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Immunophenotyping of A20 haploinsufficiency by multicolor flow cytometry

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ABSTRACT

Haploinsufficiency of A20 (HA20) causes inflammatory disease resembling Behçet's disease; many cases have been reported, including some that are complicated with autoimmune diseases. This study aims to clarify the immunophenotype of patients with HA20 by analyzing lymphocyte subsets using multicolor flow cytometry. The patients with HA20 previously diagnosed in a nationwide survey were compared by their cell subpopulations. In total, 27 parameters including regulatory T cells (Tregs), double-negative T cells (DNTs), and follicular helper T cells (TFHs) were analyzed and compared with the reference values in four age groups: 0–1, 2–6, 7–19, and ≥20 years. The Tregs of patients with HA20 tended to increase in tandem with age-matched controls at all ages. In addition, patients ≥20 years had increased DNTs compared with controls, whereas TFHs significantly increased in younger patients. In HA20 patients, the increase in DNTs and TFHs may contribute to the development of autoimmune diseases.

1. Introduction

A20, the protein encoded by the *TNFAIP3* gene, is a negative regulator of the tumor necrosis factor (TNF)-nuclear factor (NF)-κB signaling pathway. A20 may also regulate the JAK-STAT signaling pathway via modulation of signal transducer and activator of

Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; CRP, C-reactive protein; CTLA4, cytotoxic T lymphocyte antigen 4; DNT, double-negative T cell; EBV, Epstein–Barr virus; HA20, haploinsufficiency of A20; ICOS, inducible T cell co-stimulator; iNKT, invariant natural killer T; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, and X-linked; LRBA, lipopolysaccharide-responsive and beige-like anchor protein; NF, nuclear factor; NK, natural killer; PBMC, peripheral blood mononuclear cell; PIDJ, Primary Immunodeficiency Database in Japan; PSL, prednisolone; RTE, recent thymic emigrant; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription; Tc, cytotoxic T; TCM, central memory T cell; TEM, effector memory T cell; TFH, follicular helper T cell; Th, helper T; TNF, tumor necrosis factor; Treg, regulatory T cell

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transcription (STAT) 1 expression [1]. Haploinsufficiency of A20 (HA20) results from a defect in the *TNFAIP3* gene that causes an early-onset autoinflammatory disorder that resembles Behçet's disease; HA20 promotes aberrant activation of the NF-κB signaling pathway and increased production of proinflammatory cytokines [2]. Numerous cases of HA20 have been described in the literature [3–12]; some cases were directly associated with autoimmune diseases, including systemic lupus erythematosus (SLE) [2], type 1 diabetes [7], autoimmune thyroiditis [2,3,5,8], idiopathic thrombocytopenic purpura [2], nephrotic syndrome [3], and autoimmune hepatitis [3]. Other cases of HA20 were associated with specific immunodeficiency states, including immunoglobulin deficiency and persistent Epstein–Barr virus (EBV) infection [3,10,13]. Because the clinical features of HA20 are quite varied, it would be helpful to have a more focused and comprehensive view of the immunopathology associated with this disorder.

We recently reported that lymphocyte subset analysis with multicolor flow cytometry is useful for the diagnosis and pathological analysis of disorders of immune regulation [14]. In this study, we performed multicolor flow cytometry to determine the immune status and immunological characteristics of peripheral blood lymphocytes from 18 patients diagnosed with HA20.

2. Materials and methods

2.1. Patients and diagnosis of HA20

Our study included all volunteers regardless of age or sex from the cohort of patients diagnosed with HA20 who were enrolled in the Primary Immunodeficiency Database in Japan (PIDJ). HA20 was diagnosed with genetic and functional *in vitro* analyses. The current study included 16 patients from our previous cohort and two newly diagnosed patients, one of whom was added from a new independent family group [3,15,16].

2.2. Serum immunoglobulin levels

Serum IgG, IgG2, IgA, IgM, IgD, and IgE levels in patients with HA20 were evaluated and compared with age-matched historical controls that provide Japanese standards for these values [17–19]. IgG, IgA, and IgM were measured by a latex agglutination turbid metric assay, whereas IgG_2 was evaluated by turbid metric immunoassay. IgD was evaluated by latex agglutination, and IgE, by chemiluminescent enzyme immunoassay.

