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HLA alleles predisposing to autoimmunity are linked to impaired immunoregulation in patients with juvenile autoimmune liver disease and in their first-degree relatives

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Abbreviations: AILD: autoimmune liver disease; AIH: autoimmune hepatitis; ASC: autoimmune sclerosing cholangitis; ANA, anti-nuclear antibody; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein-4, SMA, smooth muscle antibody; Treg, regulatory T cell, Teff, effector T cell; PD-1 programmed cell death-1; PSC, primary sclerosing cholangitis; CYP2D6/FTCD, cytochrome P450 2D6 protein and formiminoyltransferase cyclodeaminase

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Abstract

Background & Aims: Juvenile autoimmune liver disease (JAILD) comprises autoimmune hepatitis and autoimmune sclerosing cholangitis. JAILD-predisposing genes include HLA-DR3, DR7, DR13 and haplotype A1-B8-DR3. Mechanisms leading to liver autoimmunity remain elusive, though JAILD patients have aberrated immunoregulation. We investigated the influence of HLA genes on immune cells, focusing on T-cells and frequency and function of T regulatory cells (Tregs) in JAILD patients, their first-degree-relatives (FDRs) and healthy controls (HCs). **Methods:** HLA class I and II genotypes were defined by PCR and peripheral blood mononuclear cells were immunophenotyped by FACS in 82 patients, 72 FDRs, 50 HCs. Treg function was tested by inhibition of CD4^{pos}CD25^{neg} T-cell proliferation. Links between HLA genes, Treg frequency/function, pro-inflammatory/immunoregulatory cytokines, soluble and membrane-bound programmed cell death-1 (PD-1) were investigated. **Results:** Proportion of subjects carrying HLA DR3/DR7/DR13 was 88%, 92%, 64% in patients, FDRs and HCs. Circulating Treg frequency was lower in patients and FDRs than HCs. Inhibitory capacity of Tregs was lower in patients but similar in FDRs compared to HCs. FDRs possessing HLA DR3/DR7/DR13 genes had Treg frequencies lower than those without. PD-1^{pos}CD4^{pos} T-cells were fewer in patients than HCs; PD-1^{pos}CD8^{pos} T-cells were fewer in patients and FDRs than HCs. Patient plasma levels of IFN- γ were higher, and ratios of IFN- γ /IL-10 and IFN- γ /IL-2 lower than in HCs. All nine FDRs with autoimmune disorders had HLA DR3/DR7/DR13 genes and lower Treg frequency than those without autoimmune disorders and HCs. **Conclusion:** We show a link between HLA disease-predisposing genes and defective immunoregulation not only in JAILD patients, but also in their FDRs, who are prone to autoimmune disorders.

Keywords: immunogenetics, autoimmunity, hepatitis, sclerosing cholangitis, regulatory T cells, human study

Introduction

Juvenile autoimmune liver diseases (JAILD), comprising autoimmune hepatitis (AIH) and autoimmune sclerosing cholangitis (ASC) are chronic and rare liver disorders. In AIH there is a strong female preponderance, while in ASC males and females are equally affected. Their features include: (i) increased aminotransferase levels, (ii) presence of autoantibodies such as antinuclear antibody (ANA)/anti-smooth muscle (SMA) in type 1 AIH and ASC, and anti-liver kidney microsomal antibody (anti-LKM1) and/or anti-liver cytosol (anti-LC1) in type 2 AIH (AIH-2), and anti-soluble liver antigen (SLA) in both conditions, (iii) hypergammaglobulinemia; and histologically (iv) interface hepatitis [1-6]. About 50% of children with laboratory and histological features of AIH-1 (i.e. ANA and/or SMA positivity and interface hepatitis) have bile duct damage on cholangiography at disease onset and are diagnosed as having ASC [7].

