Autoimmune Hepatitis and Autoimmune Hepatitis Overlap With Sclerosing Cholangitis: Immunophenotype Markers in Children and Adolescents

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ABSTRACT

Objective: The pathophysiology of autoimmune hepatitis (AIH) may involve the activation of immune cells and changes in the expression of cellular markers. The aim of the present study was to characterize the immunophenotype markers of lymphocytes and monocytes in the peripheral blood of children and adolescents with type 1 AIH and AIH overlap with sclerosing cholangitis (overlap syndrome [OS]).

Methods: This is a cross-sectional study of 20 children and adolescents diagnosed with type 1 AIH and 19 with OS. Fifteen healthy subjects were included as controls. Flow cytometric analysis was used to identify markers of inflammation and autoimmunity.

Results: The total number of $CD4^+$ T cells was higher in the AIH patients compared with the controls. The number of $CD4^+$ T cells expressing CCR3 and CD28 was higher in the AIH group than in the control group. CD45RO was more highly expressed in the AIH group, whereas CD45RA was more highly expressed in the OS group. In regard to $CD8^+$ T lymphocytes, the CCR3 expression was higher in both groups of patients. Patients with OS had the highest expression of CD45RA and CD25. In monocytes, human leukocyte antigen DR (HLA-DR) was less expressed in both groups of patients.

Conclusions: Complex phenotype features may be involved in the pathophysiology of AIH, accounting for changes in immune system regulation mechanisms. In conclusion, even after good response to treatment, patients still have immune activity signals at the cellular level.

Key Words: autoimmune disease, autoimmune hepatitis, children, immunophenotype

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What Is Known

- The pathophysiology of autoimmune hepatitis and autoimmune hepatitis overlap with sclerosing cholangitis involves activation of immune cells and changes in the expression of cellular markers.
- Autoimmune hepatitis and overlap syndrome have genetic associations with HLADR subtypes.
- $CD4^+$ T cells have important role in the autoimmune mechanism.

What Is New

- Patients with autoimmune hepatitis and patients with overlap syndrome exhibit persistent activation of immune system cells despite clinical and laboratory response.

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disease more prevalent in females between 10 and 30 years of age (1–3). AIH is characterized with positive autoantibodies including antinuclear antibodies (ANAs), antismooth muscle antibody (ASMA) and antiliver/kidney microsome type 1. Patients with AIH also present with increased transaminases and immunoglobulin G (IgG) (3). Histologically, AIH is characterized by interface hepatitis (3), developing hepatic regeneration with ''rosette'' formation, and piecemeal necrosis with periportal/periseptal lymphocyte infiltrates (4). Clinically, AIH ranges from asymptomatic disease to hepatic failure (2,3,5,6). AIH overlap with sclerosing cholangitis (overlap syndrome [OS]) is more common among children and adolescents, including almost 30% to 50% of the pediatric hepatitis population. OS is characterized by AIH with features of primary sclerosing cholangitis (2,3,7,8).

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The susceptibility to AIH has been associated with genes related to major histocompatibility complex II, and specifically the human leukocyte antigen (HLA) genes, including *HLA-DR3*, *HLA-DR4*, and *HLA-DRB1*1301* (9,10). The pathophysiology of AIH involves activation of lymphocytes, mostly T helper but also B lymphocytes, macrophages, and natural killer cells (11,12). Changes in the activation or functional state of immune cells may lead to an imbalance of the immune system, resulting in increased production of cytokines and loss of regulatory mechanisms (13–17). Despite the understanding of several pathways related to AIH, more studies are still needed to investigate the relationship between immune markers and the clinical course of AIH.

The purpose of the present study was to characterize immunophenotype markers of lymphocytes and monocytes of patients with AIH in comparison to healthy individuals. We also divided AIH patients in two groups according to presence or absence of sclerosing cholangitis. In addition, we have only included patients with good control of the disease to investigate whether immune system changes can still be detected.

PATIENTS AND METHODS

Patients

Among a group of 134 patients with AIH, we selected 39 children and adolescents up to the age of 18 years at diagnosis who had a good response to treatment with prednisone and azathioprine. Good therapeutic response in AIH was defined as normalization of aminotransferases and serology, and, in OS, it was defined as normalization of aminotransferases and reduction in gamma-glutamyl transferase (GGT).

The result was a cross-sectional cohort of 39 pediatric patients with AIH, 20 of whom had type 1 AIH and 19 of whom had AIH overlap with sclerosing cholangitis (OS). Patients were followed-up from January 1986 to January 2014 at a Reference Center of Pediatric Hepatology (Hospital das Clínicas, Universidade Federal de Minas Gerais).

Controls

We also included 15 healthy individuals who were age and sex matched with the patients to serve as a control group. Health status was determined through the subjects' medical history and either a parental report or self-report to rule out the presence of autoimmune diseases.

The study was approved by the National and Local Ethics Committee (ETIC number 0419.0.203.000-10). The consent form was read and signed by each of the researchers and patients.

Diagnosis of AIH was established according to the criteria of the International Group for the Study of AIH, published in 1993 and revised in 1999 and 2008 (1,2). Diagnosis of OS was based on the presence of abnormalities in the biliary tract, including stenosis and/or dilatation of the intra- and/or extrahepatic ducts on magnetic resonance imaging (MRI) of the biliary tract. MRI was performed in patients with persistent elevation of GGT and/or poor response to immunosuppressive treatment. Three pediatric hepatologists and 2 radiologists independently reviewed the biliary tract images. Liver histology was also assessed.

