Sample blood was diluted with an equal volume of phosphate-buffered saline (PBS). Mononuclear cells were separated by centrifugation using Lymphprep®, aliquoted in Cellbanker®, and subsequently stored in a −80°C refrigerator until analysis. All patients and control subjects were free from any infectious episodes at the time of

venipuncture.

Examination of Tregs

Total Tregs ($CD4^+CD25^+FOXP3^{high}$ cells) and activated Tregs ($CD4^+CD25^+FOXP3^{high}CD45RA^-$ cells) were analyzed by four-color flow cytometric analysis. We used fluorescein isothiocyanate (FITC) conjugated anti-CD4 antibody (Cat. 555346; BD Pharmingen), phycoerythrin (PE) conjugated anti-CD25 antibody (Cat. 555432; BD Pharmingen), Alexa Fluor 647 conjugated anti-FOXP3 antibody (Cat. 560045; BD Pharmingen), and VioBlue conjugated anti-CD45RA antibody (Cat. 130-113-360; Miltenyi Biotec) for cell staining. One million PBMCs were initially stained with the FITC-CD4 antibody, PE-CD25 antibody, and V450-CD45RA antibody for 20 min. Subsequently, intracellular detection of FOXP3 was performed on fixed and permeabilized cells using the Human FOXP3 Buffer Set (Cat. 560098; BD Pharmingen) according to the protocol provided by the manufacturer. After staining with Alexa Fluor 647 conjugated anti-FOXP3 antibody for 20 min, PBMCs were washed twice with PBS containing 2% fetal bovine serum (FBS). More than 20,000 $CD4^+$ T cell events were analyzed using a FACS Verse flow cytometer and the FACSuite software (BD Biosciences, San Jose, CA, USA).

Analysis of the TCR-Vβ repertoire

We evaluated the usages of the TCR-Vβ family of T cell subsets through five-color flow cytometric analysis. For the separation of T cell subsets ($CD4^+$ T cell, $CD4^+CD25^-$ T cell, $CD4^+CD25^+CD127^{low}$ T cell), we used PerCP/Cy5.5 conjugated anti-CD4 antibody (Cat. 217428; Biolegend), V450 conjugated anti-CD25 antibody (Cat. 560355; BD Horizon), and allophycocyanin (APC) conjugated anti-CD127 antibody (Cat. 351316; Biolegend). TCR Vβ staining was determined using the IOTest Beta Mark TCR Repertoire Kit® (Beckman Coulter, Marseille, France). This kit consists of monoclonal antibodies designed to identify 24 distinct TCR-Vβ families, covering approximately 70% of the normal human $CD4^+$ T cells. Each set consisted of three different

anti-Vβ family-specific monoclonal antibodies labeled with FITC, PE, or both. Thawed PBMCs obtained from each patient and control subject were washed with cold PBS containing 2% FBS, and 5×10^5 PBMCs were stained for surface antigens in room temperature for 60 min in the dark. After staining, PBMCs were washed thrice with PBS containing 2% FBS. At least 5,000 $CD4^+CD25^+CD127^{low}$ T cell events were collected for analysis.

Furthermore, we calculated the means and standard deviations (SD) of TCR-Vβ family usage using the control group as standard to compare difference in the usages of TCR-Vβ families between patients with AIN and control subjects (**Table II**). The increased/decreased Vβ subfamilies were defined for each TCR-Vβ family as above the mean +2 SD or below the mean −2 SD. The increased or decreased numbers indicated the numbers of Vβ families exceeding the limits of normal values of the TCR-Vβ family (mean +2 SD or mean –2 SD). We created heat maps of TCR-Vβ usages of conventional T cells and Tregs among patients and control subjects. In those heat maps, pink/red and pale green/green colors indicate values exceeding the upper (> +2 SD / > +3 SD, respectively) and lower (< −2 SD / < −3 SD, respectively) limits of the normal value of the TCR-Vβ family.

Statistical analysis

Frequencies of lymphocyte subsets and usage of each TCR-Vβ family between patients with AIN and age-matched control subjects were compared using the Wilcoxon rank-sum test. In the analysis of the skew of usage of TCR-Vβ families, we used the χ^2 test and Wilcoxon rank-sum test. The Wilcoxon signed-rank test was used to analyze the different usages of TCR-Vβ families between conventional T cells and Tregs. Two-sided p-values < 0.05 denoted statistically significant differences. Statistical analysis was performed using the JMP12 (SAS Institute Inc., Cary, NC, USA) software.

Results

Circulating Treg subpopulation

We first analyzed the frequencies of total Tregs and activated Tregs in peripheral blood using FOXP3 intracellular staining in patients with AIN (**Figure 1a**). The frequencies of both total Tregs and activated Tregs in patients with AIN were significantly lower than those observed in age-matched control subjects, as shown in **Figure 1b** (4.49 ± 0.99% vs. 5.87 ± 1.44% and 0.78 ± 0.17% vs. 1.00 ± 0.33%; $p = 0.0123$ and $p = 0.0123$, respectively). This result was partly consistent with those reported in our previous study (9). The low frequency of total Tregs may be caused by the decrease in activated Tregs that play an important role in suppressive function to avoid autoantibody production in autoimmune disease (including AIN).

For the analysis of the TCR repertoire, Tregs were defined as $CD4^+CD25^+CD127^{low}$ T cells substituted for FOXP3 intracellular staining using a flow cytometer (**Figure 1c**). When the frequency of $CD4^+CD25^+CD127^{low}$ Tregs was compared between patients with AIN and control subjects, Tregs in the former group showed a lower frequency than that noted in control subjects (6.32 ± 1.59% vs. 7.69 ± 1.36%, respectively; $p = 0.0113$) (**Figure 1d**). There was no difference in the frequency of $CD4^+CD25^-$ conventional T cells between patients with AIN and control subjects.