Among Caucasoids, JAILD are strongly associated with genes encoding for alleles of the human leukocyte antigen (HLA). *HLA B*08*, *-DRB1*03*, and the *A1-B8-DR3* haplotype predispose to all three forms of JAILD, homozygosity for *DRB1*03* representing the strongest risk factor. *HLA DRB1*04*, which independently confers susceptibility to AIH-1 in adults, is infrequent in juvenile AIH-1 and ASC. Distinct HLA class II alleles predispose to the different subgroups of JAILD: *DRB1*03* to AIH-1, *DRB1*13* to ASC, and *DRB1*07* to AIH-2 [8]. An association between JAILD and HLA-*DRB1*03*, *DRB1*04* and *DRB1*13* has been reported in Arab patients; and with HLA-*DRB1*03* in Indian patients [9, 10].

Besides HLA predisposition to autoimmunity, a numerical and functional impairment of total ($CD4^{pos}CD25^{high}CD127^{low/neg}FOXP3^{pos}$) regulatory T cells (Tregs) and effector ($CD4^{pos}CD25^{+}CD45RA^{neg}FOXP3^{high}$) Tregs has been unambiguously demonstrated in JAILD, both AIH and ASC [11-15], suggesting that failure of regulatory mechanisms may underlie the development of overt autoimmunity in these conditions.

Family studies are useful tools to investigate the role of the environment versus the genetic makeup in determining susceptibility to autoimmune disorders, though few have been performed in JAILD. A family study from our group investigating the relationship between HLA genes and JAILD-related autoantibody positivity in 28 children with AIH or ASC and in 45 of their first degree relatives (FDRs) [16] confirmed that homozygosity for DR3 plays a major role in the predisposition to JAILD and showed that autoantibodies diagnostic for this condition were also present in 6% of their FDRs, though not linked to clinical manifestations of autoimmune liver disease.

The aim of the present study was to extend our previous observation by investigating the genetic influence on the immune system of children with JAILD and their FDRs, most of whom HLA typed. To this end we have investigated the frequency of circulating CD4^{pos}, CD8^{pos} T-cells, Tregs (CD4^{pos}CD25^{high}CD127^{low/neg}FOXP3^{pos}) and T cell membrane bound programmed cell death-1 (PD-1), soluble PD-1 (sPD-1), and pro- and anti-inflammatory cytokines in the plasma of both patients and FDRs.

Subjects and Methods

Patients, their first-degree relatives and healthy controls

In a cross-sectional study, 82 patients unrelated to each other, were recruited from October 2006 to October 2016 at diagnosis or during follow up (Table 1). Seventy (85.3%) were Caucasoid, six (7.3%) Indian, four Black (4.8%), and 2 (2.4%) Arab (Table 1). Diagnosis of JAILD was based on the European Society of Gastroenterology, Hepatology and Nutrition (ESPGHAN) diagnostic criteria [17] and all patients were treated according to

the ESPGHAN protocol. A liver biopsy performed at the time of, or close to, diagnosis showed histological features of interface hepatitis in all patients. Twenty-four of the 82 patients had bile duct changes characteristic of sclerosing cholangitis on endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) and were diagnosed as having ASC (Supplementary Table 1). Twenty-five patients had at least one concomitant extra hepatic autoimmune disorder (Supplementary Table 1); for one patient with Duchenne muscular dystrophy, alanine transaminase (ALT) levels were used to assess liver damage. Seventy-two first-degree relatives (FDRs) from the families of 57 patients were recruited, 61 parents and 11 siblings (Table 1). Eleven of the 72 FDRs had an autoimmune disorder; 1 primary sclerosing cholangitis (PSC) and the remaining 10 extra hepatic disorders; 5 thyroiditis, 2 type-1 diabetes, 1 rheumatoid arthritis, 1 coeliac disease and 1 ulcerative colitis. Additionally, 50 healthy controls (HC) were enrolled (Table 1). The study was approved by the Ethics Committee of King's College Hospital, London (04/Q0703/12) and written consent was obtained from each AIH/ASC patient or legal guardian, FDRs and HCs. The age of HCs is significantly higher compared to patients due to legal prohibition of consenting HC younger than 18 years in the United Kingdom.