Clinical and Laboratory Monitoring

Follow-up consisted of clinical and laboratory evaluations every 1 to 6 months, according to the protocol and patients' needs. Patients were classified according to the type of AIH (type 1, if ANA positive and/or ASMA positive, and type 2 when the anti-liver/kidney microsome type 1 was positive) and the severity of AIH or OS.

Laboratory findings included complete blood count, prothrombin reaction time, prothrombin activity, activated partial thromboplastin time, electrophoresis of plasma proteins, and serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, GGT, and bilirubin. Other causes of liver diseases were excluded. Liver biopsies were performed in all patients for disease staging. MRI and histological findings confirmed OS.

Immunophenotype Markers

All patients selected for the present study had complete response to treatment, fulfilling the criteria of remission of the disease. Immune marker evaluations did not interfere with the treatment. The profile of immune markers was not compared before and after treatment. To evaluate immunophenotype markers, a total of 10 mL of peripheral venous blood was collected in heparinized tubes from each participant on 1 occasion.

Flow cytometry analysis was performed as described elsewhere (18). Mononuclear cells were obtained from peripheral blood using a Ficoll gradient (Sigma Chemical Co., St. Louis, MO). Cells were stained with monoclonal antibodies for the markers to be analyzed with fluorochromes. Samples were acquired in a flow cytometer BD FACSCanto II (BD Biosciences, San Jose, CA) and analyzed by FlowJo software 7.5 (FlowJo Co, Ashland, OR). Percentages of $CD4^+$ and $CD8^+$ lymphocytes and of $CD14^+$ monocytes were evaluated, as was the percentage of expression of the different surface markers. The surface markers analyzed included CD45RA, CD45RO, CTLA-4, CD69, HLA-DR, CD28, CD40L, CD25, CD95, CD95L, CCR3, CCR5, and CD80, and the antibodies used were anti-CD4-APCCy7, anti-CD4-Cy7, anti-CD8-FITC, anti-CD8-APC, anti-CD14-FITC, anti-CD45RA-FITC, anti-CD45RO-PE, anti-CTLA4-Cy5, anti-CD69-Cy7, anti-CD28-PE, anti-CD40L-Cy5, anti-HLA-DR-Cy7, anti-CD95L-PE, anti-CD95-Cy5, anti-CD25-Cy7, anti-CCR3-PE, anti-CCR5-Cy5, anti-CD80-Cy5, and anti-CD86-APC.

Treatment Protocol

The treatment consisted of daily doses of prednisone (1–2 $mg \cdot kg^{-1} \cdot day^{-1}$, maximum 60 mg/day) and azathioprine (1.5 $mg \cdot kg^{-1} \cdot day^{-1}$, maximum 100 mg/day) (4,5). After 4 weeks of treatment, the dose of prednisone was reduced by one third of the initial dose and then, every 2 weeks, the dose was decreased by 25% until reaching 5 mg, according to clinic protocol. Low-dose prednisone (5 mg) was maintained in cases of clinical and laboratory remission. Azathioprine was maintained at the initial dose. For patients with leukopenia (leukocyte overall <3000) and/or thrombocytopenia ($<50{,}000$), only prednisone was administered. Azathioprine was only added to treatment if there was improvement in the hematological parameters. Treatment response was evaluated according to the International Group for the Study of AIH criteria (1).

Statistical Analysis

Statistical analysis was performed using SPSS 17 (IBM Co, New York). Descriptive analysis, using mean, median, standard deviation, interquartile range (25–75th) and percentages were used to characterize the study group, and a chi-square test was used to compare categorical variables.

Immunophenotype markers were analyzed using the GraphPad Prism (GraphPad Software Inc, San Diego, CA) software 5.03.

The normality of the distribution was checked by the Shapiro-Wilk test. For data with nonparametric distribution, comparisons between 2 groups were done using the Mann-Whitney test, and the Kruskal-Wallis statistical test was used to compare 2 or more groups. Dunn multiple comparison test was used to compare the pairs in groups. Differences were considered significant when $P \leq 0.05$. Confidence intervals (CIs) were calculated for the comparisons between groups.

The total number of patients in each analysis for each different marker varied between 3 and 20 in the AIH and OS groups and 9 and 15 in the control group due to loss of samples during experimental procedures. Sample losses are inherent to the experimental method used.

RESULTS

Patient Descriptions

Characteristics of the patients are displayed in Table 1.

At first histopathological evaluation, 9 patients (45%) in the AIH group and 10 (52.6%) patients in the OS group exhibited liver cirrhosis, a result that was not significantly different ($P = 0.87$). In the OS group, 2 (10.5%) patients also had inflammatory bowel disease. All patients were receiving the combination of prednisone and azathioprine at the time of immunophenotype marker evaluations. The OS group was also receiving ursodeoxycholic acid. No other medications were given to our patients.

Immunophenotype Markers

Immunophenotype markers were evaluated in the lymphocytes and monocytes of patients and controls. Because of technical problems with flow cytometry (loss of samples during preparation or reading), the number of patients in each group was variable for each marker.

There was no statistically significant difference between the AIH group and the control group in lymphocyte ($P = 0.080$) and monocyte counts ($P = 0.395$). The OS and control groups, however, differed significantly in lymphocyte counts ($P = 0.037$), although there were no differences in monocyte counts ($P = 0.368$).

As shown in Figure 1, the first evaluation performed was the frequency of T $CD4^+$, T $CD8^+$, and $CD14^+$ cells. Concerning $CD4^+$ T cells, there was a significant difference between the AIH and control groups ($P < 0.001$) and between the OS group and the controls ($P = 0.025$). Both groups of patients had higher numbers of these cells than the controls (Fig. 1). CIs of these comparisons are shown in Supplemental Tables 2, 3, and 4 (Supplemental Digital Content 1–3, *http://links.lww.com/MPG/B140*, *http://links.lww.com/MPG/B141*, *http://links.lww.com/MPG/B142*).