Usage of TCR-Vβ families in $CD4^+$ T cells

We examined the TCR-Vβ family distribution through flow cytometric analysis to detect any skewed distributions in the TCR repertoire in patients with AIN. A summary of the normal range of the distribution of 24 TCR-Vβ families, estimated from 22 control subjects, is presented in **Table II**. When the usage of 24 TCR-Vβ families was compared between control subjects and patients with AIN, the TCR-Vβ 9 and 17 families in $CD4^+$ T cells significantly increased in patients with AIN ($p = 0.0252$ and $p = 0.0361$, respectively) (**Figure 2**).

Usage of TCR Vβ families in conventional T cells and Tregs

Next, we compared the usage of Tregs and conventional T cells between the control group and AIN group. As shown in **Figure 3**, the usage of TCR-Vβ 9, 17, 20, and 21.3 families in Tregs, and that of the TCR-Vβ 17 family in conventional T cells were significantly increased in patients with AIN. There was no significant difference noted in the remaining TCR-Vβ families between patients with AIN and control subjects.

Skewed usage of TCR-Vβ families in conventional T cells and Tregs of individual patients with AIN

Based on the standard value of the usage of 24 TCR-Vβ families in age-matched control subjects (**Table 3**), the usages of TCR-Vβ families in conventional T cells and Tregs were individually compared in patients with AIN. The number of patients with AIN who showed skewed usages of TCR-Vβ families in conventional T cells was significantly higher than that of age-matched control subjects (76.5% vs. 40.9%, respectively; $p = 0.024$) (**Figure 4a**). Similarly, the number of $CD4^+CD25^+CD127^{low}$ T cells was significantly higher in patients with AIN than age-matched control (76.5% vs 45.5%, respectively; $p = 0.047$) (**Figure 4a**). There were strongly skewed usages in several TCR-Vβ families that exceeded the expression of the mean +3 SD of age-matched control subjects. Three of 17 patients in conventional T cells (patients 4, 10, and 16) and eight of 17 patients in Tregs (patients 2, 4, 5, 7, 10, 14, 16, and 17), respectively showed values higher than the mean + 3 SD in some TCR-Vβ families. In addition, we compared the increased/decreased usage of TCR-Vβ families in conventional T cells or Tregs. Patients with AIN showed more increased numbers than age-matched control subjects in both conventional T cells and Tregs ($p < 0.01$ and $p < 0.0001$, respectively) (**Figure 4b**). In contrast, there was no significant difference in the decreased numbers of TCR-Vβ families in conventional T cells or Tregs (**Figure 4b**). Thus, it appears that patients with AIN show variational and skewed usage of TCR-Vβ families in both conventional T cells and Tregs compared with those recorded in age-matched control subjects.

Change of preferent usage of TCR-Vβ families between conventional T cells and Tregs

Recently, it has been reported that the TCR repertoire in Tregs is highly as diverse as one in conventional T cells and the usages of TCR-Vβ families overlapped between these T cell subsets. Therefore, the usage of 24 TCR-Vβ families was individually assessed between conventional T cells and Tregs in both control subjects and patients with AIN (**Figure 5**). Increased usages of TCR-Vβ 3, 7.1, 8, 9, 13.1, 16, 17, 20, and 22 were observed in conventional T cells compared with Tregs in both control subjects and patients with AIN. In contrast, the usages of TCR-Vβ 5.1, 5.2, 5.3, 12, 13.2, and 13.6 in Tregs were significantly increased compared with those observed in conventional T cells. These results were summarized in **Figure 6**. Although there was a certain preference of usages of the TCR-Vβ repertoire between conventional T cells and Tregs in both control subjects and patients with AIN, usage of the TCR-Vβ 9 family was prominently different between control subjects and patients with AIN. All control subjects showed that the usage of TCR-Vβ 9 in conventional T cells was higher than that observed in Tregs. In contrast, patients with AIN did not demonstrate consistent variation between conventional T cells and Tregs. The highly increased usage of TCR-Vβ 9 in Tregs was observed in four patients with AIN in whom neutropenia persisted (Patient 2,3,4,7). There were no significantly different usages noted in the remaining 23 TCR-Vβ families between control subjects and patients with AIN.

Discussion

Tregs play the most important role in the tolerance of immune response in the peripheral environment (10). Tregs express biomarkers CD4, CD25, and FOXP3, and maintain tolerance to self-antigens, resulting in the prevention of autoimmune diseases. Several studies have found depletion of Tregs in autoimmune diseases producing autoantibodies, such as SLE, idiopathic thrombocytopenic purpura, and autoimmune thyroid disease (22–24). This study demonstrated low frequencies of activated Tregs ($CD4^+CD25^+FOXP3^{high}CD45RA^-$ T cells) and decrease in total Tregs ($CD4^+CD25^+FOXP3^{high}$ T cells) of peripheral blood in patients with AIN. Previously, we reported low levels of Tregs ($CD4^+CD25^+FOXP3^+$) in peripheral blood during the neutropenic period and natural restoration after the recovery of neutropenia in patients with AIN (9). Activated Tregs exhibited the most suppressive function among Tregs (12). Thus, the significant decrease in activated Tregs in patients with AIN may play an important role in autoimmune response to HNA due to the deficiency of suppressive function in children with AIN.

It has been reported that the percentage of Tregs in peripheral blood during the period of infancy are the lowest across the lifetime of an individual, despite the abundance noted in the neonatal period. After infancy, the frequency of Tregs gradually increases to the adult levels (25, 26). Infants with a low frequency of Tregs encounter various external antigens, such as foods, microorganisms, vaccinations, and/or other environmental factors, for the first time in their life. Furthermore, maternal antibodies gradually disappear during that period (27). When infants receive stimulations from various antigens, the activation of the immune system may lead to the development of self-reactive T cells. In addition to the decrease in activated Tregs, patients with AIN showed significantly skewed usage of several TCR-Vβ families in CD4+, conventional T cells, and Tregs compared with those noted in age-matched control subjects. Furthermore, the number of patients with AIN who displayed expanded usage of TCR-Vβ families was significantly higher than that reported in control subjects in both conventional T cells and Tregs. The mouse model study showed that depletion of Tregs led to the expression of a more diverse TCR repertoire in $CD4^+$ T cells. TCR-Vβ transgenic mice with depletion of Tregs

tended to produce some undesired self-reactive clonotypes (28). Taken together, the undesired T cell clonotype that reacts against HNA could expand to develop a self-reactive immune response under the low level of Tregs in patients with AIN.