HLA Typing

HLA genotyping was performed in 68 of 82 AIH/ASC patients, 60 of 72 FDRs and 48 of 50 HCs, in whom enough cells were available, and DNA quality was high enough to perform polymerase chain reaction using sequence-specific primers (SSP) according to the manufacturer's instructions using the HLA SSP typing kit (ROSE Europe GmbH, Frankfurt, Germany). Detailed method has been described by Ma Y, et al. 2021 [8].

Cell separation

Peripheral blood mononuclear cells (PBMCs) were obtained from 10 to 20 mls of blood by venepuncture. The blood was layered over Ficoll-Paque Plus lymphocyte isolation sterile solution (GE Healthcare, Uppsala, Sweden) as previously described [18]. Mononuclear cell viability, determined by Trypan blue exclusion, was $\geq 93\%$.

Flow cytometry

The frequency and phenotypic properties of T cells were determined by flow cytometry using a Becton Dickinson fluorescent activated cell sorter-II (BD FACS CANTO II, Oxford, UK). Cell surface markers on PBMCs were stained with allophycocyanin (APC)-cyochrome (Cy)-7-conjugated anti-CD4, peridinin-chlorophyll protein cyanine 5.5 (PERCP-Cy5.5) conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD127, PE-conjugated anti-CD25, FITC-conjugated anti-PD1 (all from Biolegend, London, UK). Cells were incubated at 4°C in the dark for 30 minutes, washed with phosphate buffered saline (PBS)/1% foetal calf serum (FCS), resuspended and analysed by flow cytometry; FlowJo (TreeStar Inc. Ashland, USA) software was used for analysis. A minimum of 50,000 gated events were acquired for each sample. Gating strategy is shown in supplementary figure 1A.

Intranuclear staining: following the cell surface staining, the frequency and phenotype of cells positive for FOXP3 was determined by intranuclear staining after cell fixation with a mixture (1:3) of FOXP3 staining buffer concentrate and diluent for 1 hour followed by 1 minute permeabilization with 1 X permeabilization buffer (all from eBioscience, Hatfield, UK) and counterstaining with APC-conjugated anti-FOXP3 (clone PCH101) monoclonal antibodies (from eBioscience, Hatfield, UK) for 30 minutes. Cells were then washed (1xPBS) and acquired. To determine the background signal, hence position of the gating for each fluorochrome, the fluorescence minus one (FMO) was acquired.

Purification of T effectors and Tregs: Purified CD4^{pos}CD127^{pos} T effectors and CD4^{pos}CD25^{high}CD127^{low/neg} Tregs used in suppression assays were purified from PBMCs using immunomagnetic beads (DynaL Invitrogen, Oslo, Norway) as previously described [19, 20]. CD4^{pos}CD25^{high}CD127^{low/neg} cells were purified according to the expression of CD127. Briefly, CD4^{pos}CD25^{high} cells were incubated with PE-conjugated anti-CD127 for 30 minutes, then with microbeads conjugated with monoclonal anti-PE antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 15 minutes at 4°C. The purity of CD4^{pos}CD25^{neg} target cells exceeded 95%; that of CD4^{pos}CD25^{high}CD127^{low/neg} Treg was consistently between 92% and 95%.

Suppression Assay

CD4^{pos}CD25^{high}CD127^{low/neg} cells were added at a 1:8 ratio to CD4^{pos}CD25^{neg} target T-cells. Parallel cultures of CD4^{pos}CD25^{neg} cells on their own were set up under identical conditions. Cells were cocultured at 37 °C and 5% CO₂ for 5 days in the presence of anti-CD3/anti-CD28 T-cell expander (ratio bead/cell: 1:2; Dynal Invitrogen) and IL-2 (30 U/mL). All experiments were performed in duplicate. For the last 18 hours, cells were pulsed with 0.25 µCi/well of ³H-thymidine and harvested using a multichannel harvester (PerkinElmer, Buckinghamshire, UK). Percentage inhibition was calculated using the formula: count per minute (cpm) in the presence of CD4^{pos}CD25^{high}CD127^{low/neg} cells divided by the cpm in the absence of CD4^{pos}CD25^{high}CD127^{low/neg} cells. As we have previously shown that the extent of inhibition of cell proliferation measured by either ³H-thymidine incorporation or carboxy fluorescein succinimidyl ester staining is comparable [21] the ³H-thymidine incorporation method was selected because it requires fewer lymphocytes and is therefore suitable when dealing with low-yield cell samples often obtained from JAILD patients.