There was no statistically significant difference between groups with regard to the percentage of $CD8^+$ T cells ($P = 0.314$ and $P = 0.630$, respectively). On the other hand, only the AIH group had a significantly reduced percentage of $CD14^+$ cells (monocytes) compared to the control group ($P = 0.004$).

Following this initial analysis, we assessed specific cell markers. Markers with statistically significant differences in $CD4^+$ T cells were CCR3 ($P = 0.003$ for AIH vs control and $P = 0.047$ for OS vs control); CD45RA (only for OS vs control, $P = 0.023$); CD45RO (only for AIH vs control, $P = 0.040$) and CD28 (only for AIH vs control, $P = 0.027$). Markers with statistically significant differences for $CD8^+$ T cells included: CCR3 l ($P = 0.044$ for AIH vs control and $P = 0.001$ for OS vs control),

TABLE 1. Comparison of the main clinical, laboratory, and evolution features of 2 groups of patients with AIH and AIH in association with autoimmune cholangitis (overlap syndrome)

	Autoimmune hepatitis ($n = 20$ patients)	Autoimmune hepatitis associated with autoimmune cholangitis ($n = 19$ patients)	Statistical significance (p value)
Median (IQ 25th–75th) of age at clinical onset (years)	10.7 (7.2–12.7)	10.2 (7.6–11.2)	0.90
Median (IQ 25th–75th) of age at the time of evaluation	15 (13.5–20.5)	15 (12.3–18)	1.00
Sex (females, n)	13 (65%)	10 (52.6%)	0.52
Median (IQ 25th–75th) of follow-up time, mo	78 (29–143)	38.5 (24–73.3)	0.14
Median (IQ 25–75th) of time into remission, mo	48 (24–96)	24 (24–48)	0.13
Forms of presentation (number of patients)			
Acute onset	9 (45%)	4 (21%)	0.18
Chronic liver disease	7 (35%)	6 (31.6%)	1.00
Hepatic failure	2 (10%)	3 (15,8%)	0.66
Fortuitous finding	2 (10%)	4 (21%)	0.41
Other forms	0 (0%)	2 (10.5%)	0.23
Autoantibodies positivity			
ASMA	14 (70%)	15 (78.9%)	0.72
ANA	9 (45%)	14 (73.7%)	0.10
Laboratory findings at first consultation (median and IQ range)			
AST*	12.3 (2.4–17.0)	11.4 (5.6–27.0)	0.88
ALT*	11.8 (4.6–20.0)	7.3 (3.2–22.0)	0.59
GGT*	**2.5 (1.7–4.4)**	**4.2 (2.6–6.4)**	**0.02**
Albumin	3.5 (3.3–4.0) g/dL	3.6 (3.2–3.9) g/dL	0.90
Gammaglobulinemia	**2.3 (1.3–2.8) g/dL**	**3.2 (2.2–4.1) g/dL**	**0.04**
Prothrombin activity	75 (54–87) %	59 (42–74) %	0.24

ALT = alanine aminotransferase, ANA = antinuclear antibody; ASMA = antismooth muscle antibody; AST = aspartate aminotransferase; GGT = gamma-glutamyl transferase.

*AST, ALT, and GGT results are show as mean of numbers of times the upper reference value (RV). P values < 0.05 are indicated in bold characters.