TCR repertoire skewing has been studied in several autoimmune diseases in children and adults. The analyses of TCR repertoire in SLE have been performed using several techniques, such as flow cytometry, spectratyping, and high throughput sequencing. Studies report increased TCR-Vβ 16 CD4 T cells (17); prominent usages of TRBV 2, BV 8, BV 11, BV 14, BV 16, BV 19, and BV 24 of T cells (29); and highly expressed TRBV 10-2 and BV 23-1 (30), whereas preferential Vβ usage with the reduced diversity was not reported among patients with SLE (31). Studies on other autoimmune diseases reported the clonal expansion of TCR-Vβ 21 T cells in patients with ITP (32), increased TCR-Vβ 4 CD4 T cells in patients with type 1 diabetes mellitus (17), clonal expansion of TCR-Vβ 5.2 T cells in patients with multiple sclerosis (33), and persistent expansions of TCR Vβ correlated with clinical severity in patients with myasthenia gravis (34). Thus, skewed or divergent repertoire usages in T cells may be associated with the pathogenesis of autoimmune diseases. However, the consistent and common usages of TCR-Vβ subfamilies in T cells were not noted among autoimmune disorders, including AIN, in this study.

Our study also presented the skewed usage of two TCR-Vβ families (Vβ 9 and Vβ 17) in $CD4^+$ cells of patients with AIN. Furthermore, skewed usages of four TCR-Vβ families in Tregs (Vβ 9, 17, 20, and 21.3) and one TCR-Vβ family conventional T cells (Vβ 17) were observed when CD4 T cells were divided into conventional T cells and Tregs based on the cell surface expression of CD127. The expansion of Vβ 17 was observed in both Treg and T conventional cells in patients with AIN as shown in **Figure 3**. A prior study found that the usage of TCR-Vβ families overlapped in both Treg and T conventional cells in healthy children, which suggests the sharing of dominant clones (35). It is likely that the abnormal expansion of Vβ 17 in the Treg of patients with AIN may be associated with the response to the expansion of Vβ 17 in T conventional cells. Yu et al. noted that two patients with SLE showed expanded TRBV 2, BV 19 (equivalent of Vβ 17), BV 20-1, and BV

28 in the active phase during a longitudinal analysis of the TCR repertoire (36). The predominant usage of TRBV 19 in patients with SLE was also shown by Luo et al. (29). Moreover, some patients with SLE could exhibit secondary neutropenia due to anti-HNA antibodies. Analyses of a large number of patients may be necessary to determine the specificity of skewed TCR-Vβ in autoimmune diseases, including AIN. Moreover, the high frequency in the expansion of TCR-Vβ families appeared in Tregs of patients with AIN, as shown in **Figure 4b**. The several skewed usages of TCR-Vβ families in regulatory and conventional T cells of patients with AIN may affect the development of autoreactive clones against HNA. A previous study on the TCR-Vβ repertoire using CDR3 spectratyping in patients with immune thrombocytopenia reported that less expansion of the TCR-Vβ repertoire was associated with good response to splenectomy; in contrast, patients with more expansion exhibited poor response (20). It is likely that the frequency of expansion of the TCR-Vβ repertoire is associated with tolerance to immune response in autoimmune diseases. Collectively, these findings suggest that skewed usages of TCR-Vβ families of Tregs and low frequencies of activated Tregs in patients with AIN may be involved in the development of antineutrophil antibodies in children with AIN. However, the precise mechanism between the skewed usages of TCR Vβ families and low frequency of activated Tregs remains unclear.

Human Tregs have shown a very high TCR diversity compared with other T cell subsets including naïve T cells. This evidence suggested an important role in the immune-regulatory function of Tregs (37, 38). These results led us to individually study the difference in the usage of the TCR repertoire between regulatory and conventional T cells. The different usage of TCR-Vβ families in conventional T cells and Tregs has been reported in healthy children. The results indicated a significant preferential usage for five Vβ families and decreased usage for two Vβ families in Tregs (35). A summary of the results of our current study is shown in **Figure 6**. Preferential and decreased usages were similarly observed in several TCR Vβ families. Collectively, these results implied that reactivity to self-antigens is an important feature of the TCR repertoire in Tregs.

Quantitative differences in the usage of TCR Vβ repertoire of Tregs were more observed in patients with AIN than in control subjects (**Figure 4b**). Among the different usages of the TCR Vβ repertoire in

conventional T cells and Tregs, the usage of the TCR-Vβ 9 family observed in patients with AIN was prominently different from that in control subjects (**Figure 6**). Animal models of autoimmunity and immunodeficiency demonstrated that a diverse Treg repertoire is essential to maintain Treg function (28). Thus, the findings of TCR Vβ diversity in Tregs may be associated with abnormalities of Tregs observed in patients with AIN. Recently, abnormalities in the Treg repertoire have been reported in juvenile idiopathic arthritis (39). The restricted and clonotypic expansion of the Treg repertoire engendered antigenic triggers for disease pathogenesis in juvenile idiopathic arthritis. Furthermore, hematopoietic stem cell transplantation ameliorated the autoimmune diseases through the functional renewal and TCR diversification of Tregs (40).

This study had some limitations. First, this analysis with flow cytometry informed us the abnormal usage of TCR- Vβ families of T cells in patients with AIN, which do not indicate expansion of autoreactive clone directly. Considering that many Tregs have TCRs against self-antigen, the change of repertoire of Treg in patients with AIN could indicate disruption of homeostasis in repertoire of Tregs. The results of this study suggest that future research for the pathogenesis of AIN also require the deeper analysis using high throughput sequencing. Second, we could not follow up most patients from disease onset to recovery. There is no study on the individual and longitudinal change in the TCR-Vβ repertoire in children, although data on the cross-sectional frequency of TCR-Vβ in each age of healthy children or atopic children are available (17, 35, 41). We could not precisely conclude whether the expansions of the TCR-Vβ repertoire in patients with AIN has individually continued during the course of the disease. Therefore, longitudinal Tregs and TCR repertoire analyses in each patient with AIN are warranted because neutropenia spontaneously resolves within several years in the majority of patients with AIN.