ELISA for the measurement of cytokine levels

The concentrations of IFN- γ , IL-2, IL-4, IL-17 and IL-10 in patients' plasma were measured by ELISA using a standard protocol as previously described [22]. In addition, IFN- γ and IL-10 in the supernatant collected from the suppression assay were measured by ELISA. Soluble programmed cell-death-1 (sPD-1) levels were determined in accordance to the manufacturer protocol (R&D Systems, Minneapolis, MN, Cat. No.: DY1086).

Statistical analysis

The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness-of-fit-test; once the hypothesis of normality was accepted ($P > 0.05$), comparisons were performed by Chi-square, unpaired Student t-test or a one-way ANOVA. Tukey's multiple comparisons test was used for multiple samples. When appropriate, Spearman correlation test was used to correlate sPD-1 values with pro-inflammatory cytokines. Results are expressed as mean $\pm$ standard error of the mean (SEM), unless otherwise stated, and P values < 0.05 were considered significant. Data were analysed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA).

RESULTS

Distribution of HLA class I/II alleles

The frequency of HLA-DR3 was 69%, 55.7% and 24.5% in JAILD, FDRs and HCs, respectively (Fig 1A), being significantly higher in patients ($p < 0.001$) and FDRs ($p < 0.01$) than in HCs. The frequency of HLA-DR7 was similar in patients (15.5%) and FDRs (29.1%), and non-significantly lower than in HCs (24.5%). The frequency of HLA-DR13 was similar in patients (22%), FDRs (16.9%) and HCs (11.6%). Non-JAILD associated HLA alleles (DR1, DR2, DR4, DR5, DR6, DR8, DR9, DR10, DR11, DR12, DR14, DR15, DR16) were significantly lower in patients (11.2%, $p < 0.001$) and FDRs (8.1%, $p < 0.001$) than in HCs (35.4%). The combined frequency of HLA-DR3/DR7/DR13 was comparable in patients

(88%) and FDRs (92%) (Fig 1B), being significantly higher than in HCs (64%, $p<0.001$ for both).

Similarly, the frequency of the ancestral haplotype (A1-B8-DR3), linked to susceptibility to autoimmune liver diseases and comprising class I and II alleles, was 44% in patients and 37% in FDRs, being significantly higher than in HCs (15%, $p<0.001$ for both, Fig. 1B). [The HLA profile of the non-Caucasoid patients tested is shown in supplementary table-2.](#)

At diagnosis 7 A1-B8-DR3 positive patients had a tendency towards higher levels of AST (1084.7 IU/L versus 354 IU/L), bilirubin (88.8 $\mu\text{mol/L}$ versus 40.5 $\mu\text{mol/L}$) and IgG (31.8 g/L versus 21.3 g/L) levels compared to 10 non-A1-B8-DR3 patients (Supplementary Fig. 1B). AST, IgG and bilirubin levels in treated patients were similar in A1-B8-DR3 carriers and non-carriers (Supplementary Fig. 1B).

HLA profile versus total T cells and Tregs

The percentage of total CD4^{pos} T-cells was significantly higher in JAILD (42.5%) and in FDRs (42.3%) than in HCs (34.7%, $p<0.01$) (Fig. 2A), without being affected by possession of specific HLA genes (Fig. 2B, C). CD8 T-cells (Fig. 2A-2C) as well as the CD4/CD8 ratio (Supplementary Fig. 1C) were not affected by the genetic background.

