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Running title: Th1/Th17 polarization in severe Hemophilia A

Circulating Th1 and Th17 Cells in Severe Hemophilia A: Associations with Factor VIII Inhibitor Status and Titers

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Patient consent: Written informed consent was obtained from all individuals participated in this study.

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Abstract

Purpose: Factor VIII (FVIII) inhibitor development is a major complication of replacement therapy in severe hemophilia A (HA). This study investigated circulating T-helper 1 (Th1) and T-helper 17 (Th17) cell frequencies according to inhibitor status and their associations with inhibitor titers.

Methods: This case-control study included 32 male patients with severe HA (8 inhibitor-positive and 24 inhibitor-negative) and 24 healthy controls. Flow cytometry was used to assess $CD4^{+}IFN\text{-}\gamma^{+}$ Th1 and $CD4^{+}IL\text{-}17^{+}$ Th17 cells and intracellular cytokine mean fluorescence intensity (MFI).

Results: Overall, Th1 and Th17 cell frequencies and the cytokine MFIs did not differ significantly between HA patients and healthy controls (P>0.05). Patients with FVIII inhibitors exhibited a significantly lower proportion of Th1 lymphocytes than those without inhibitors (median [IQR]: 2.77% [1.37–2.81] vs. 5.83% [3.47–6.30], *P=0.003) and healthy controls (2.77% [1.37–2.81] vs. 4.19% [2.88–5.57], *P = 0.014). No significant differences in Th1-cell frequency were observed between inhibitor-negative patients and healthy controls (P>0.05). Th17-cell percentage and IL-17 MFI did not differ significantly among all groups (P>0.05). Among inhibitor-positive patients, Th1-cell frequency correlated positively with Th17-cell frequency (r=0.494, *P=0.032) and IFN-γ MFI (r=0.542, *P=0.016). The proportion of Th1 lymphocytes had a strong inverse correlation with FVIII inhibitor titer (r=-0.708, *P<0.001). Neither Th1 nor Th17 cell frequency was significantly associated with white blood cell and platelet count, or hemoglobin level.

Conclusion: Reduced Th1-cell frequency was associated with FVIII inhibitor positivity and higher inhibitor titers. Further studies are needed to validate these results and clarify their clinical significance.

Keywords: Severe hemophilia A; Inhibitors; Immune system; T helper 1 (Th1); T helper 17 (Th17)

Introduction

Hemophilia A (HA) is an X-linked recessive bleeding disorder caused by a congenital deficiency of coagulation factor VIII (FVIII), with an incidence of approximately 1 in 4,000–5,000 male live births [1, 2]. Patients with severe HA experience recurrent spontaneous bleeding episodes, particularly into joints and soft tissues, which can lead to progressive arthropathy. Management relies on prophylactic or on-demand hemostatic treatment, including FVIII replacement therapy [3].

Advances in treatment and disease management over recent decades have substantially improved the quality of life of individuals with HA [4]. The standard treatment for HA patients involves FVIII replacement therapy using plasma-derived or recombinant products for prophylaxis or the management of bleeding episodes [5]. However, a major complication of FVIII replacement therapy is the development of neutralizing anti-FVIII antibodies, known as FVIII inhibitors. These antibodies develop in approximately 25–30% of patients with severe HA and can compromise treatment efficacy, increasing bleeding risk, morbidity, and healthcare costs while impairing quality of life [6, 7]. Inhibitor management may require costly bypassing agents or other specialized therapies. Immune tolerance induction (ITI), which involves repeated FVIII administration over months or years, is an established approach to eradicating inhibitors in eligible patients [6-8]. Although the underlying immunological mechanisms remain incompletely understood, a failure to establish or maintain immune tolerance to FVIII is considered an important factor in inhibitor development [9].

It is well established that the production of anti-FVIII antibodies is T-cell dependent, as $CD4^+$ T cells provide essential signals for the activation and differentiation of FVIII-specific B cells, including antibody class switching and affinity maturation [10]. Previous studies have suggested

that $CD4^+$ T-cell responses, particularly the balance between T-helper 1 (Th1) and Th2 responses, may influence the development and magnitude of FVIII inhibitor responses [9-12]. However, the association between circulating Th1-cell frequency and inhibitor status remains incompletely characterized.

T helper 17 (Th17) cells represent another subset of $CD4^+$ T lymphocytes, characterized by the production of interleukin-17A (IL-17A), and play important roles in inflammatory and autoimmune processes [13, 14]. Although Th17 cells have been implicated in the pathogenesis of several antibody-mediated diseases [15], their potential association with FVIII inhibitor status and inhibitor titers in patients with HA remains unclear.

Therefore, this study aimed to investigate the frequencies of circulating Th1 and Th17 cells in patients with severe HA according to FVIII inhibitor status and to assess their associations with inhibitor titers.

Patients and methods

Study design and patients' selection criteria

This case–control study was conducted at the Comprehensive Hemophilia Center affiliated with Shiraz University of Medical Sciences between January and December 2021. The study enrolled 32 male patients with severe HA, comprising 8 individuals with factor VIII (FVIII) inhibitors and 24 without inhibitors. A group of 24 age-matched healthy male volunteers without a history of infection, malignancy, autoimmune or inflammatory disorders served as controls.