2.3. Multicolor flow cytometry analysis

Peripheral blood samples from patients with HA20 were analyzed by multicolor flow cytometry using a BD LSRFortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Lymphoprep (Axis-Shield Diagnostics Ltd., Dundee, Scotland) gradient centrifugation. Lymphocytes were analyzed via six panel designs, including PBMC, T1, T2, T3, B1, and B2 panels. The PBMC panel was designed to identify T cells ($CD3^+CD19^-$), B cells ($CD3^-CD19^+$), and natural killer (NK) cells ($CD16^+CD56^+$) within the lymphocyte gate. The aforementioned $CD3^+$ T cells were subdivided into $CD4^+$ helper T (Th) cells and $CD8^+$ Tc cells. Naïve Th and Tc cells were defined with the antigen profiles $CD3^+CD4^+CD45RA^+CD45RO^-$ and $CD3^+CD8^+CD45RA^+CD45RO^-$, respectively. Memory B cells were identified as $CD19^+CD27^+$ cells within the lymphocyte gate.

The T1 panel was designed to identify invariant natural killer T (iNKT) cells ($CD3^+TCRV\alpha24^+V\beta11^+$) and recent thymic emigrants (RTEs: $CD3^+CD4^+CD45RA^+CD31^+$). Moreover, memory Th cells ($CD3^+CD4^+CD45RA^-CD45RO^+$) were subdivided into $CD4^+$ central memory T cells (TCMs: $CCR7^+CD62L^+$) and $CD4^+$ effector memory T cells (TEMs: $CCR7^-CD62L^-$). Memory Tc cells as defined above ($CD3^+CD8^+CD45RA^-CD45RO^+$) were subdivided into $CD8^+$ TCMs and $CD8^+$ TEMs using the aforementioned surface markers.

The T2 panel was designed to identify αβ T cells ($CD3^+TCR\alpha\beta^+$) and γδ T cells ($CD3^+TCR\gamma\delta^+$). Double-negative T cells (DNTs) were defined as the $CD4^-CD8^-$ population within the αβ T cell gate. Furthermore, regulatory T cells (Tregs) were defined as the $CCR4^+CD25^+CD127^{low}$ population within the Th cell gate.

The T3 panel was designed to identify Th1 cells ($CCR6^-CXCR3^+$), Th2 cells ($CCR6^-CXCR3^-$), and Th17 cells ($CCR6^+CXCR3^-$) within the $CD3^+CD4^+CD45RO^+$ cell gate. Moreover, follicular helper T cells (TFHs) and activated T cells were defined as $CD45RO^+CXCR5^+$ and $CD38^+HLA\text{-}DR^+$ populations within the Th cell gate, respectively.

Within the $CD19^+$ B cell gate, the B1 and B2 panels were designed to identify transitional B cells ($CD24^{bright}CD38^{bright}$), plasmablasts ($CD24^-CD38^{bright}$), IgM memory B cells ($CD27^+IgM^+IgD^+$), and switched memory B cells ($CD27^+IgM^-IgD^-$). The details of the lymphocyte surface markers, fluorescent antibodies, staining method, and the normal range of control subjects are described in full in our previous publication [14].

2.4. Ethics

Written informed consent was obtained from patients or their guardians. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics boards of Gifu University and Tokyo Medical and Dental University.

2.5. Statistical analysis

All data were analyzed by Student's *t*-test using Prism 7 (GraphPad Software, San Diego, CA); $p < .05$ was considered statistically significant.

3. Results

3.1. Patients characteristics

Table 1 includes the characteristics of patients diagnosed with HA20 who participated in this study; these variables included the sex and age at the time of this study together with information on specific *TNFAIP3* gene mutations, ongoing treatment with immunomodulatory agents, levels of serum C-reactive protein (CRP), autoantibodies detected, and developed autoimmune diseases. The *TNFAIP3* gene mutations and their impact on the domain structure of the A20 protein are shown in Supplementary Appendix Fig. 1. In total, 18 participants were enrolled with an age range of 9 months to 69 years old. Five patients (27.8%) were undergoing treatment with biologicals including anti-TNF-α agents or rituximab at the time of analysis. In addition, seven patients (38.9%) had autoantibodies and/or were diagnosed with autoimmune diseases including Hashimoto's disease, nephrotic syndrome, and SLE.