In conclusion, this study showed low frequencies of total and activated Tregs in patients with AIN. Furthermore, it is the first investigation of the skewed uses of the TCR-Vβ repertoire in patients with AIN. The low frequencies of total and activated Tregs and the skew of TCR-Vβ families would allow the development of HNA-reactive T cell clones. Further studies are necessary to clarify the involvement of Tregs and the TCR-Vβ

repertoire in the pathogenesis of AIN in children.

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Disclosure

The authors have no conflicts of interest to disclose.

Data Availability Statement

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

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Table 1. Clinical characteristics of 25 patients with autoimmune neutropenia

Patient number	Sex*	Age at onset (months)	Initial manifestation for the diagnosis of neutropenia	Human neutrophil antigens against antineutrophil antibodies
1	F	6	Pyoderma	1a
2	M	10	Acute upper respiratory infection	1a
3	F	12	Pyrexia	1a
4	M	9	Lymphadenitis	1a/1b
5	F	14	Pyrexia	1a
6	M	22	Pyoderma	1a
7#	M	17	Adeno Virus infection	1a
8#	M	5	Pyrexia	1a/1b
9	F	22	Acute upper respiratory infection	1a
10#	M	14	Lymphadenitis	1a
11	F	38	Pyrexia	1a
12#	F	12	Pyrexia	1a
13	M	3	Pyrexia	1a/1b
14	M	5	Pyoderma	1a/1b
15#	F	6	Pyrexia	1a/1b
16	F	6	Not doing well	1a
17	F	21	Acute bronchitis	1a/1b
18	M	10	Pyrexia	1a/1b

19	F	6	Acute upper respiratory infection	1a
20	F	11	Pyrexia	1a
21	F	7	Acute bronchitis	1a
22	F	4	Respiratory syncytial virus infection	1a
23	M	10	Lymphadenitis	1a
24	F	31	Acute bronchitis	1a
25	F	20	Pyrexia	1a

* M, male; F, female

[#] Patient examined for both the frequency of total/activated regulatory T cells and T cell receptor (TCR)-Vβ repertoire.

Patients 18–25: analysis of frequency of total and activated regulatory T cells.

Table 2. Standard value of TCR-Vβ family usage in $CD4^+$ T cells

Vβ family	%Mean	95%CI	SD	%Median	IQR
Vβ 1	3.08	2.93, 3.24	0.35	3.09	0.54
Vβ 2	10.84	10.1, 11.6	1.66	11.10	2.46
Vβ 3	6.75	5.78, 7.72	2.19	7.13	2.79
Vβ 4	2.10	2.01, 2.19	0.20	2.08	0.47
Vβ 5.1	4.73	4.10, 5.36	1.43	5.00	1.22
Vβ 5.2	0.98	0.93, 1.03	0.12	0.97	0.20
Vβ 5.3	0.86	0.82, 0.90	0.08	0.88	0.12
Vβ 7.1	2.06	1.88, 2.24	0.40	2.05	0.57
Vβ 7.2	0.40	0.20, 0.59	0.44	0.07	0.75
Vβ 8	3.44	3.16, 3.72	0.63	3.46	0.88
Vβ 9	3.12	2.94, 3.30	0.41	3.17	0.60
Vβ 11	0.79	0.73, 0.84	0.13	0.79	0.20
Vβ 12	1.78	1.59, 1.96	0.43	1.72	0.67
Vβ 13.1	4.07	3.68, 4.45	0.88	4.13	1.32
Vβ 13.2	1.84	1.46, 2.21	0.84	1.60	1.33
Vβ 13.6	1.78	1.70, 1.86	0.18	1.76	0.25
Vβ 14	2.70	2.53, 2.86	0.37	2.69	0.46
Vβ 16	0.83	0.77, 0.89	0.13	0.84	0.17
Vβ 17	5.20	4.96, 5.45	0.55	5.27	0.86
Vβ 18	2.00	1.87, 2.13	0.29	2.06	0.39
Vβ 20	3.27	2.87, 3.66	0.89	3.34	1.48

Vβ 21.3	1.54	1.41, 1.66	0.28	1.55	0.33
Vβ 22	3.80	3.57, 4.03	0.52	3.78	0.67
Vβ 23	0.46	0.42, 0.49	0.08	0.43	0.12

TCR, T cell receptor; 95%CI, 95% confidential interval; SD, standard deviation; IQR, interquartile range (75th–25th percentile).

Table 3. Standard value of TCR-Vβ family usage in $CD4^+CD25^-$ T cells and $CD4^+CD25^+CD127^{low}$ T cells