Severe HA was defined as baseline FVIII activity below 1 IU/dL, as measured by a one-stage clotting assay. Eligible patients had clinically or radiologically documented arthropathy affecting

at least two target joints. Patients receiving prophylactic treatment were required to have maintained a stable treatment regimen for at least 3 months before enrollment.

Patients were classified according to their FVIII inhibitor status. The inhibitor-positive group included individuals with a documented history of inhibitor development and an inhibitor titer of ≥0.6 Bethesda units (BU)/mL, confirmed by three separate measurements. The inhibitor-negative group comprised patients with no previous history of FVIII inhibitor development or immune tolerance induction (ITI), a negative inhibitor assay at enrollment, and no documented inhibitor development during subsequent clinical follow-up.

Blood samples were obtained when patients were clinically stable, at least 10 days after the most recent FVIII infusion. Patients experiencing an acute bleeding episode at the time of collection were excluded. Healthy controls were recruited from hospital staff and university personnel. All were age-matched to the patient population and had no known bleeding disorders, malignancies, autoimmune or inflammatory diseases, acute or chronic infections, or other recognized immunological disorders. Individuals receiving immunosuppressive medication were excluded from the control group.

Additional exclusion criteria for patients included a history of collagen vascular diseases, such as systemic lupus erythematosus or rheumatoid arthritis; autoimmune or chronic inflammatory conditions, including inflammatory bowel disease and psoriasis; and the use of immunosuppressive treatments during the preceding 6 months. In addition, patients with active or chronic infections were also excluded, including hepatitis B virus infection (positive hepatitis B surface antigen), hepatitis C virus infection (detectable HCV RNA), or HIV infection. Further exclusion criteria were bacterial infections requiring antibiotic treatment within the preceding 8

weeks and febrile illness, defined as a body temperature above 38°C, within 2 weeks before blood collection.

The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (approval number: IR.SUMS.REC.1398.1204). Written informed consent was obtained from all participants or their legal guardians, as applicable.

Factor VIII activity and inhibitor titration

Plasma FVIII activity was quantified using a one-stage clotting assay on an automated coagulation analyzer (ACL-TOP CTS 300; Instrumentation Laboratory, USA). FVIII-neutralizing antibodies were assessed using the Bethesda assay, with and without prior heat treatment of the samples at 56°C for 30 minutes. Inhibitor levels were reported as Bethesda units per milliliter (BU/mL).

Sample Collection and PBMC Isolation

Peripheral venous blood (5 mL per participant) was collected from patients with HA and healthy controls into tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Hypaque reagent (Inno-Train, Germany) at 400×g for 20 minutes. The isolated PBMCs were washed with 1× phosphate-buffered saline (PBS) and prepared for subsequent flow cytometric staining.

Flow cytometry staining of Th1 and Th17 lymphocytes

To assess Th1 and Th17 cell frequencies, PBMCs were subjected to pharmacological stimulation followed by intracellular cytokine staining and flow cytometric analysis. Briefly, 5×10^5 PBMCs were suspended in 1 mL of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100

U/mL penicillin, and 100 µg/mL streptomycin. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich, Germany) and ionomycin (1 µg/mL; Sigma-Aldrich, Germany). Monensin (1 µL; BD Biosciences, USA) was added to inhibit intracellular cytokine transport and promote cytokine accumulation. Cells were incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2.

Following stimulation, cells were stained using a three-color antibody panel comprising PerCP/Cy5.5-conjugated mouse anti-human CD4 (clone SK3), FITC-conjugated mouse anti-human interferon-γ (IFN-γ; clone B27), and PE-conjugated mouse anti-human IL-17 (clone N49-653), all obtained from BD Biosciences (USA). Th1 cells were identified as $CD4^+IFN\text{-}\gamma^+$ cells, whereas Th17 cells were identified as $CD4^+IL\text{-}17^+$ cells, as previously described [16]. After staining, the cell pellets were resuspended in 200–300 µL of staining buffer and analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA). Data analysis was performed using FlowJo software (version 7.6) with a sequential gating strategy. First, lymphocytes (R1) were identified according to their forward-scatter (FSC-A) and side-scatter (SSC-A) profiles. Within this population, $CD4^+IFN\text{-}\gamma^+$ cells were assigned to the Q2 quadrant and classified as Th1 cells, whereas $CD4^+IL\text{-}17^+$ cells were assigned to the Q6 quadrant and classified as Th17 cells. Representative flow cytometry plots illustrating the identification of IFN-γ-producing Th1 cells and IL-17-producing Th17 cells are shown in Figure 1. Intracellular cytokine expression was additionally assessed by measuring the mean fluorescence intensity (MFI) of IFN-γ and IL-17.

Statistical analysis

Statistical analyses were performed using SPSS Statistics, version 22.0 (IBM Corp., Armonk, NY, USA). Quantitative variables were expressed as median and interquartile range (IQR). The Mann–

Whitney U test was used to compare quantitative variables between two independent groups, whereas the Kruskal–Wallis test was used for comparisons among more than two groups. When the Kruskal–Wallis test showed a statistically significant difference, post hoc pairwise comparisons were performed. Spearman's rank correlation coefficient was used to assess associations between quantitative variables. A two-sided P value <0.05 was considered statistically significant.