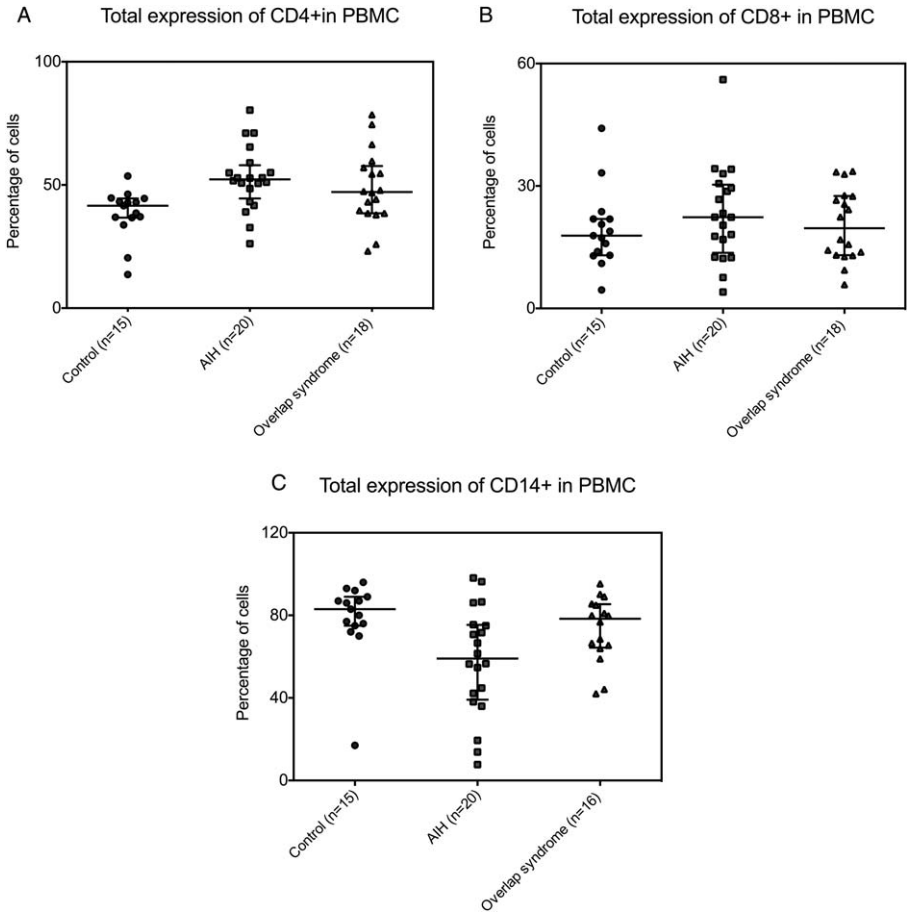

FIGURE 1. Percentage of cells expressing $CD4^+$ (Panel A), $CD8^+$ (Panel B), and $CD14^+$ (Panel C) in PBMC with representation of the median and interquartile range (percentile 25–75). AIH = autoimmune hepatitis; PBMC = peripheral blood mononuclear cell.

CD45RA (only for OS vs control, $P = 0.017$), CD45RO (only for OS vs control, $P = 0.005$), and CD25 (only for OS vs control, $P = 0.044$). In regard to monocytes ($CD14^+$ cells), percentages of HLADR were statistically significantly different between the patients and the controls, being $P = 0.0001$ for AIH versus control and $P = 0.029$ for OS versus control.

P values and CIs for the percentages of expression of all surface markers are shown in Tables 2, 3, and 4 as Supplemental Digital Content. Medians and interquartile ranges for all markers are also displayed in Supplemental Tables 5, 6, and 7 (Supplemental Digital Content 4–6, *http://links.lww.com/MPG/B143*, *http://links.lww.com/MPG/B144*, *http://links.lww.com/MPG/B145*).

Results of the markers that showed statistically significant differences are presented in Figures 2 and 3. The bar graphs show medians with interquartile range of the percentage of cells.

We also compared the combined data of both groups of patients versus the control group. In this analysis, markers with statistically significant differences for $CD4^+$ T cells were CCR3 ($P = 0.004$; CI: 0.94–33.9), CD45RA ($P = 0.04$; CI: 0.12–16.5), CD28 ($P = 0.03$; CI: 0.75–17.4), and total CD4 ($P = 0.001$; CI: 4.6–17.8). For $CD8^+$ T cells, the significant results were CCR3 ($P = 0.002$; CI: 0.19–1.22), CD45RA ($P = 0.012$; CI: 2.53–13.3), CD45RO ($P = 0.008$; CI: −5.04 to −0.54) and CD25 ($P = 0.02$; CI: −0.04 to −0.5). For $CD14^+$ cells, HLADR ($P = 0.0008$; CI: −49.1 to −15.3) and total CD14 ($P = 0.01$; CI: −26.1 to −2.24) had statistically significant differences.

DISCUSSION

First, both groups have similar clinical and laboratory characteristics to those described in other samples of patients with AIH (6,19–26). Adolescents and females were the most prevalent. The most common clinical forms at disease onset were acute onset and chronic liver disease. The autoantibodies ASMA and ANA were the most prevalent, confirming that type 1 AIH was the most frequent type, as previously reported (6,19–26). At first histopathological evaluation, liver cirrhosis was present in >50% of patients, as already described elsewhere (6,19,21–26).

There was no statistically significant difference in laboratory variables for the AIH and OS groups, except for GGT and gamma globulin levels. The difference in GGT was expected, because patients in the OS group had a bile duct disease. Indeed, persistent elevations in GGT justify the investigation of associated cholangitis (6,8,20,23,25,26). Before treatment, IgG levels were high in AIH and in OS patients, being even higher in OS group. After treatment, IgG levels returned to normal values in AIH and in OS patients.

Patients presented with higher percentages of $CD4^+$ T cells when compared with the controls. Other studies have already demonstrated the role of these cells in the immune response of AIH (12,14,27–29). In the AIH group, we also observed a reduced percentage of $CD14^+$ T cells compared with the control group. This finding is in opposition to the results reported by Longhi et al (30) in children and young adults diagnosed with AIH, even without clinical and laboratory disease activity. The authors showed an