	Conventional T cells ($CD4^+CD25^-$T cells)					Regulatory T cell ($CD4^+CD25^+CD127^{low}$ T cells)				
Vβ family	**%Mean**	**95%CI**	**SD**	**%Median**	**IQR**	**%Mean**	**95%CI**	**SD**	**%Median**	**IQR**
Vβ 1	3.06	2.89, 3.24	0.40	3.11	0.62	2.92	2.77, 3.07	0.34	3.01	0.36
Vβ 2	10.80	10.0, 11.6	1.70	10.70	2.74	10.9	10.2, 11.7	1.65	11.10	2.44
Vβ 3	6.85	5.86, 7.83	2.20	7.28	2.77	5.74	4.87, 6.61	1.97	5.81	2.68
Vβ 4	2.10	2.01, 2.19	0.04	2.09	0.32	2.00	1.85, 2.15	0.33	1.96	0.50
Vβ 5.1	4.59	3.97, 5.21	1.40	4.92	1.23	6.03	5.21, 6.84	1.84	6.43	1.44
Vβ 5.2	0.97	0.92, 1.03	0.12	0.96	0.18	1.13	1.06, 1.20	0.16	1.12	0.22
Vβ 5.3	0.84	0.80, 0.88	0.08	0.84	0.11	1.03	0.95, 1.11	0.18	1.03	0.22
Vβ 7.1	2.04	1.85, 2.22	0.41	2.07	0.58	1.83	1.67, 1.99	0.35	1.88	0.60
Vβ 7.2	0.40	0.20, 0.59	0.44	0.07	0.76	0.40	0.23, 0.58	0.39	0.23	0.68
Vβ 8	3.47	3.18, 3.75	0.65	3.49	0.85	3.22	2.97, 3.46	0.55	3.25	0.75
Vβ 9	3.14	2.96, 3.32	0.41	3.19	0.64	2.83	2.65, 3.00	0.39	2.78	0.56
Vβ 11	0.78	0.72, 0.84	0.13	0.77	0.19	0.73	0.64, 0.81	0.19	0.76	0.29
Vβ 12	1.74	1.54, 1.94	0.44	1.66	0.63	2.26	2.05, 2.47	0.48	2.41	0.75
Vβ 13.1	4.12	3.73, 4.52	0.90	4.20	1.39	3.72	3.38, 4.06	0.76	3.94	1.05
Vβ 13.2	1.79	1.41, 2.16	0.85	1.51	1.33	1.98	1.59, 2.36	0.87	1.46	1.38
Vβ 13.6	1.75	1.67, 1.82	0.18	1.75	0.25	2.15	2.00, 2.30	0.34	2.16	0.46
Vβ 14	2.70	2.53, 2.87	0.38	2.65	0.52	2.74	2.55, 2.93	0.42	2.70	0.42
Vβ 16	0.83	0.77, 0.89	0.14	0.85	0.14	0.73	0.64, 0.81	0.19	0.73	0.31

Vβ 17	5.21	4.96, 5.47	0.58	5.26	0.87	4.86	4.58, 5.12	0.61	4.87	1.21
Vβ 18	1.99	1.86, 2.13	0.30	2.02	0.41	1.99	1.85, 2.11	0.30	2.02	0.39
Vβ 20	3.37	2.95, 3.78	0.93	3.45	1.56	2.61	2.34, 2.89	0.63	2.65	0.80
Vβ 21.3	1.55	1.67, 1.42	0.28	1.57	0.33	1.49	1.38, 1.60	0.25	1.48	0.33
Vβ 22	3.83	3.59, 4.06	0.52	3.81	0.71	3.32	3.03, 3.61	0.65	3.28	1.16
Vβ 23	0.45	0.42, 0.48	0.07	0.43	0.12	0.41	0.35, 0.47	0.14	0.39	0.12

TCR, T cell receptor; 95%CI, 95% confidential interval; SD, standard deviation; IQR, interquartile range (75th–25th percentile).

Figure 1. Flow cytometric analysis of regulatory T cells (Tregs) and Treg subpopulations

$CD4^+CD25^+FOXP3^{high}$ T cells are counted as total Tregs and $CD4^+CD25^+FOXP3^{high}CD45RA^-$ T cells in $CD4^+$ T cells (Fraction II in the Treg subpopulations) are counted as activated Tregs (a). Data are presented in box-plots which display the minimum value, 25th, 50th, 75th, maximum value, and describe means as X (b). For the evaluation of T cell receptor (TCR)-Vβ usage of each T cell subsets by flow cytometry, $CD4^+CD25^-$ T cells in $CD4^+$ T cell are classified as conventional T cells (Tcons) and $CD4^+CD25^+CD127^{low}$ T cells are classified as Tregs. Each T cell subset IS sorted with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated anti-TCR-Vβ family antibodies (c). The data for the percentage of each T cell subset (Tcons in $CD4^+$ T cell, Tregs in $CD4^+$ T cell) are presented as box-plots (d). **p* = 0.0123, ***p* = 0.0123, ****p* = 0.0113. n.s; not significant. (Wilcoxon rank-sum test).

Figure 2. The usages of 24 TCR-Vβ families in $CD4^+$ T cells

Data are represented as box-plots. Analysis was performed in the control group (Cnt, n=22) and autoimmune neutropenia (AIN) group (AIN, n = 17). *Vβ 9, *p* = 0.0252, **Vβ 17, *p* = 0.0361 (Wilcoxon rank-sum test).

Figure 3. The usages of 24 TCR- Vβ families in regulatory T cells (Tregs) ($CD4^+CD25^+CD127^{low}$ T cells) and conventional T cells (Tcons) ($CD4^+CD25^-$ T cells)

Data are represented as box-plots. Analysis was performed in the control group (Cnt, n = 22) and autoimmune neutropenia (AIN) group, (AIN, n = 17). Upper clonograph: T cell receptor (TCR) in Tregs. Lower clonograph; TCR in Tcons. *Vβ9-Treg; *p* = 0.0031, **Vβ17-Treg; *p* = 0.0234, ***Vβ20-Treg; *p* = 0.0218, ****Vβ21.3-Treg; *p* = 0.0262, *****Vβ17-Tcon; *p* = 0.0325 (Wilcoxon rank-sum test).

Figure 4. The increased/decreased usage of T cell receptor (TCR)-Vβ families in $CD4^+CD25^-$ T cells and $CD4^+CD25^+CD127^{low}$ T cells of patients with autoimmune neutropenia (AIN) and control subjects

Means and standard deviations (SDs) of TCR-Vβ family usage of conventional T cells ($CD4^+CD25^-$ T cells) and regulatory T cells ($CD4^+CD25^+CD127^{low}$ T cells) were calculated in the control group and used as normal values. Heat map classifies the degrees of each usage of TCR-Vβ families using five colors; within normal range: −2 SD – +2 SD (white), > +2 SD (pink), > +3 SD (red), < −2 SD (pale green), < −3 SD (green) (a). The increased and decreased numbers of $CD4^+CD25^-$ T cell and $CD4^+CD25^+CD127^{low}$ T cells per person were compared. Data are represented as box-plots (b). *$p = 0.0022$, **$p = 0.0001$ (Wilcoxon rank-sum test). n.s; not significant.