Results

A total of 32 male patients with severe hemophilia A (HA), including 8 inhibitor-positive and 24 inhibitor-negative patients, and 24 healthy male controls were enrolled. The mean age was 23.84 ± 15.89 years in patients with HA and 25.23 ± 14.46 years in healthy controls. The clinical and laboratory characteristics of the patient cohort have been described previously [17].

Determination of Th1/Th17 polarization in HA patients and controls

The frequency of Th1 and Th17 lymphocytes was determined by flow cytometry and compared between HA patients and healthy controls (Figure 2). The results showed that the percentages of $CD4^{+}IFN\text{-}\gamma^{+}$ Th1 lymphocytes were not different between HA patients and healthy controls (median [IQR]: 3.87% [2.79-6.21] *vs.* 4.19% [2.88-5.57]; P=0.9) (Figure 2). In addition, there was no significant difference in the MFI of IFN-γ between HA patients and healthy controls (median [IQR]: 18 [12.7-27.9] *vs.* 15.25 [9.75-35.82]; P=0.627).

In case of Th17, the results demonstrated that the frequency of $CD4^{+}IL17^{+}$ Th17 lymphocytes as well as the MFI of IL-17 did not differ between HA patients and healthy controls (Median [IQR]: 0.91% [0.57-1.19] *vs.* 0.72% [0.59-1.06]; P=0.059 and 2.33 [1.98-2.56] *vs.* 2.2 [1.92-2.33]; P=0.977, respectively).

Th1/Th17 subsets in HA patients with and without inhibitors

The frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 lymphocytes as well as IFN-γ and IL-17 MFI was compared between HA patients with and without inhibitors as well as healthy controls (Figure 3). The results revealed that the proportion of $CD4^+IL\text{-}17^+$ Th17 lymphocytes and IL-17 MFI was not significantly different between HA patients with and without inhibitors (median [IQR]: 0.75% [0.34-0.86] *vs.* 0.67% [0.51-1.11]; P=0.462 and 2.81 [2.23-3.18] *vs.* 2.43 [2.13-3.04]; P=0.561, respectively), as well as between inhibitor positive patients and healthy controls (median [IQR]: 0.75% [0.34-0.86] *vs.* 0.86% [0.6-1.32]; P=0.157 and 2.81 [2.23-3.18] *vs.* 2.33 [2.05-3.3.72]; P=0.798, respectively) (Figure 3). Moreover, no significant difference was observed in the frequency of $CD4^+IL17^+$ Th17 lymphocytes frequency or IL-17 MFI between inhibitor negative patients and healthy controls (median [IQR]: 0.67% [0.51-1.11] *vs.* 0.86% [0.6-1.32]; P=0.094 and 2.43 [2.13-3.04] *vs.* 2.33 [2.05-3.3.72]; P=0.946) (Figure 3).

However, analysis of our results showed that HA patients with inhibitors had a lower frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 lymphocytes compared to inhibitors negative ones (median [IQR]: 2.77% [1.37-2.81] *vs.* 5.83% [3.47-6.3]; *P=0.003). as well as healthy controls (median [IQR]: 2.77% [1.37-2.81] *vs.* 4.19% [2.88-5.57]; *P=0.014). No significant differences were observed in the frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 lymphocytes between HA patients without inhibitors and healthy controls (median [IQR]: 5.83% [3.47-6.3] *vs.* 4.19% [2.88-5.57]; P=0.178) (Figure 3). The MFI of IFN-γ did not differ between HA patients with and without inhibitors (median [IQR]: 16.7 [9.59-26.5] *vs.* 18.35 [15.55-29.65]; P=0.559), between HA patients with inhibitors and healthy controls (median [IQR]: 16.7 [9.59-26.5] *vs.* 15.25 [9.75-35.82]; P=0.823), and between HA patients without inhibitors and healthy controls (median [IQR]: 18.35 [15.55-29.65] *vs.* 15.25 [9.75-35.82]; P=0.454).

There was a positive correlation between the frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 lymphocytes (r=0.494, *P=0.032) and the frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 subsets and IFN-γ MFI (r=0.542, *P=0.016) in severe HA patients (Figure 4).

Association between Th1/Th17 population, inhibitor titers, and laboratory and clinical parameters

The correlation between the percentages of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 subsets and laboratory findings of patients, including WBC and platelet counts as well as the levels of hemoglobin, was determined. The results delineated that there was not a significant correlation between the frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 lymphocytes with WBC and platelet counts, and the level of hemoglobin (P>0.05).

Furthermore, we checked whether the polarization of Th1/Th17 subsets was associated with inhibitor titers. The results demonstrated that the proportion of $CD4^+IFN\text{-}\gamma^+$ Th1 cells is negatively correlated with inhibitor titers (r=-0.97, *P<0.001) (Figure 4).

We further explored the potential confounding effects of age, the type of received FVIII concentrate (plasma-derived *vs.* recombinant), and type of exogenous FVIII treatment (on-demand *vs.* prophylaxis) on the primary immunological outcomes.

Accordingly, in an exploratory age-adjusted analysis restricted to inhibitor-positive patients, variables were rank-transformed, and Spearman partial correlations were computed. The inverse correlation between Th1-cell frequency and FVIII inhibitor titer remained significant after adjustment for age (partial Spearman correlation, $r_s = -0.999$, *P = 0.001). However, given the small number of patients included in this analysis, this finding should be interpreted cautiously.