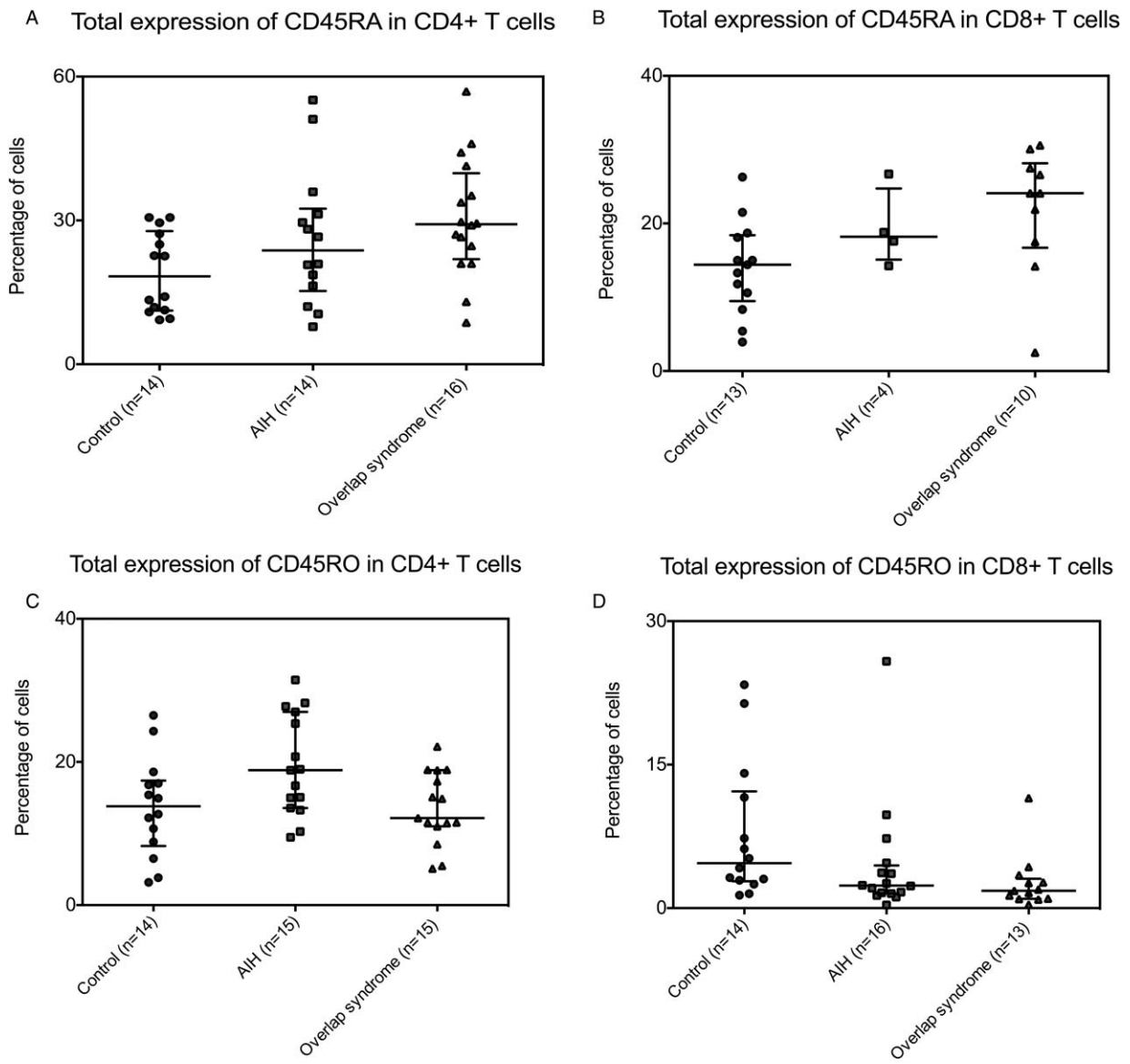

FIGURE 2. Percentage of T cells expressing CD45RA and CD45RO in PBMC of patients and controls with median and interquartile range (percentile 25–75) representation. A, Percentage of cells expressing CD45RA in $CD4^+$ T cells. B, Percentage of cells expressing CD45RA in $CD8^+$ T cells. C, Percentage of cells expressing CD45RO in $CD4^+$ T cells. D, Percentage of cells expressing CD45RO in $CD8^+$ T cells. AIH = autoimmune hepatitis; PBMC = peripheral blood mononuclear cell.

increase in the number and activity of monocytes, with impairment of immune regulatory mechanisms.

CD45 surface marker was evaluated as $CD45RA^+$ for naive T cells and as $CD45RO^+$, a memory T cell marker. In terms of $CD4^+$ T lymphocytes, $CD45RA^+$ had an increased expression in AIH, and it was even higher in OS. On the contrary, $CD45RO^+$ was increased only in AIH. In the OS group, this marker was similar to that of the control subjects. This finding is suggestive of a chronic and persistent activation of lymphocytes in AIH, as previously described (17). Both the AIH and OS groups seemed to have a continuous activation of the immune system. In $CD8^+$ T cells, $CD45RA^+$ and $CD45RO^+$ were highly expressed only in the OS group in comparison to the controls. These findings suggest that only patients with OS had activation of a memory marker of $CD8^+$ T cells.

The CCR3 receptor belongs to the G protein–coupled receptor superfamily of membrane proteins. The stimulation of CCR3 receptors promotes an inflammatory response and is associated with the release of reactive oxygen species. Autoimmune conditions may correspond with high expression of this receptor (31–33). Landi et al (34) showed that eotaxin-3 (CCL26), which is a chemokine produced in the vascular endothelium that is responsible for the recruitment of eosinophils to sites of inflammation, can bind to CCR3. Eotaxin-3 is significantly increased in the serum of patients with autoimmune liver disease compared with normal individuals or patients with chronic hepatitis C. In addition, high expression of CCR3 in peripheral T lymphocytes was observed in adult individuals with ulcerative colitis when compared with patients with Crohn disease and controls (35). In our study, both the AIH and OS groups presented more $CD4^+$ and $CD8^+$ cells expressing CCR3 than