3.2. Serum immunoglobulin analysis

Serum immunoglobulin levels of patients diagnosed with HA20 are shown in Table 2. There were no cases that showed a reduction in immunoglobulin, with the exception of two cases (P7 and P9) in which IgA and IgM decreased, but were not deficient. By contrast, serum IgG, IgA, and IgM levels were above the normal limits in nine (50.0%), six (33.3%), and one (5.6%) patient, respectively, compared with age-matched historical controls. Interestingly, serum IgE levels were above normal limits in 11 (61.1%) HA20 patients compared with age-matched controls.

3.3. Multicolor flow cytometry analysis using the PBMC panel

Analysis of lymphocyte subpopulations using the PBMC panel is

Table 1
Characteristics of patients with HA20.

Patient No. ([a])	Sex	Age at the time of analysis	*TNFAIP3* mutation	Immunomodulatory treatment at the time of analysis	CRP level (mg/dL) at the time of analysis	Autoantibody	Developed autoimmune disease
P1 (1)	female	17 y	c.252delC	Adalimumab + PSL 7 mg + MTX + NSAID	0.04	No	No
P2 (2)	female	38 y	c.252delC	PSL 5 mg	0.12	Low titer anti-TPO antibody	No
P3 (3)	male	3 y 9 mo	c.2088 + 5G > C	Etanercept + PSL 5 mg + MTX + NSAID	2.07	No	No
P4 (4)	female	4 y 6 mo	c.2209delC	Etanercept (twice a month)	0.04	No	No
P5 ([b])	female	9 mo	c.2209delC	No treatment	0.45	No	No
P6 (5)	female	28 y	c.2209delC	No treatment	1.13	Anti-TPO antibody	Hashimoto's disease
P7 (6)	female	69 y	c.2209delC	No treatment	0.09	Anti-Tg antibody	Hashimoto's disease
P8 (8)	female	9 y 6 mo	c.2209delC	No treatment	4.27	Anti-Tg antibody	Hashimoto's disease
P9 (9)	female	2 y 8 mo	c.2209delC	Colchicine	0.03	No	No
P10 (10)	male	6 y	c.1906 + 1G > A	Cimetidine + MTX	4.34	No	No
P11 (11)	male	33 y	c.1906 + 1G > A	No treatment	0.59	Not analyzed	No
P12 (12)	male	19 y	c.728G > A	Rituximab + MZB + CyA + PSL 5 mg + Colchicine	0.48	No	Nephrotic syndrome
P13 (17)	female	7 y 7 mo	c.1345delA	No treatment	1.72	No	No
P14 (19)	male	4 y 6 mo	c.1760_1770del11	No treatment	4.88	Not analyzed	No
P15 (20)	male	33 y	c.1760_1770del11	No treatment	0.18	Anti-Tg antibody Anti-TPO antibody TSH receptor antibody	Graves' disease
P16 (21)	male	1 y 3 mo	c.1245_1248del4	CyA + PSL 4 mg	2.73	Anti-DNA antibody Anti-GBM antibody	SLE, AIH, ALPS-U, Nephrotic syndrome
P17 (22)	male	7 y 11 mo	c.133C > T	Colchicine	0.9	No	No
P18 ([b])	male	11 y	deletion of exon 2 and 3	Infliximab	7.39	No	No

AIH = autoimmune hepatitis, ALPS-U = autoimmune lymphoproliferative syndrome undefined, Anti-GBM antibody = anti-glomerular basement membrane antibody, Anti-Tg antibody = anti-thyroglobulin antibody, Anti-TPO antibody = anti-thyroid peroxidase antibody, CyA = cyclosporine A, MTX = methotrexate, MZB = mizoribine, NSAID = nonsteroidal anti-inflammatory drug, PSL = prednisolone, SLE = systemic lupus erythematosus.

[a] Patient number in our previous report [3].

[b] Additional patients not included in our previous report.

Table 2
The serum immunoglobulin data of HA20 patients.