Moreover, no significant differences in Th1 and Th17 cell frequency was seen regarding type of received FVIII concentrate (plasma-derived *vs.* recombinant) and type of exogenous FVIII treatment (on-demand *vs.* prophylaxis) (both $P > 0.05$). These findings suggest that neither age nor type of FVIII product or type of treatment had a detectable influence on the primary immunological outcomes in this cohort.

Discussion

The development of neutralizing anti-factor VIII (FVIII) antibodies, known as inhibitors, remains one of the most serious complications of replacement therapy in patients with severe congenital HA, affecting approximately 20–35% of this population [6, 7]. Inhibitor development can compromise treatment efficacy and increase the clinical and economic burden of the disease, underscoring the need for a better understanding of the immunological mechanisms underlying this response [8, 9]. In the present study, we investigated the frequencies and intracellular cytokine expression profiles of Th1 and Th17 cells in patients with severe hemophilia A according to FVIII inhibitor status and examined their associations with inhibitor titers and relevant clinical parameters.

The principal finding was a lower frequency of $CD4^{+}IFN\text{-}\gamma^{+}$ Th1 cells in inhibitor-positive patients than in inhibitor-negative patients and healthy controls. Th1-cell frequency also showed a strong inverse correlation with FVIII inhibitor titer. In contrast, Th17-cell frequency and intracellular IL-17 expression did not differ significantly among the study groups.

The role of $CD4^{+}$ T-cell subsets in FVIII inhibitor development has been investigated in several studies, although the available evidence remains limited. Reding et al. reported that high-titer FVIII inhibitor responses were predominantly associated with Th2-related anti-FVIII IgG

subclasses, whereas Th1-associated responses were more frequently linked to low inhibitor levels or successful immune tolerance induction [12]. Similarly, Hu et al. detected FVIII-specific, IFN-γ-producing $CD4^+$ T cells in both patients without inhibitors and healthy individuals, suggesting that Th1 cells may participate in the initial immune response to FVIII, while Th2 cells may contribute more substantially to high-titer inhibitor production [11]. Further evidence comes from a murine model of hemophilia A, in which anti-CD3 therapy prevented FVIII inhibitor formation and was associated with a shift toward a Th1 immune response, supporting the possibility that Th1 responses may have a protective role in inhibitor development [18]. However, these findings should be interpreted cautiously because animal models and studies of FVIII-specific responses do not directly correspond to measurements of circulating Th1-cell frequencies in patients.

Several explanations may account for the lower Th1-cell frequency observed in inhibitor-positive patients. One possibility is that prolonged antigen exposure alters T-cell differentiation or function, potentially involving functional exhaustion or activation-induced cell death. These mechanisms were not assessed in our study and remain speculative. Ettinger et al. examined T-cell clones at different stages of anti-FVIII immune responses and identified lineages exhibiting both Th17 and Th1 characteristics, suggesting that T-helper cell polarization may change as the immune response evolves [19]. This raises the possibility that the immune response in patients with established inhibitors may differ from that observed during its initial development. The inverse association between Th1-cell frequency and inhibitor titer is consistent with a relationship between inhibitor burden and circulating Th1-cell frequency. Nevertheless, this correlation should be interpreted cautiously because of the small sample size and the limited number of inhibitor-positive patients. Moreover, the cross-sectional design does not establish whether reduced Th1-cell frequency precedes inhibitor development or occurs as a consequence of an established immune response.

The balance between effector T cells and regulatory T cells populations may also contribute to the development of FVIII inhibitors. Regulatory T cells (Tregs) are thought to contribute to immune tolerance to FVIII, although clinical studies have reported inconsistent associations between Treg frequency and inhibitor status. Abo El-Asrar et al. and Jorge Vela-Ojeda et al. reported reduced frequencies of $CD4^+CD25^+CD127^-$ Tregs in patients with FVIII inhibitors [20, 21], whereas Arreola-Díaz et al. found no significant difference in Treg frequency between inhibitor-positive and inhibitor-negative patients [22]. In our previous study in the same population, we reported that $CD4^+CD25^-FOXP3^+$ Tregs might be involved in preventing FVIII inhibitor formation in patients with severe HA [23]. However, we found no significant correlations between $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^-FOXP3^+$ Treg populations and Th1 or Th17 cell subsets in the current study ($P>0.05$) (data are not shown). In the present study, the lower Th1-cell frequency observed in inhibitor-positive patients may reflect a distinct aspect of immune dysregulation. Nevertheless, the relationship between regulatory and effector T-cell populations in FVIII inhibitor development remains incompletely understood and requires further investigation. Among patients with severe HA, we observed positive correlations between Th1- and Th17-cell frequencies and between Th1-cell frequency and IFN-γ MFI. These associations may reflect coordinated regulation or overlapping features of these T-helper-cell populations. Although Th1 and Th17 cells exhibit functional plasticity and may share certain differentiation signals, the biological significance of these correlations and their relevance to FVIII inhibitor development remain unclear.

Several limitations should be considered when interpreting our findings. The small number of inhibitor-positive patients limits statistical power and precludes robust subgroup analyses. Furthermore, the cross-sectional design does not allow causal inferences or determine whether reduced Th1-cell frequency precedes or follows inhibitor development. In addition, peripheral

blood T-cell frequencies may not adequately reflect immune responses within lymphoid organs, where FVIII-specific responses are initiated and maintained. Although an exploratory age-adjusted analysis suggested that the inverse association between Th1-cell frequency and inhibitor titer persisted after adjustment for age, the small sample size makes this result hypothesis-generating rather than conclusive. Larger studies are needed to validate this association.