Tregs were defined as CD4^{pos}CD25^{high}CD127^{low/neg}FOXP3^{pos} T-cells within total CD4^{pos} T-cells (Supplementary Fig. 1A). The frequency of Tregs in JAILD patients (2.7%, $p<0.01$) and FDRs (2.9%, $p<0.05$) was lower than in HCs (3.4%) (Fig. 3A). HLA-DR3/DR7/DR13 positive JAILD patients tended to have a lower Treg frequency (2.6%) than non-HLA-DR3/DR7/DR13 carriers (3.3%, $p=0.11$) (Fig. 3B). Among FDRs, HLA-DR3/DR7/DR13 positive subjects had lower Treg frequency than those negative for these alleles (2.9% vs 3.7%,

p<0.05) (Fig. 3B). The same class II alleles had the opposite effect in HCs: HLA-DR3/DR7/DR13 positive HCs had more Tregs (3.72%) compared to HLA-DR3/DR7/DR13 negative HCs (2.98%, p<0.05) (Fig. 3B). A1-B8-DR3 positive JAILD patients had significantly lower percentage of Tregs (2.2%) compared to A1-B8-DR3 negative ones (2.9%, p<0.05) (Fig. 3C). In FDRs possession of A1-B8-DR3 did not affect Treg frequency (3.0% vs 3.1%, p=ns) (Fig. 3C). while in HCs, A1-B8-DR3 carriers had higher Tregs frequency (4.25%) in comparison to non-carriers (3.31, p<0.05) (Fig. 3C).

Treg function studies across the three groups showed that suppression of CD4^{pos}CD25^{neg} T effector proliferation by CD4^{pos}CD25^{high}CD127^{low/neg} Tregs was significantly lower in JAILD patients (13.6%) than in FDRs (28.8%, p<0.05) and HCs (36.9%, p<0.01) (Fig. 3D). Levels of the pro-inflammatory cytokine IFN-γ in the supernatant of the suppression assays were significantly higher in JAILD (469 pg/ml, p<0.01) than in FDRs (48 pg/ml) and HCs (51 pg/ml) (Supplementary Fig. 1D). Supernatant levels of the immunomodulatory cytokine IL-10 were lower in JAILD (86 pg/ml) than in FDRs (193 pg/ml) and HCs (1,000 pg/ml), though the difference did not achieve statistical significance owing to the high variation in the values of HCs (Supplementary Fig. 1E).

When the frequencies of CD4^{pos} T cells, CD8^{pos} T cells, and Tregs were compared between AIH-1, AIH-2, and ASC and between treated and untreated (i.e. at diagnosis) patients no differences were observed (Supplementary Fig. 2A,B), though among patients with AIH-1 and AIH-2 those treated (tended to have higher Tregs compared to those tested at diagnosis (Supplementary Fig. 2C).

PD-1 expression on CD4^{pos}, CD8^{pos} T cells and Tregs

In JAILD patients both CD4^{pos} (1.6%) and CD8^{pos} (1.2%) T-cells expressed lower levels of the immune checkpoint cell surface marker PD-1 than in HCs (CD4^{pos}: 2.5%, $p < 0.01$, CD8^{pos}: 2.6%, $p < 0.001$) (Fig. 4A). FDRs had levels of PD-1 expression on CD4^{pos} and CD8^{pos} similar to JAILD patients, and CD8^{pos} T-cell PD-1 (1.7%) significantly lower than CD8^{pos} PD-1 T-cells from HCs (2.6%, $p < 0.05$) (Fig. 4A). There was no difference in PD-1 expression on T cells among patients, FDRs and HCs positive or negative for JAILD predisposing HLA alleles (Fig. 4B, C). PD-1 expression on Tregs was similar among JAILD, FDRs and HCs (Supplementary Fig. 32A).

To investigate whether the reduced expression of PD-1 on T-cells in JAILD could be due to increased shedding, we measured plasma levels of sPD-1. sPD-1 levels in the plasma of JAILD patients was more than double (2,583 pg/ml) compared to FDRs (1,197 pg/ml, $p < 0.05$) and HCs (1,105 pg/ml, $p < 0.05$) (Fig. 5A left panel).