Patient No.	IgG (mg/dL)	IgG2 (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	IgD (mg/dL)	IgE (IU/mL)
[a]9 months of age	360–1010	50.8–224.0	10–56	55–200	≤ 12	< 10
P5	615	116	36	113	< 0.6	**12.4**
[a]1 year of age	465–1215	62.2–275.1	15–113	69–287	≤ 12	< 20
P16	**2432**	**312**	72	154	< 0.6	**60.5**
[a]2 years of age	500–1280	58.5–292.1	19–136	72–297	≤ 12	< 20
P9	696	167	*17*	88	< 0.6	< 5.0
[a]3 years of age	535–1340	58.5–292.1	24–167	75–306	≤ 12	< 40
P3	**1784**	233	**413**	186	1.6	**991**
[a]4 years of age	565–1395	106.4–381.9	29–190	78–315	≤ 12	< 40
P4	692	336	54	171	1.1	**2360**
P14	1374	177	**248**	122	< 0.6	**364.1**
[a]6 years of age	640–1510	110.4–412.5	42–248	82–329	≤ 12	< 100
P10	**1644**	226	195	248	0.2	40
[a]7 years of age	670–1560	110.4–412.5	48–276	85–337	≤ 12	< 100
P13	1380	238	**280**	103	**16.7**	**402**
P17	**1873**	**519**	**322**	146	< 0.6	**322**
[a]9 years of age	715–1625	147.7–459.9	56–314	86–341	≤ 12	< 100
P8	**1871**	377	314	203	< 0.6	**1310**
[a]11 years of age	760–1685	190.3–501.7	65–349	87–346	≤ 12	< 100
P18	1642	303	348	**570**	< 0.6	11.8
[a]Adult	680–1620	208–754	84–438	57–288	≤ 12	< 170
P1	**1893**	652	417	139	1.2	**341**
P2	**1748**	644	364	192	8	**451**
P6	1558	509	**455**	102	< 0.6	94.5
P7	1237	*201*	258	*37*	**69.9**	19.4
P11	**1648**	384	397	132	**50.3**	142
P12	**1623**	225	**574**	104	1.3	**19741**
P15	1392	257	220	65	< 0.6	32.2

If the value is higher than normal range, it is shown in bold type, and if it is lower, it is shown in italic type.

[a] Normal range at each age (2.5–97.5% tile).

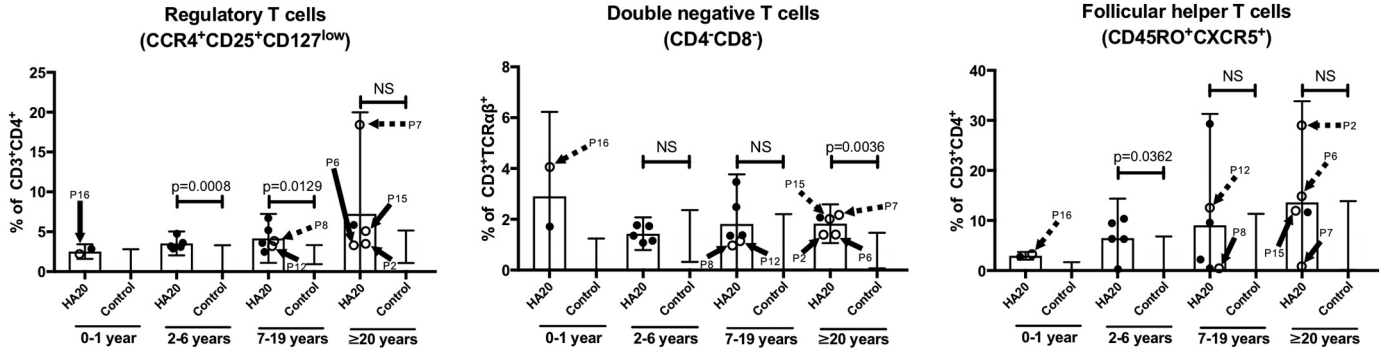

Fig. 1. Multicolor flow cytometry analysis of the regulatory T cells, double-negative T cells, and follicular helper T cells.
The bottom of each figure shows the age range based on previous reports [14]; the vertical axis indicates the cell proportion. The error bars denote ± 2 standard deviations (SD). Open circles indicate the patients who are autoantibody-positive and/or developed autoimmune disease. Each arrow shows the patient number of the open circle, and among these, the dotted arrows indicate the cases with proportions that are equal to or greater than two standard deviations (≥2 SD) from the values from the age-matched controls. As shown, more regulatory T cells were detected in peripheral blood of patients with HA20 in the 2–6 year and 7–19 year age brackets. The double-negative T cells tended to be higher among patients diagnosed with HA20 who were ≥ 20 years old, whereas follicular helper T cells were higher among the HA20 patients at 2–6 years old.