The complex immunological mechanisms underlying FVIII inhibitor development also warrant investigation beyond conventional Th1 and Th17 subsets. In particular, the T follicular helper (Tfh)/T follicular regulatory (Tfr) axis may contribute to the regulation of FVIII-specific antibody responses. Jing et al. reported an increased Tfh/Tfr ratio in FVIII inhibitor-producing mice, suggesting a potential imbalance between follicular helper and regulatory responses [24]. Future studies should examine Tfh and Tfr populations alongside Th1 and Th17 cells to provide a more comprehensive understanding of the cellular mechanisms associated with FVIII inhibitor development.

Conclusion

In summary, our findings indicate that lower Th1-cell rather than Th17-cell frequency is associated with FVIII inhibitor positivity and higher inhibitor titers in patients with severe HA, providing new insights into the relationship between Th1-cell responses and FVIII inhibitor status. Th1-cell frequency warrants further investigation as a potential biomarker, although its clinical utility remains unestablished. Larger, longitudinal studies are needed to validate these findings, clarify the underlying mechanisms, and determine whether changes in Th1 and Th17 responses are associated with the development or persistence of FVIII inhibitors.

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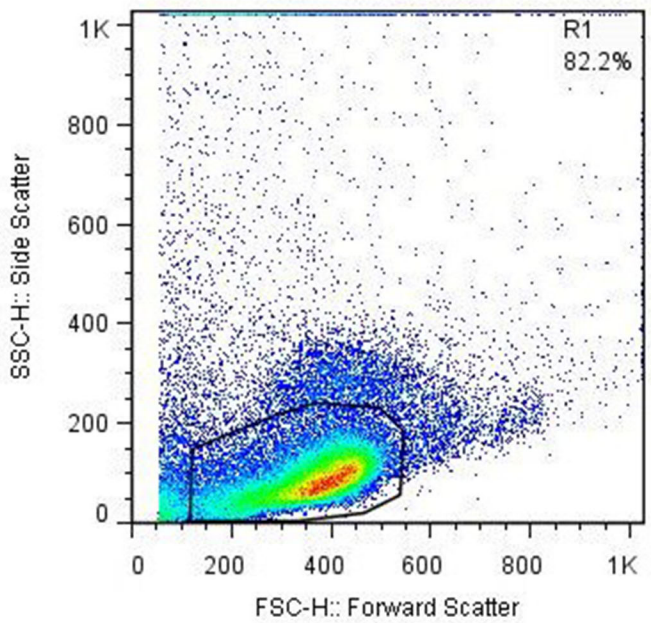
Figure legends

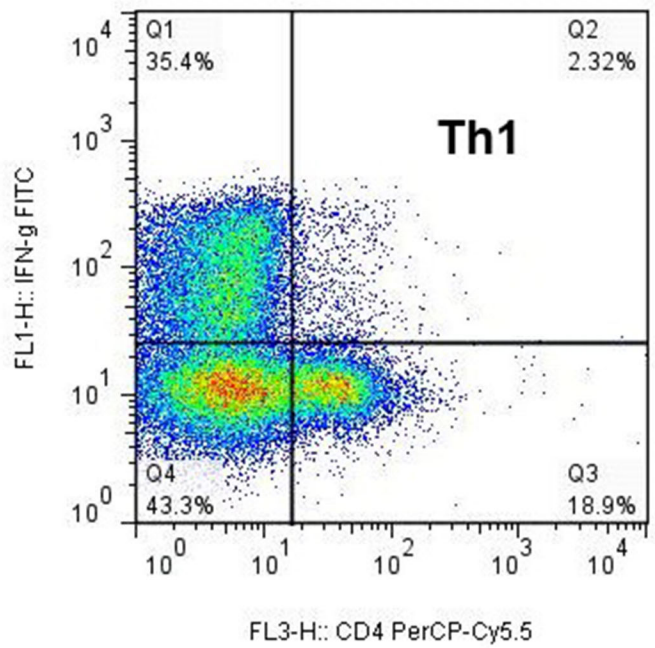
Figure 1. Flow cytometry analysis of IFN-γ-producing Th1 and IL-17-producing Th17 subsets. First, lymphocytes (R1) were identified according to their forward-scatter (FSC-A) and side-scatter (SSC-A) profiles. Within this population, $CD4^+IFN\text{-}\gamma^+$ cells were assigned to the Q2 quadrant and classified as Th1 cells, whereas $CD4^+IL\text{-}17^+$ cells were assigned to the Q6 quadrant and classified as Th17 cells. Data were analyzed using FlowJo software version 7.6.