There was no difference in plasma sPD-1 levels according to HLA make up in any of the three groups (Fig. 5A middle and right panel). Plasma levels of sPD1 and frequencies of PD1+ CD4^{pos} T cells/ and PD1+ CD8^{pos} T cells were similar in AIH-1, AIH-2, and ASC and in -treated versus untreated patients (Supplementary Fig. 3B,C); treatment effect on sPD-1 could not be investigated as almost all samples were from treated patients (Supplementary Fig. 3D). The effect of disease/treatment on PD1+ Tregs frequency was not evaluated owing to the small sample size (10 AIH-1, 2 AIH-2, 0 ASC).

Pro-inflammatory and regulatory cytokines

Both IFN- γ and IL-17 plasma levels were higher in patients (423 pg/ml and 470 pg/ml, respectively) than in FDRs (189 pg/ml, $p < 0.05$ and 93 pg/ml, $p < 0.05$) and HCs (94 pg/ml, $p < 0.01$ and 169 pg/ml, $p < 0.05$) (Fig. 5B, C left panel). DR3/DR7/DR13 positive JAILD patients had IFN- γ levels of 610pg/ml as compared to 208 pg/ml in patients with no HLA-

DR3/DR7/DR13 $p<0.05$) whereas other comparisons showed no differences (Fig. 5B, C middle and right panel).

sPD-1 levels positively correlated with IFN- γ and IL-17 levels in patients, FDRs and HCs (IFN- γ : Fig. 5D left, middle and right panel; IL-17: Supplementary Fig. [4A-4C](#)).

Evaluation of the immunomodulatory cytokines IL-10 and IL-4 and of IL-2, which has dual immunoregulation and pro-inflammation function showed that levels of all three cytokines were significantly higher in JAILD patients than in FDRs and HCs (Fig. 6A-6C): IL-4 in patients 88 pg/ml, in FDRs 15.7 pg/ml ($p<0.05$) and in HCs (9.9 pg/ml ($p<0.01$); IL-10 in patients 759 pg/ml, in FDRs 231 pg/ml ($p<0.05$) and in HCs 168 pg/ml ($p<0.01$); IL-2 in patients 345 pg/ml, in FDRs 45 pg/ml ($p<0.05$), and in HCs 17 pg/ml ($p<0.05$). Levels were not affected by the genetic make-up apart for IL-4, which was significantly lower (5.18 pg/ml) in HLA-DR3/DR7/DR13 positive compared to negative FDRs (25.4 pg/ml, $p<0.05$) (Fig. 6A).

The ratio between the pro-inflammatory IFN- γ and the immunoregulatory IL-10 was higher in JAILD patients (1.83) than in HCs (0.49, $p<0.01$), and tended to be higher in FDRs compared to HCs, without reaching statistical significance ($p=0.14$) (Fig. 6D). IFN- γ /IL-2 ratio was higher in JAILD patients (49) than in FDRs (15, $p<0.05$) and HCs (7.7, $p<0.05$) (Fig. 6E). IFN- γ /IL-4 and IFN- γ /IL-17 ratios were comparable in the three groups (Supplementary Fig [4D, E](#)). There was no influence of the HLA make-up on any of the cytokine ratios (Supplementary Fig [5A, H](#)). Cytokine levels were similar among the disease groups (Supplementary Fig. 6A, E). Since only 3 patients (2 AIH-1, 1 ASC) were tested at diagnosis the effect of treatment could not be established (Supplementary Fig. 6A, E).

Association between FDR HLA profile and presence of autoimmune disorders

Of the 60 FDRs tested only 5 did not possess HLA-DR3/DR7/DR13. All 9 FDRs with autoimmune disorders had HLA-DR3/DR7/DR13. FDRs with autoimmune disorders had a lower percentage of Tregs than those without autoimmune disorders ($p < 0.05$) (Fig. 7A), while no difference was observed for the other immune parameters tested (data not shown). Distribution of the A1-B8-DR3 haplotype was similar in FDRs with (30%) and FDRs without autoimmune disease (33%) with also indifferent immune phenotype (data not shown).