Figure 5. The different usage of T cell receptor (TCR)-Vβ families in individual conventional T cells (Tcons) and regulatory T cells (Tregs)

Each line presents individual usage of TCR-Vβ families in Tcon and Treg. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (Wilcoxon signed-rank test)

Figure 6. Preferential T cell subsets with higher usage of T cell receptor (TCR) Vβ families

Usage of TCR Vβ families was compared between conventional and regulatory T cells, and the T cell subsets with higher usage of TCR Vβ families were defined as preferential T cell subsets. Red color or pink color indicates a preferential subset, while blue color or light blue color indicates a decreased subset. White color indicates no preference between conventional and regulatory T cells. Con T, conventional T cell; Reg T, regulatory T cell. Wilcoxon signed-rank test: pink-light blue, $p < 0.05$; red-blue, $p < 0.001$.

Figure1.

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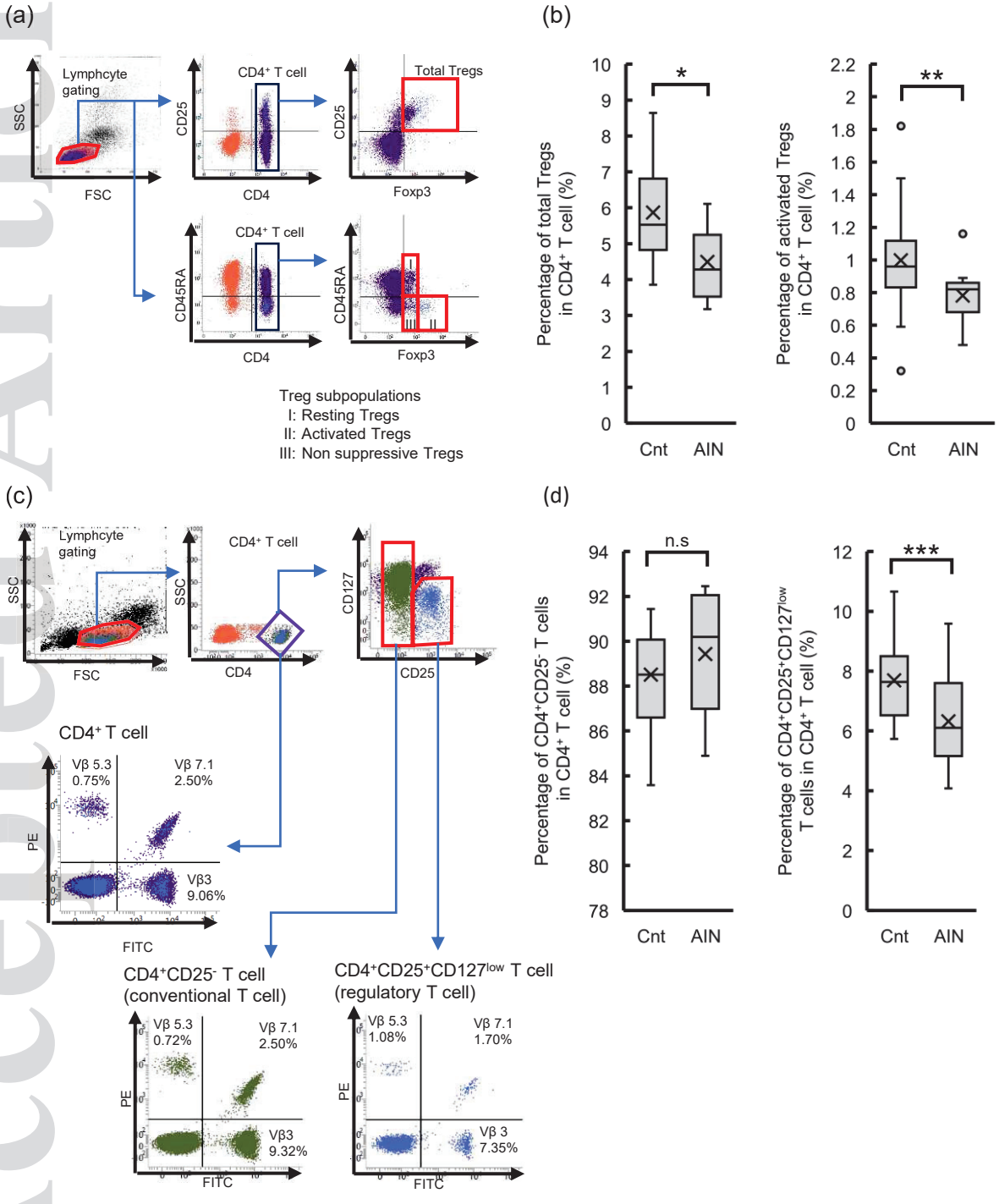