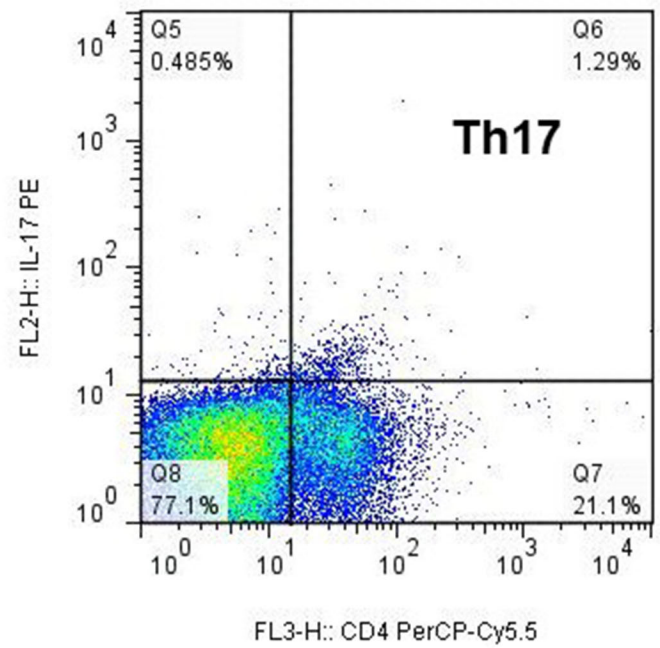
Figure 2. Comparison of Th1 and Th17 cell frequencies between patients with severe HA and healthy controls. Th1 cells were defined as $CD4^+IFN\text{-}\gamma^+$ cells, and Th17 cells as $CD4^+IL\text{-}17^+$ cells, as determined by flow cytometry and compared between two groups using the Mann–Whitney U test. Graphs were plotted using GraphPad Prism software version 10. A P-value <0.05 was considered significant.

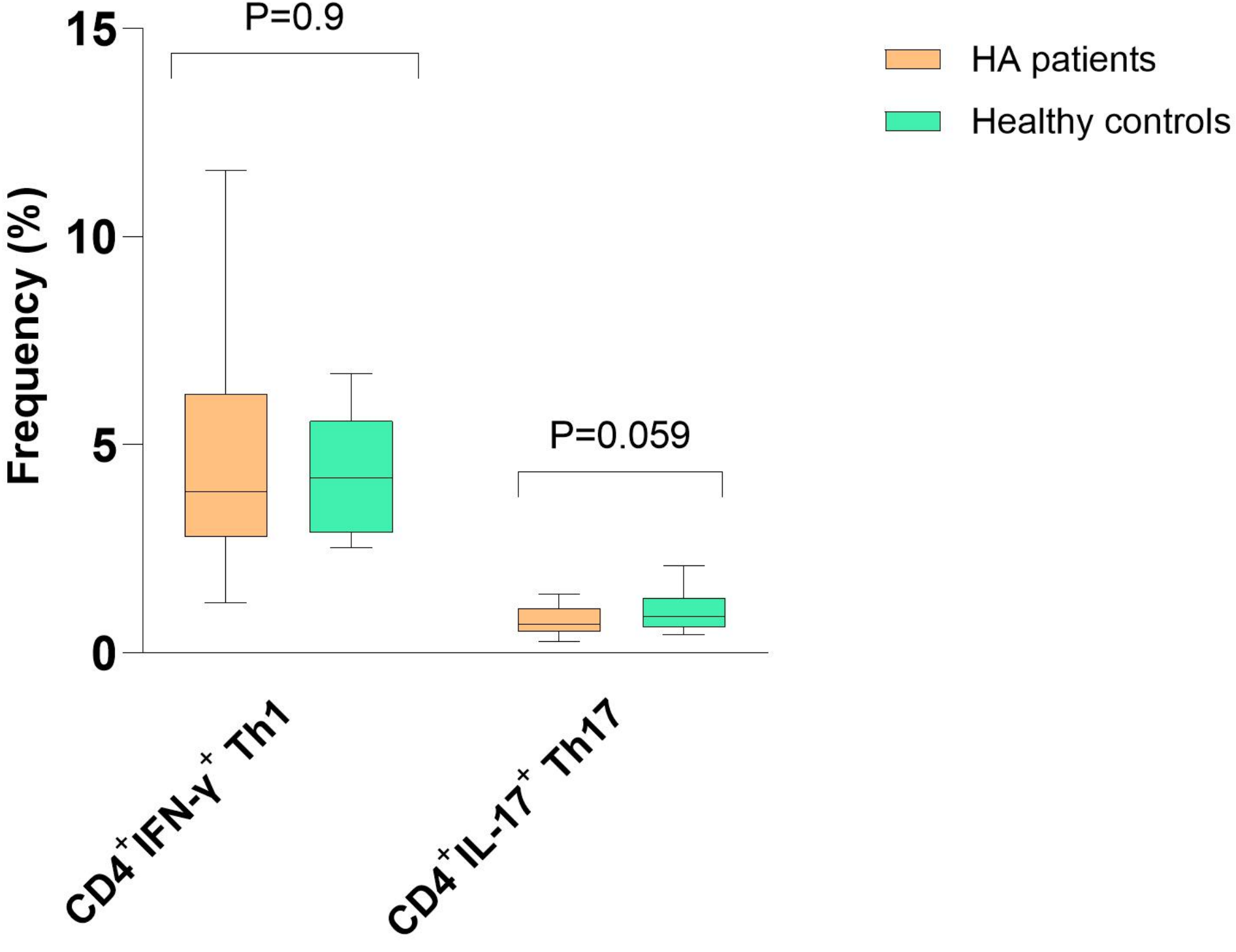
Figure 3. Comparison of Th1 and Th17 cell frequency among severe HA patients with and without FVIII inhibitors and healthy controls. The frequency of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 cells was assessed by flow cytometry and compared between three groups using the Kruskal–Wallis test. Graphs were plotted using GraphPad Prism software version 10. A P-value <0.05 was considered significant.