Discussion

HLA genes are implicated in the predisposition to autoimmune liver disease, as shown in HLA association and GWAS studies [23, 24]. [Autoimmunity predisposing HLA alleles such as HLA-DR3 and DR4 have low affinity for class II-associated invariant chain peptide \(CLIP\), which regulates antigen binding in the thymus leading to elimination of autoreactive T cells \[25-27\]. Low affinity HLA class II alleles-peptide complexes are formed that are recognized by T cells with an affinity strong enough to cause T cell activation but not to trigger deletion in the thymus, leading to the escape of these autoreactive T cells in the circulation, with resulting autoimmune manifestations. In this context, DR3 and DR4 are instrumental in the development of AIH in humanised murine models \[28, 29\].](#) In this family study, we confirm that possession of the JAILD predisposing HLA DR3, DR7 or DR13 genes is associated to defective immunoregulation [8, 16]. In addition, we show that defective immunoregulation not only affects the patients, but also their FRDs, as reflected by a low frequency of $CD4^{pos}CD25^{high}CD127^{low/neg}$ Tregs in both. Of note, in FDRs decreased Treg numbers are present in those with associated autoimmune disorders, but not in those without. In the patients, decreased Treg numbers are accompanied by defective Treg function that is likely to be a key factor for the transition from predisposition to overt liver disease. In addition, reduced numbers of effector Tregs ($CD4^{pos}CD25^{pos}CD45RA^{neg}FOXP3^{high}$), which

are especially numerous in the liver tissue, have been reported in blood and liver of children with AIH-1 and 2 [14, 30]. Unexpectedly, possession of the JAILD predisposing HLA-I/II alleles had the opposite effect in healthy controls. The frequency of Tregs was higher in the group positive for HLA DR3, DR7 or DR13 and the same effect was observed in A1-B8-DR3 positive HCs, having higher frequencies of Tregs.

JAILD patients have increased IFN γ and decreased IL10 production in the culture supernatants of the suppression assays; decreased PD-1 expression on CD4 and CD8 T cells; increased plasma levels of IFN γ and IL-17, particularly in patients carrying HLA DR3, DR7 or DR13, which correlate with levels of sPD-1, confirming a link between sPD-1 shedding and inflammatory status [31]. In this context, a recent paper reports higher serum sPD1 in adult AIH patients as well as in patients with primary biliary cholangitis (PBC) and AIH/PBC variant syndromes compared to age matched HCs [32]. In our series, all investigated pro and/or anti-inflammatory serum cytokine levels were higher in patients compared to HCs, but were also higher in FDRs, suggesting ongoing inflammatory activity. Additionally, we observed that patients, and to some extent FDRs, had relatively higher serum IFN- γ than IL-10 and IL-2, suggesting an imbalance between immune activation and compensatory immunoregulation.

Thirty percent of JAILD patients had associated non-hepatic autoimmune disorders, a percentage similar to previous series [17]. As expected by the high frequency of autoimmunity predisposing HLA class I/II alleles within their families, autoimmune disorders were present also in 11% of their FDRs, but only one of them had an autoimmune disease affecting the liver, PSC. Interestingly, a nationwide population study from Denmark shows that though FDRs of index patients with AIH are at increased risk of developing AIH over time, the absolute risk is very low [33]. In contrast, FDRs of patients with PSC have a PSC

prevalence of 0.7%, which represents a nearly 100-fold increased risk of developing PSC compared with the general population, indicating a stronger role for genetic factors [34], possibly because of the association between PSC and inflammatory bowel disease, which also recurs frequently within families [35].

Besides possession of HLA predisposing genes, the autoimmune phenotype is influenced by other factors. Interestingly, and somewhat surprisingly, a Swedish nationwide population study in AIH patients reports a lower role for genetics, expressed as familial risk of developing the disease, than environmental factors [36, 37], as the risk of developing AIH in siblings was 3.83x times higher than in the normal population, whereas it was 6x times higher for the partner of a patient with AIH, though no clinical information on the 8 couples concordant for AIH was provided. In the Danish nationwide population study mentioned above, proband concordance for AIH in monozygotic twins was only 8.1% [37], in contrast with the reported proband concordance in monozygotic twins of >50% for type 1 diabetes [38], again implicating factors other than genetic in the development of AIH. In Graves disease, the reported FDR risk of developing the disease is some 4x higher than in the general population, but only 1.9x for the unaffected spouse [39]. These findings suggest that the role of genetic versus environmental risks depends on the organ of interest. A potential explanation as to why environmental factors may prevail over genetic risks in autoimmune liver disease is the liver unique anatomical location, which continuously exposes hepatocytes to a variety of toxins, xenobiotics, gut-derived microbial products, and hepatotropic viruses, with consequent release of intracellular proteins [40]. Liver autoimmunity is likely to be the result of a complex interplay between HLA, non-HLA (SH2B3, CARD10, CTLA-4, GATA2) and environmental (diet, vitamin D, gut microbiota) factors [14, 23, 24, 29, 41-43]. In the context of non-HLA factors, it is of note that in paediatric AIH specific genetic immune defects associated to liver autoimmune attack have been reported, including