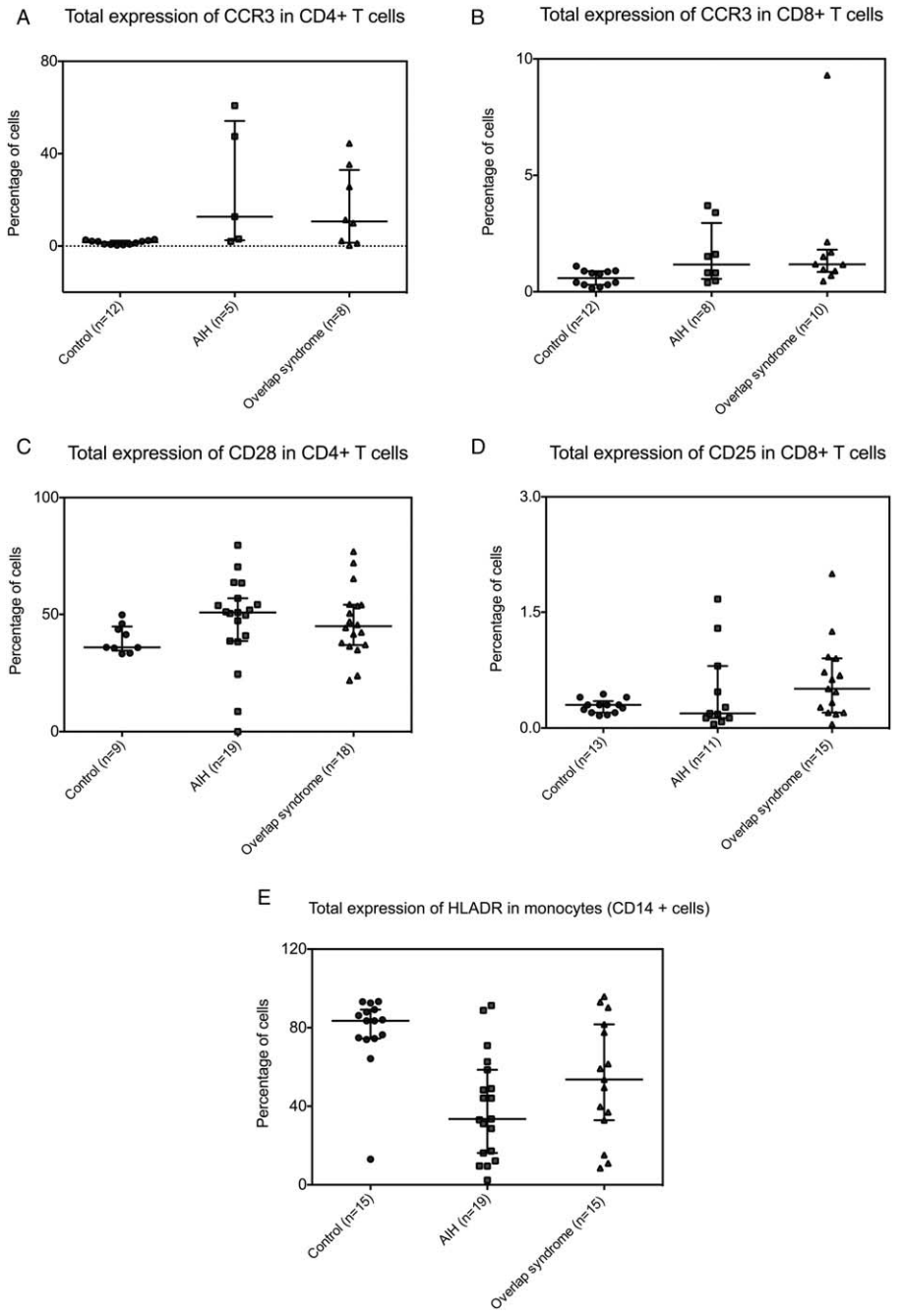

FIGURE 3. Expression of specific markers in PBMC of patients and controls with median and interquartile range (percentile 25–75) representation. A, Percentage of cells expressing CCR3 in $CD4^+$ T cells. B, Percentage of cells expressing CCR3 in $CD8^+$ T cells. C, Percentage of cells expressing CD28 in $CD4^+$ T cells. D, Percentage of cells expressing CD25 in $CD8^+$ T cells. E, Percentage of monocytes expressing HLADR. AIH = autoimmune hepatitis; PBMC = peripheral mononuclear cell.

controls. Considering that CCR3 may be a potential therapeutic target, further studies of this marker are important (36).

CD28 ligation is the major costimulatory signal for the activation of naive T cells, promoting T cell viability and expansion, cytokine production and regulating long-term T cell survival (37). Some authors considered that, among $CD8^+$ T cells, $CD28^+$/$CD28^-$ cells reflect replicative senescence and may signify immune exhaustion (38). When compared with $CD28^+$ T cells, activated $CD28^-$ T cells produce high levels of interferon-γ and tumor necrosis factor-α, enhancing inflammation (39). In the study by Kurokohchi et al (40), the numbers of $CD28^+CD4^+$ T cells in peripheral blood mononuclear cell (PBMC) were significantly reduced in patients without corticosteroid treatment, whereas in treated patients, the numbers were comparable to the control individuals, probably indicating response to treatment. In contrast, $CD28^+CD8^+$ T cells in peripheral blood mononuclear cell (PBMC)

were decreased in both treated and untreated patients. Numbers of $CD28^{-}CD4^{+}$T and $CD28^{-}CD8^{++}$ T cells in PBMC were comparable between AIH patients and controls.

In our study, an increased expression of CD28 in $CD4^{+}$ T cells was found in AIH compared to controls. We hypothesized that children and adolescents may have a better response to treatment with corticosteroids, increasing $CD4^{+}CD28^{+}$ T cells. Similar to the findings of Kurokohchi et al (40), $CD28^{-}CD4^{+}$ and $CD28^{-}CD8^{+}$ T cells percentages in PBMC were comparable between AIH patients and controls.

CD25 has been recognized as an important regulator of T-cell proliferation by inducing cell death and by the actions of both the regulatory (Treg) and effector T cells (41). Longhi et al (12,27) described reductions in the number and function of Treg cells in autoimmune liver disease. In addition, by using a more specific profile for Treg cells ($CD4^{+}CD25^{high}CD127^{-}FOXP3^{+}$), Peiseler et al (28) and Liberal et al (29) showed conflicting results. The first author reported a higher frequency of Treg cells in AIH patients with active disease, whereas the second author demonstrated numerical and functional impairment of Treg cells in autoimmune liver disease (28,29). In our data, $CD25^{+}$ was significantly more expressed in $CD8^{+}$ T lymphocytes in the OS group compared to the controls. We believe that, in view of what has already been described for $CD4^{+}$ T cells in the literature, this marker may play a major role in the pathophysiology of AIH.