shown in Supplementary Appendix Fig. 2. The bottom of each figure shows the range of ages as described in our previous report [14]; the vertical axis presents the cell proportion. The error bars denote ± 2 standard deviations (SD), and the open circles denote the patients who were autoantibody-positive and/or who were diagnosed with autoimmune diseases. Among our findings, we detected a higher proportion of Tc cells among HA20 patients in the 2–6 and 7–19 year age brackets compared with those detected among the age-matched controls. In addition, we detected lower levels of naïve Th cells among HA20 patients in groups except within the 0–1 year age bracket. We also noted an increase in the proportion of memory B cells in three out of 18 patients, although this finding did not reach statistical significance. Finally, we detected a significant decrease in NK cells among the HA20 patients in the ≥20 years of age.

3.4. Multicolor flow cytometry analysis using the T1, T2, and T3 panels

Comparisons among the proportions of Tregs, DNTs, and TFHs are shown in Fig. 1; other results from the T1, T2, and T3 flow cytometry panels are included in Supplementary Appendix Figs. 3, 4, and 5, respectively. Among our findings, the proportion of Tregs among patients diagnosed with HA20 tended to be higher than those among the age-matched controls at all age groups; these differences reached statistical significance in the 2–6 and 7–19 year age brackets (Fig. 1). As steroids may have the immunomodulating effects for Tregs, additional analyses were performed after exclusion of the five patients who were undergoing treatment with prednisolone (PSL). Interestingly, the increase in the proportion of Tregs remained after these cases were excluded (Supplementary Appendix Fig. 6). An increase in the proportion of DNTs was observed in seven out of 18 patients, and statistically significant differences were detected between the HA20 patients and the age-matched controls among those in the ≥20 years age bracket. Finally, we observed that the proportion of TFHs had a tendency to increase, especially among the younger HA20 patients. Moreover, all patients who were autoantibody-positive or who were diagnosed with an autoimmune disease as a comorbidity (except for P8), had higher proportion of DNT and/or TFH of more than two SD from the values determined for the age-matched control cohorts. There were no significant differences in the Th1 and Th2 cell proportions between the HA20 patients and age-matched controls (Supplementary Appendix Fig. 5). Interestingly, three out of 18 patients with HA20 showed a higher proportion of Th17 cells than the ± 2 SD value of age-matched control group, but no statistically significant differences were identified.

3.5. Multicolor flow cytometry analysis using the B1 and B2 panels

The results from the B1 and B2 panels are included in Supplementary Appendix Figs. 7 and 8, respectively. There were no significant differences when comparing transitional B cells and plasmablasts between HA20 patients and controls (Supplementary Appendix Fig. 7). However, the proportions of IgM memory B cells and switched memory B cells in HA20 patients were significantly decreased in ≥20 years age and 2–6 age bracket compared with age-matched control, respectively (Supplementary Appendix Fig. 8).

3.6. Multicolor flow cytometry analysis of the Tregs, TFHs, and DNTs in the remission and during disease flares

The lymphocyte profiles reported here for HA20 patients might vary in response to disease activity; as such, the HA20 patients were divided into those experiencing a disease flare and those in remission. The flare phase was defined as those with serum CRP levels > 1.0 mg/dL; those with serum CRP levels < 1.0 mg/dL were defined as in remission. This resulted in 8 and 10 patients in the flare group and the remission group, respectively. Interestingly, even during the remission phase, the proportion of Tregs was significantly elevated among HA20 patients in the 7–19 year age bracket (Supplementary Appendix Fig. 9). The proportion of DNTs among HA20 patients was also significantly increased in ≥20 years age bracket compared with age-matched control during this phase. By contrast, the proportion of TFHs was significantly increased in among the 2–6 year-old patients during a disease flare (Supplementary Appendix Fig. 10).

4. Discussion

In this study, we performed immunophenotyping of PBMCs from patients diagnosed with HA20. Increases in the proportions of Tregs, TFHs, and DNTs, and the decrease in the proportion of naïve Th cells were observed among HA20 patients compared with those identified among age-matched controls. It is critical to note that the patients who participated in this study, as well as those featured in previous reports, had not only autoinflammatory findings but also autoimmune diseases, and/or persistent EBV infection.