Figure2

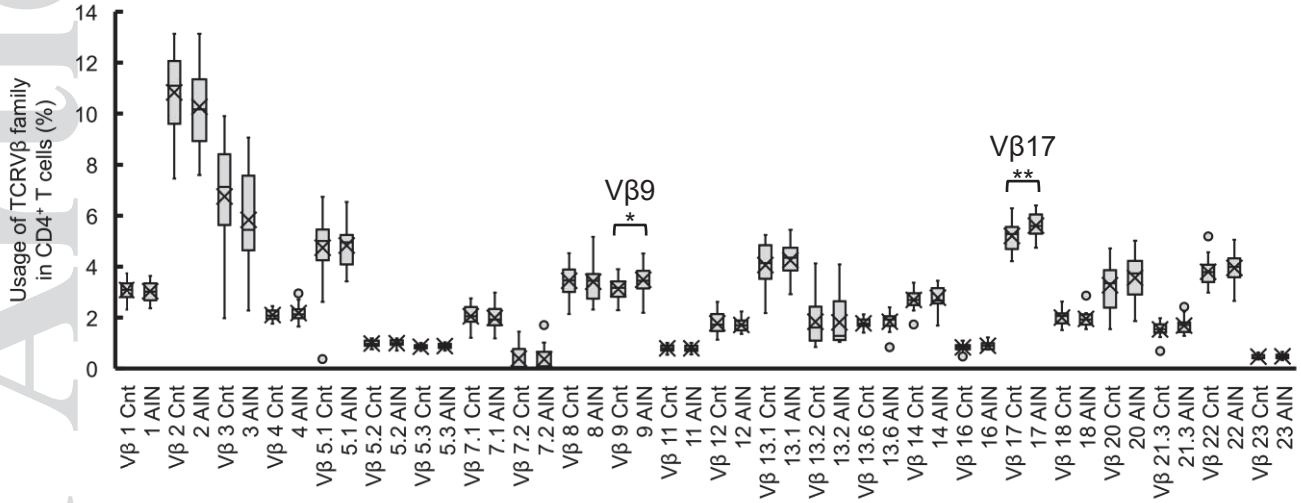


Figure3

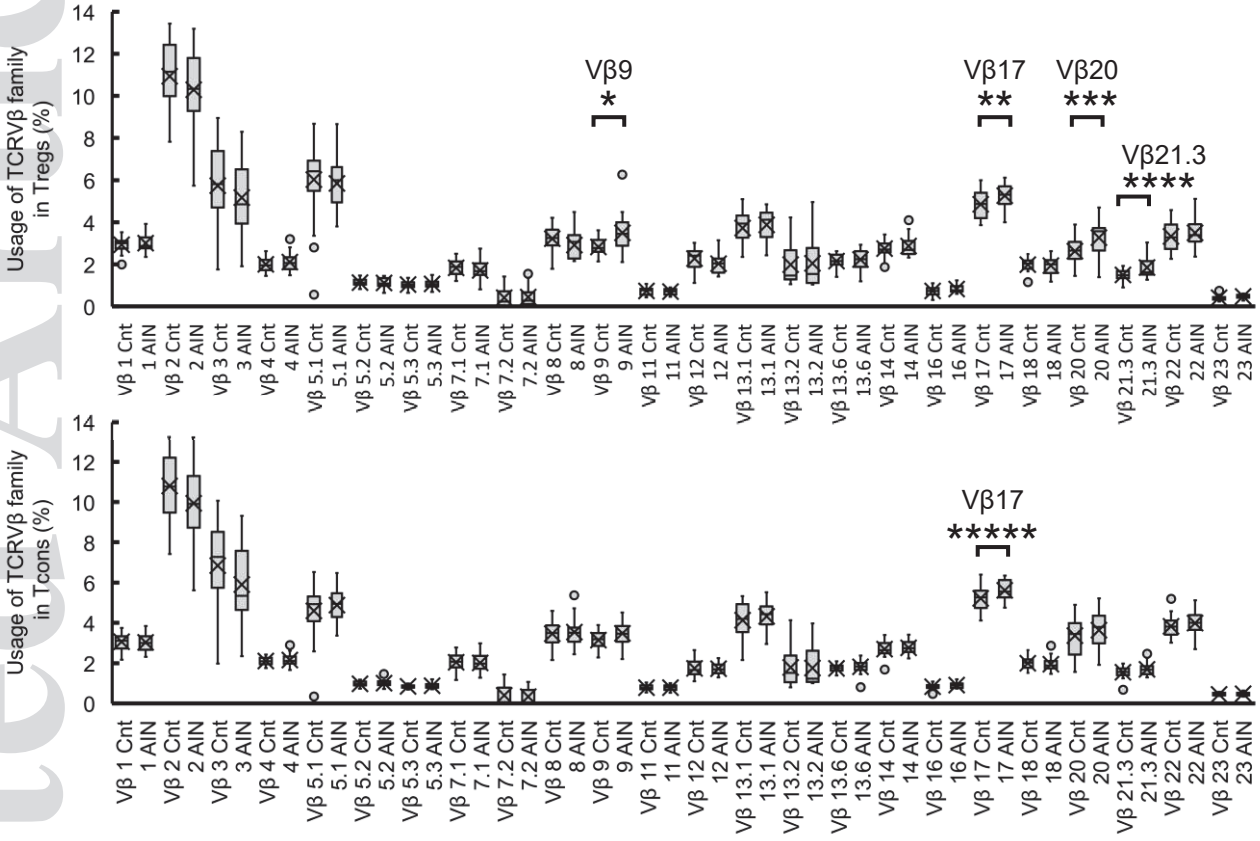
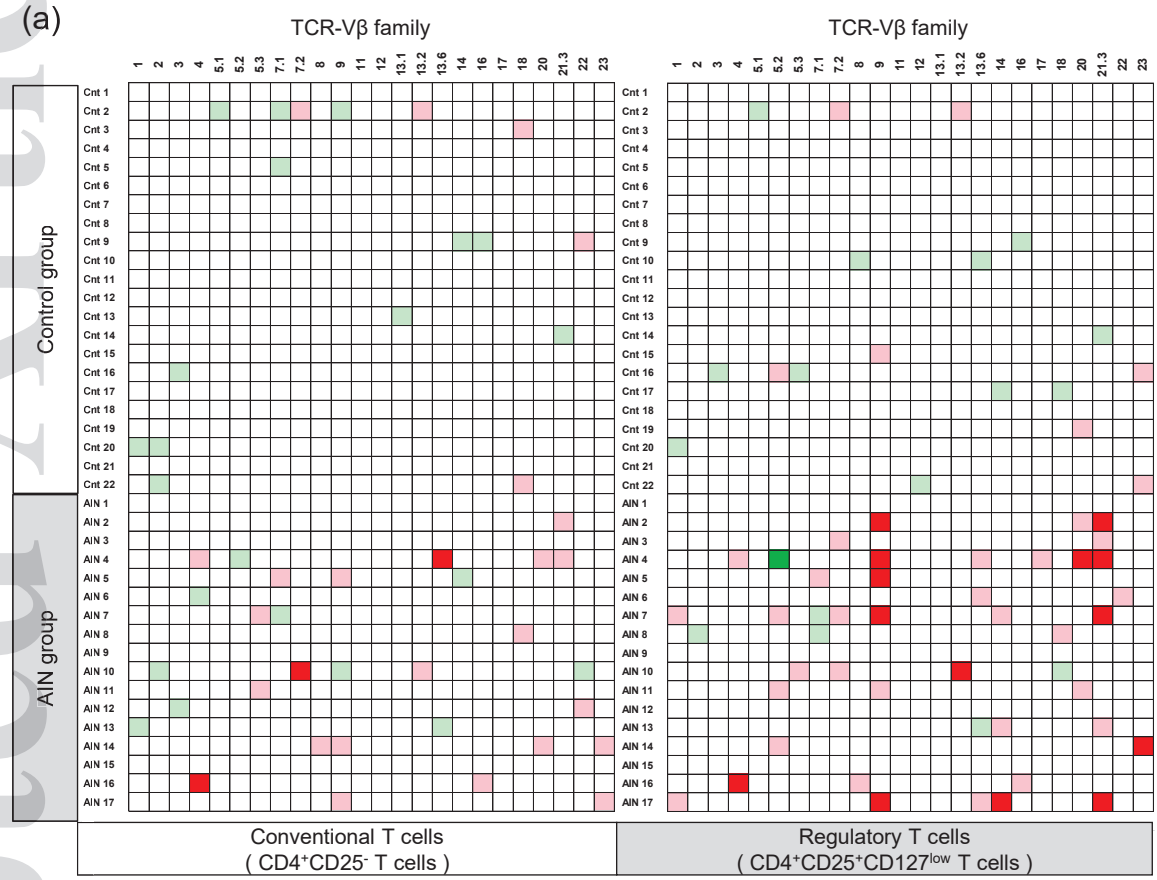