In monocytes, we evaluated the expression of HLA-DR. HLA-DR haplotypes have already been described as susceptibility factors for AIH in children, but few studies have investigated the surface cell expression of HLA-DR (9–11,42–44). In our study, HLA-DR was less expressed in the AIH and OS groups, with even lower expression in the AIH group when compared to the OS group. Longhi et al (30) reported the same result when assessing children and young adults with AIH. Hiasa et al (45) also showed a similar finding in dendritic cells of adult patients with AIH and primary biliary cirrhosis. We believe that low levels of expression of HLA-DR may contribute to the breakdown of tolerance to self-antigen in AIH and OS.

The cross-sectional design and the relatively small sample size are limitations of our study. A longitudinal design might allow us to investigate changes in inflammatory markers according to the stage of the disease. In addition, the selection of patients who only had a good response to treatment could make our sample less heterogeneous, whereas it also precludes the analysis of the relationship of our findings with the therapeutic response. Another limitation is that disease remission was not confirmed by histology, and hence, a degree of persistence of liver tissue inflammation as a cause of increased expression of inflammatory markers cannot be ruled out. Nevertheless, some aspects may increase the strength of our findings, including the strict inclusion criteria and well-established protocol for flow cytometry.

In conclusion, we described a complex immunophenotype in pediatric patients with AIH and OS. The main findings were related to CCR3, CD45RA/RO, CD25 in $CD4^{+}$ and $CD8^{+}$T lymphocytes, and HLA-DR in monocytes as markers of disease activity with persistent inflammatory findings. Our data showed that signs of immune system activation and autoimmunity at the cellular level still persist, even in patients undergoing immunosuppressive treatment and in the presence of proper disease control.

REFERENCES

1. Alvarez F, Berg PA, Bianchi FB, et al. International autoimmune hepatitis group report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999;31:929–38.
2. Mieli-Vergani G, Vergani D. Autoimmune hepatitis in children: what is different from adult AIH? *Semin Liver Dis* 2009;29:297–306.
3. Mieli-Vergani G, Heller S, Jara P, et al. Autoimmune hepatitis. *J Pediatr Gastroenterol Nutr* 2009;49:158–64.
4. Ferreira AR, Roquete ML, Toppa NH, et al. Effect of treatment of hepatic histopathology in children and adolescents with autoimmune hepatitis. *J Pediatr Gastroenterol Nutr* 2008;46:65–70.
5. Bogdanos DP, Mieli-Vergani G, Vergani D. Autoantibodies and their antigens in autoimmune hepatitis. *Semin Liver Dis* 2009;29:241–53.
6. Gregorio GV, Portmann B, Reid F, et al. Autoimmune hepatitis in childhood: a 20-year experience. *Hepatology* 1997;25:541–7.
7. Abdalian R, Dhar P, Jhaveri K, et al. Prevalence of sclerosing cholangitis in adults with autoimmune hepatitis: evaluating the role of routine magnetic resonance imaging. *Hepatology* 2008;47:949–57.
8. Gregorio GV, Portmann B, Karani J, et al. Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study. *Hepatology* 2001;33:544–53.
9. Pando M, Larriba J, Fernandez GC, et al. Pediatric and adult forms of type I autoimmune hepatitis in Argentina: Evidence for differential genetic predisposition. *Hepatology* 1999;30:1374–80.
10. Czaja AJ, Souto EO, Bittencourt PL, et al. Clinical distinctions and pathogenic implications of type I autoimmune hepatitis in Brazil and the United States. *J Hepatol* 2002;37:302–8.
11. Ferri Liu PM, De Miranda DM, Fagundes EDT, et al. Autoimmune hepatitis in childhood: the role of genetic and immune factors. *World J Gastroenterol* 2013;19:4455–63.
12. Longhi MS, Hussain MJ, Mitry RR, et al. Functional study of $CD4^{+}CD25^{+}$ regulatory T cells in health and autoimmune hepatitis. *J Immunol* 2006;176:4484–91.
13. Shevach EM, McHugh RS, Piccirillo CA, et al. Control of T-cell activation by $CD4^{+}$ $CD25^{+}$ suppressor T cells. *Immunol Rev* 2001; 182:58–67.
14. Ferri S, Longhi MS, De Molo C, et al. A multifaceted imbalance of T cells with regulatory function characterizes type 1 autoimmune hepatitis. *Hepatology* 2010;52:999–1007.
15. Kurokohchi K, Masaki T, Himoto T, et al. Usefulness of liver infiltrating CD86-positive mononuclear cells for diagnosis of autoimmune hepatitis. *World J Gastroenterol* 2006;12:2523–9.
16. Loetscher P, Uguccioni M, Bordoli L, et al. CCR5 is characteristic of Th1 lymphocytes. *Nature* 1998;391:344–5.
17. Ogawa S, Sakaguchi K, Takaki A, et al. Increase in CD95 (Fas/APO-1)-positive $CD4^{+}$ and $CD8^{+}$ T cells in peripheral blood derived from patients with autoimmune hepatitis or chronic hepatitis C with autoimmune phenomena. *J Gastroenterol Hepatol* 2000;15:69–75.
18. Torres KCL, Antonelli LRV, Souza ALS, et al. Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. *J Neuroimmunol* 2005;166:144–57.
19. Ferreira AR, Roquete MLV, Penna FJ, et al. Autoimmune hepatitis in children and adolescents: clinical study, diagnosis and therapeutic response. *J Pediatr (Rio J)* 2002;78:309–14.
20. Bellomo-Brandão MA, Costa-Pinto EA, De Tommaso AM, et al. Clinical and biochemical features of autoimmune hepatitis in 36 pediatric patients. *Arq Gastroenterol* 2006;43:45–9.
21. Vitfell-Pedersen J, Jørgensen MH, Müller K, et al. Autoimmune hepatitis in children in Eastern Denmark. *J Pediatr Gastroenterol Nutr* 2012;55:376–9.
22. Dehghani SM, Haghighat M, Imanieh MH, et al. Autoimmune hepatitis in children: experiences in a tertiary center. *Iran J Pediatr* 2013;23:302–8.
23. Rojas CP, Bodicharla R, Campuzano-Zuluaga G, et al. Autoimmune hepatitis and primary sclerosing cholangitis in children and adolescents. *Fetal Pediatr Pathol* 2014;33:202–9.
24. Nares-Cisneros J, Jaramillo-Rodriguez Y. Autoimmune hepatitis in children: progression of 20 cases in northern Mexico. *Rev Gastroenterol Méx* 2014;79:238–43.
25. Low AS, Tan M, Garcia A, et al. Childhood autoimmune hepatitis in a paediatric unit of a tertiary care hospital. *Singapore Med J* 2014; 55:648–51.
26. Jiménez-Rivera C, Ling SC, Ahmed N, et al. Incidence and characteristics of autoimmune hepatitis. *Pediatrics* 2015;136:e1237–4.