Tregs are essential for self-tolerance, and they play a key role in controlling the development of an allergic response or autoimmune disease. However, there is currently no literature on the relationship between HA20 and Tregs. Increased numbers of Tregs were reported in association with autoinflammatory syndromes including the active phase of familial Mediterranean fever and Behçet's disease [20,21].

Moreover, increases in Tregs have also been reported in other chronic inflammatory conditions such as rheumatoid arthritis [22]. Therefore, it is speculated that the increase in Tregs in these diseases occurs as a suppressive response against inflammation. In support of this hypothesis, an increase in Tregs was observed in both *A20*$^{-/-}$ [23,24] and *A20*$^{+/-}$ [23] mice, which are findings that are consistent with our results. There is at least one publication that reports an increase in Tregs in response to treatment with PSL [25]. We considered the possibility that our findings might be directly related to PSL administration; however, the results were no different once these five HA20 patients were excluded from the analysis. It is notable that HA20 is shown to cause not only autoinflammatory symptoms but also an autoimmune disease and immunodeficiency symptoms [3–6,8,9,11,13,26]. Primary immunodeficiency syndromes complicated with autoimmune diseases include the condition known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, *STAT3* gain of function disease, and cytotoxic T lymphocyte antigen 4 (CTLA4) haploinsufficiency; these are all immunoregulatory disorders associated with Treg deficiency. Thus, the characteristic increase in Tregs is a critical and essential feature for differentiation of HA20 from other disorders of immune regulation.

Factors underlying the association between HA20 with autoimmune disease may include impaired apoptosis and an increase in DNTs, which are the progenitors of mature T cells. Some DNTs appear in peripheral blood, having avoided negative selection in the thymus [27]. DNTs have been reported to be capable of proliferation and infiltration into inflamed tissues in association with autoimmune and inflammatory conditions such as SLE, Sjögren's syndrome, and psoriasis [28]. In a previous report, mice with a selective loss of A20 in dendritic cells (*TNFAIP3*$^{fl/fl}$Cd11c-cre^{+} mice) developed apoptotic disorders and a concomitant increase in DNTs [29]. Autoimmune lymphoproliferative syndrome (ALPS) is a primary immunodeficiency that is associated with a significant increase in DNTs; ALPS is associated with disordered apoptosis due to abnormalities in genes including *FAS*, *FASLG*, and *CASP10*; these deficiencies result in a failure of negative selection during lymphocyte differentiation. For this reason, ALPS results in lymphoproliferation, including DNTs, lymphoma, and complications of autoimmune disease via an increase in autoantibody-producing B cells. The current study included a patient diagnosed with ALPS-U (ALPS of undetermined genetic basis) in association with chronic lymphadenopathy, splenomegaly, increased DNTs, and elevated levels of interleukin (IL)-10, soluble FAS ligand, and IL-18 [30]. Our study also featured six additional cases where the DNT proportion was more than two SD away from those of the age-matched controls; two of these cases were complicated by autoimmune thyroid disease. In HA20, A20 dysfunction induces activation of the TNF-NF-κB signaling pathway, which results in the overproduction of proinflammatory cytokines. By contrast, one might anticipate suppressed apoptosis among patients with HA20 as previous reports suggest that A20 regulated apoptosis induction [31]. Taken together, we consider the possibility that HA20, similar to ALPS, might be associated with an increase in DNTs and autoantibody-producing B cells as a result of a disorder of apoptosis, which may serve to complicate the course of autoimmune disease. Biallelic somatic mutations in the *TNFAIP3* gene have been found in patients with B cell lymphoma [32]. Although we described one HA20 case associated with Hodgkin's disease in a previous report [3], it remains unclear whether individuals diagnosed with HA20 are predisposed to develop lymphoma.