Figure4



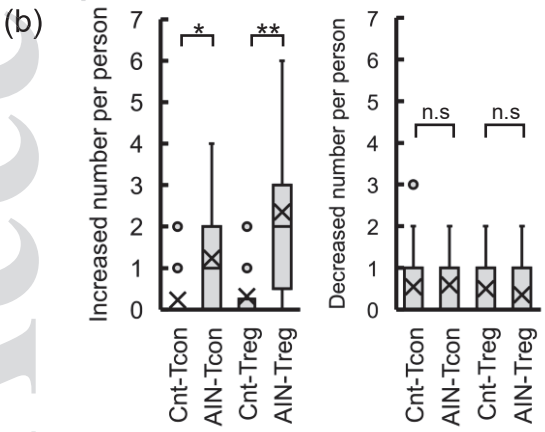


Figure5

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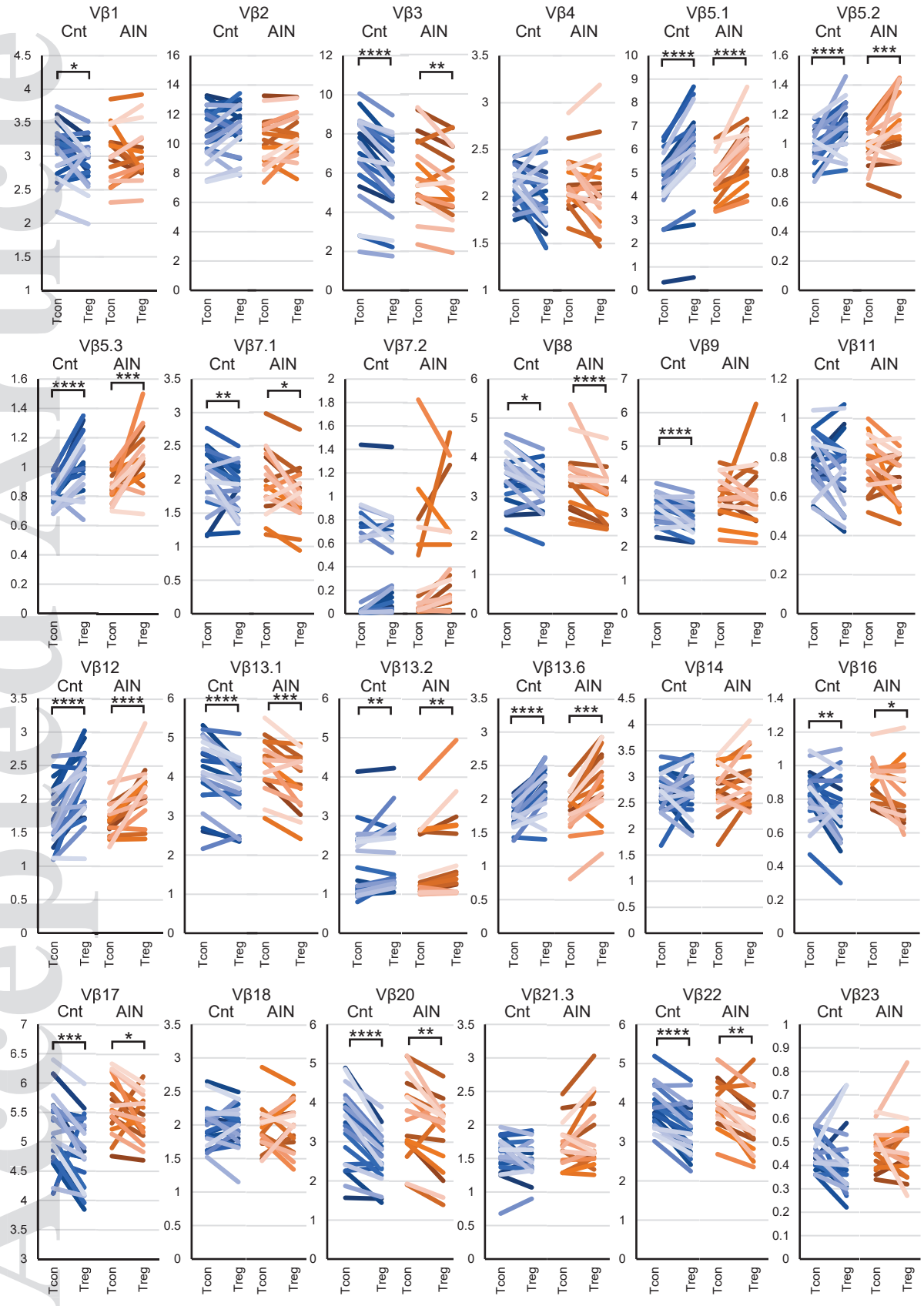

Figure 6