27. Longhi MS, Ma Y, Bogdanos DP, et al. Impairment of CD4+CD25+ regulatory T-cells in autoimmune liver disease. *J Hepatol* 2004;41: 31–7.
28. Peiseler M, Sebode M, Franke B, et al. FOXP3+ regulatory T cells in autoimmune hepatitis are fully functional and not reduced in frequency. *J Hepatol* 2012;57:125–32.
29. Liberal R, Grant CR, Holder BS, et al. In autoimmune hepatitis type 1 or the autoimmune hepatitis-sclerosing cholangitis variant defective regulatory T-cell responsiveness to IL-2 results in low IL-10 production and impaired suppression. *Hepatology* 2015;62:863–75.
30. Longhi MS, Mitry RR, Samyn M, et al. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. *Hepatology* 2009;50:130–42.
31. Gaspar K, Kukova G, Bunemann E, et al. The chemokine receptor CCR3 participates in tissue remodeling during atopic skin inflammation. *J Dermatol Sci* 2013;71:12–21.
32. Freutel S, Gaffal E, Zahn S, et al. Enhanced CCR5+/CCR3+ T helper cell ratio in patients with active cutaneous lupus erythematosus. *Lupus* 2011;20:1300–4.
33. Aloush V, George J, Elkayam O, et al. Decreased levels of CCR3 in CD4+ lymphocytes of rheumatoid arthritis patients. *Clin Exp Rheumatol* 2010;28:462–7.
34. Landi A, Weismuller TJ, Lankisch TO, et al. Differential serum levels of eosinophilic eotaxins in primary sclerosing cholangitis, primary biliary cirrhosis, and autoimmune hepatitis. *J Interferon Cytokine Res* 2014; 34:204–14.
35. Manousou P, Kolios G, Valatas V, et al. Increased expression of chemokine receptor CCR3 and its ligands in ulcerative colitis: the role of colonic epithelial cells in in vitro studies. *Clin Exp Immunol* 2010;162:337–47.
36. Pease JE, Horuk R. Recent progress in the development of antagonists to the chemokine receptors CCR3 and CCR4. *Expert Opin Drug Discov* 2014;9:467–83.
37. Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 1993;11:191–212.
38. Brzezińska A, Magalska A, Szybińska A, et al. Proliferation and apoptosis of human CD8(+)CD28(+) and CD8(+)CD28(-) lymphocytes during aging. *Exp Gerontol* 2004;39:539–44.
39. Liaskou E, Jeffery LE, Trivedi PJ, et al. Loss of CD28 expression by liver-infiltrating T cells contributes to pathogenesis of primary sclerosing cholangitis. *Gastroenterology* 2014;147:221.e7–32.e7.
40. Kurokohchi K, Arima K, Masaki T, et al. Analysis of CD28 and bcl-2 expression on peripheral blood and liver-infiltrating mononuclear cells in patients with autoimmune hepatitis. *J Clin Immunol* 2006;26:323–30.
41. Brusko TM, Wasserfall CH, Hulme MA, et al. Influence of membrane CD25 stability on T lymphocyte activity: implications for immunoregulation. *PLoS One* 2009;4:e7980.
42. Donaldson PT, Doherty DG, Hayllar KM, et al. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens HLA-DR4 and A1-B8-DR3 are independent risk factors. *Hepatology* 1991;13:701–6.
43. Van Gerven NM, De Boer YS, Zwiers A, et al. HLA-DRB1*03:01 and HLA-DRB1*04:01 modify the presentation and outcome in autoimmune hepatitis type-1. *Genes Immun* 2015;16:247–52.
44. Elfaramawy AA, Elhossiny RM, Abbas AA, et al. HLA-DRB1 as a risk factor in children with autoimmune hepatitis and its relation to hepatitis A infection. *Ital J Pediatr* 2010;36:73.
45. Hiasa Y, Akbar SM, Abe M, et al. Dendritic cell subtypes in autoimmune liver diseases; decrease expression of HLA-DR and CD123 on type 2 dentritic cells. *Hepatol Res* 2002;22:241–9.