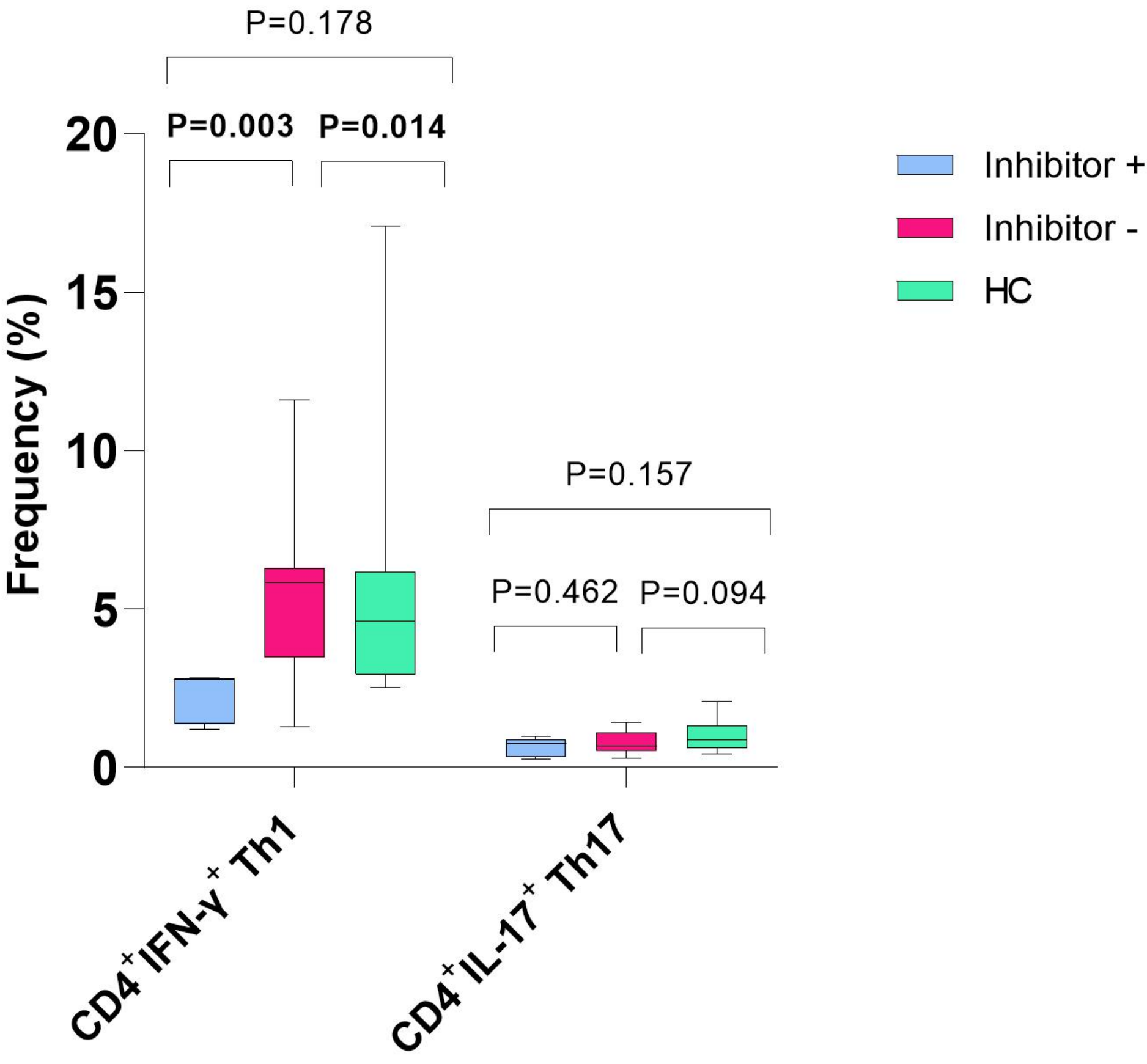
Figure 4. Correlations between Th1 and Th17 cell frequencies and FVIII inhibitor titers in patients with severe HA. The correlation between inhibitor titers and the percentages of $CD4^+IFN\text{-}\gamma^+$ Th1 and $CD4^+IL\text{-}17^+$ Th17 cells was assessed using Spearman's rank correlation test. Graphs were plotted by GraphPad Prism software version 10. A P-value <0.05 was considered significant.