TFHs localize at the germinal center of lymphoid follicles and induce immunoglobulin production from B cells by producing IL-21 [33,34]. Sanroque mice [35] have a mutation in the gene encoding Roquin that serves as a repressor of inducible T cell co-stimulator (ICOS), thereby resulting in the overexpression of ICOS; high levels of ICOS will induce TFH differentiation, which can lead to an overproduction of IL-21, germinal center hyperplasia, and SLE-like symptoms. In addition, embryonic germinal center hyperplasia could be induced by transferring TFHs derived from sanroque mice into wild-type mice [36]. Taken together, these results suggest a relationship between autoimmune disease and the biology of TFHs. In humans, it has been shown that TFHs are present at high levels in the peripheral blood of patients with SLE, Sjögren's syndrome, rheumatoid arthritis, dermatomyositis, Graves' disease, Hashimoto's disease, and type 1 diabetes [37–41]. Furthermore, the proportion of TFH to PBMCs or to Th cells correlated with the disease activity, organ failure, and autoantibody titers [37,38]. Increases in TFH are also associated with deficiencies of lipopolysaccharide-responsive and beige-like anchor protein (LRBA) and CTLA4 [42]. As described above, these diseases are all associated with Treg dysfunction as decreased expression of CTLA4 ultimately results in an increase in TFHs. These diseases which are all known to be associated with multiple autoimmune diseases and are also called ALPS-V. In patients with HA20, the activation of the TNF-NF-κB signaling pathway results in the increased production of IL-12, which is a growth factor for TFH. Taken together, we conclude that HA20 may promote pathology associated with autoimmune disease secondary to increasing proportion of TFHs and which leads to increased immunoglobulin production from B cells.

Previous reports have documented increased proportion of Th17 cells in patients diagnosed with HA20 and were specifically associated with the development of autoimmune disease [2,3]. Interestingly, our study did not feature any increases in the proportion of Th17 cells; this might be attributed to differences in the staining methods. We defined Th17 cells by the antigen profile CD3^{+}CD4^{+}CCR6^{+}CXCR3^{-}, whereas previous studies have defined these cells as intracellular IL-17A^{+} upon detection of the cytokine in permeabilized cells that were treated with phorbol myristic acid and ionomycin. It is also possible that specific drug treatments may have an influence on the Th17 cell profile. As such, it is necessary to accumulate a sufficient number of cases in order to generate a comprehensive understanding of the differences in the Th17 cell population.

In this study, the HA20 patients displayed no immunoglobulin deficiencies and exhibited no significant decrease in the proportions of B cell subsets. However, in a previous report that featured mice with selective loss of A20 in B cells, results included a decrease in memory B cells and diminished levels of IgG1 and IgG3 [23]. Furthermore, there are reports of HA20 patients with immunoglobulin subclass deficiency [13]. For this reason, further investigation of the impact of HA20 on B cell development and differentiation is most certainly warranted.

Recently, a case of HA20 associated with persistent EBV infection was reported [10]. Among our patient cohort, P14 also had persistent EBV infection in childhood. These results suggest that the immune response to EBV may be limited in patients with HA20. The results of immunophenotyping indicate that the proportion of NK cells is reduced among patients in the ≥20 years age bracket, although this finding cannot explain the childhood persistent EBV infection. Recently, our colleagues reported that *CTLA4* mutations might be associated with EBV viremia [43]. CTLA4 deficiency induces hyperactivation of signaling pathways such as NF-κB and PI3K-Akt due to impaired suppression of T cell activation [44,45]. Activation of these pathways led to T cell exhaustion and senescence, which could lead to impaired T cell function and a decrease in naïve Th cells [43,46,47]. In HA20 patients, over-activation of the NF-κB signaling pathway may also cause T cell exhaustion and senescence and thereby facilitate persistent EBV. In fact, P14 had a typical NK cell proportion (7.5%), whereas he showed a small decrease in naïve Th cells (54.3%). Interestingly, he also showed a significant decrease in CD4^{+} TCMs (2.95%) and a significant increase in CD4^{+} TEMs (63.1%). We did not explore EBV clearance in this study. We will need to identify more patients with HA20 and persistent EBV infection in order to perform a comprehensive and detailed analysis of leukocyte subsets associated with this disorder.

The main limitation of this study is that the number of patients was small and notably so in the cohort of those under 2 years of age. Furthermore, a number of our patients were undergoing treatment with

immunomodulatory agents at the time of analysis. Moreover, the timing of blood samples included both stable periods and periods of disease flares.

In conclusion, the increase in the proportion of Tregs observed among patients diagnosed with HA20 may be a regulatory response to autoinflammation. Increases in the proportions of DNTs and TFHs were also observed and might be associated with the development of autoimmune diseases. Defects of A20 can cause an ALPS-like immunophenotype in the absence of Treg deficiency. However, given the protean role of A20 in disease and at homeostasis, further detailed analysis will be needed in order to evaluate the quality and the function of Tregs and their role in promoting the pathogenesis of HA20 patients.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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