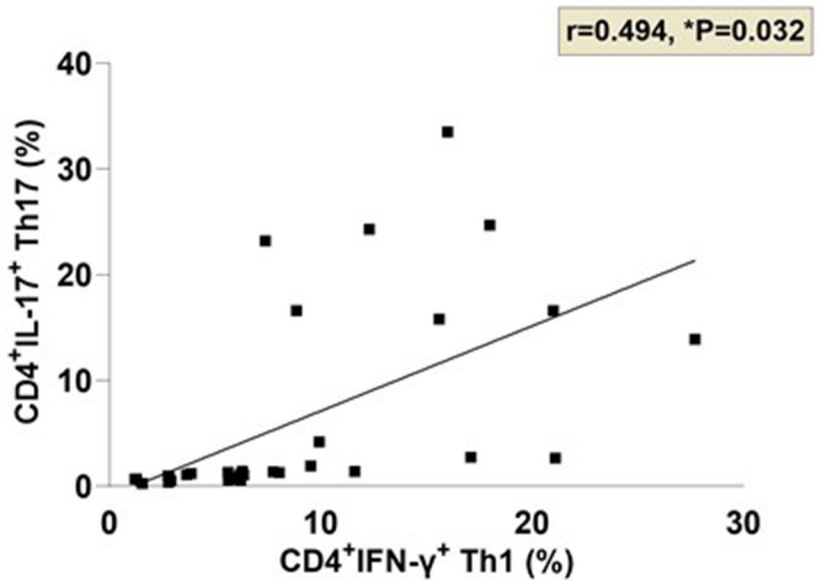

R1
82.2%
SSC-H:: Side Scatter
FSC-H:: Forward Scatter
0
200
400
600
800
1K

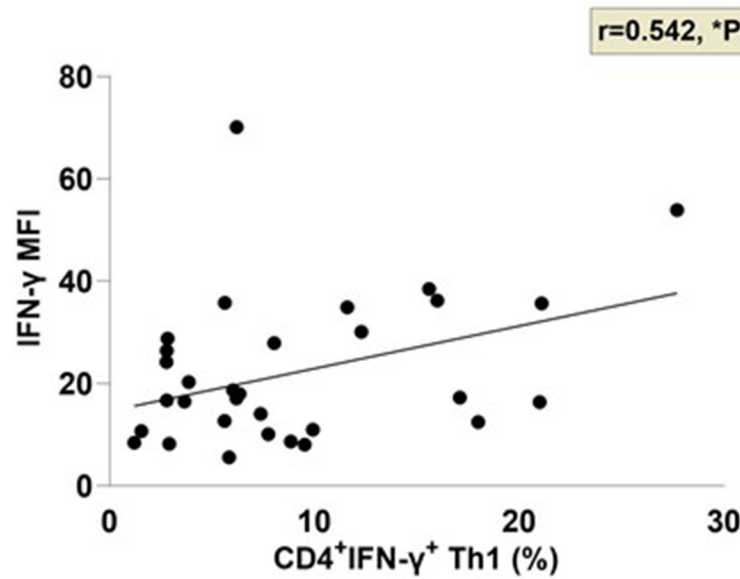

Q1
35.4%
Q2
2.32%
Th1
Q4
43.3%
Q3
18.9%
FL1-H:: IFN-g FITC
FL3-H:: CD4 PerCP-Cy5.5

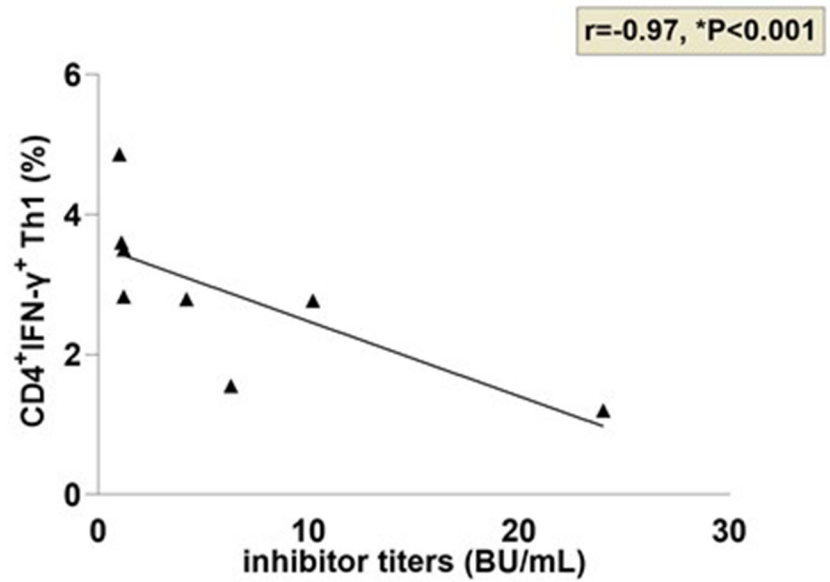

Q5
0.485%
Q6
1.29%
Th17
Q8
77.1%
Q7
21.1%
FL2-H:: IL-17 PE
FL3-H:: CD4 PerCP-Cy5.5


P=0.9
P=0.059
15
10
5
0
Frequency (%)
HA patients
Healthy controls
CD4+IFN-γ+ Th1
CD4+IL-17+ Th17


P=0.178
P=0.003
P=0.014
P=0.157
P=0.462
P=0.094
20
15
10
5
0
Frequency (%)
Inhibitor +
Inhibitor -
HC
CD4+IFN-Y+ Th1
CD4+IL-17+ Th17


r=0.494, *P=0.032
CD4+IL-17+ Th17 (%)
CD4+IFN-γ+ Th1 (%)
0
10
20
30
40


r=0.542, *P=0.016
IFN-γ MFI
CD4+IFN-γ+ Th1 (%)
0
20
40
60
80


r=-0.97, *P<0.001
CD4+IFN-γ+ Th1 (%)
inhibitor titers (BU/mL)
0
2
4
6