autoimmune regulator (*AIRE*) mutations [44], gain-of-function mutation of the FMS-like tyrosine 3 kinase (*FLT3*) receptor oncogene [45], autosomal dominant signal transducer and activator of transcription 1 (*STAT1*) gain-of-function defect [46], and variants in tumour necrosis factor alpha induced protein 3 (*TNFAIP3*) [47], stressing the involvement of diverse genetic contributions in the emergence of the autoimmune phenotype.

A limitation of our study was the inability to perform all experiments in all patients, as the yield of PBMCs was not always sufficient, owing to the small volume of paediatric blood samples, which has also prevented us from investigating the role of each AIH associated HLA molecule independently. [A further constraint is the age difference between JAILD patients and healthy controls, due to the current UK policy of not authorizing sampling healthy children.](#) Nevertheless, we demonstrate a direct link between HLA disease predisposition genes and [defective](#) immunoregulation not only in JAILD patients, but also in their FDRs, who are prone to the development of autoimmune disorders. Further investigation to explore how the interaction between HLA genes, non-HLA genes and the environment contribute to immunoregulation impairment, loss of tolerance and overt liver disease manifestation is warranted.

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Figure Legends:

Figure 1. Distribution of HLA class II alleles and ancestral haplotype. (A) Percentage of HLA class II alleles HLA-DR3, DR7, DR13 in JAILD patients (n=68), compared to FDRs (n=60) and HCs (n=48). (B, left) Frequency of the combined HLA-DR3/DR7/DR13 in the three groups. (B, right) Frequency of autoimmunity predisposing haplotype A1-B8-DR3 in the three groups.

Figure 2. Frequency of CD4^{pos} or CD8^{pos} T-cells in peripheral blood mononuclear cells according to HLA alleles. (A) Percentages of CD4^{pos} or CD8^{pos} T-cells in PBMCs from JAILD patients (n= 56), FDRs (n= 51) and HCs (n= 46); (B,C) percentages of CD4^{pos} or CD8^{pos} T-cells in HLA-DR3/DR7/DR13 positive or negative and A1-B8-DR3 positive or negative subjects.

Figure 3. Regulatory T-cell frequency versus HLA alleles. (A-C) Frequency of Tregs in JAILD patients (n= 42), FDRs (n=37) and HCs (n=47) overall, and sub-divided in HLA-DR3/DR7/DR13 positive or negative (B), and A1-B8-DR3 positive or negative (C) subjects. (D) Suppressive function of CD4^{pos}C25^{pos}CD127^{neg/low} Tregs in JAILD patients (n= 18), FDRs (n= 14) and HCs (n= 9).

Figure 4. Expression of immune check point molecule programmed cell death-1 (PD-1). (A-C) Percentages of CD4^{pos}PD-1 or CD8^{pos}PD-1 cells among total CD4^{pos} or CD8^{pos} T-cells from patients (n= 53), FDRs (n= 46) and HCs (n= 46), divided in HLA-DR3/DR7/DR13 positive or negative (B), and A1-B8-DR3 positive or negative (C) subjects.

Figure 5. Plasma soluble programmed cell death-1 (sPD-1) and pro-inflammatory cytokine levels and their correlation. (A) Left panel: plasma sPD-1 levels in JAILD patients (n=27), FDRs (n=25) and HCs (n=24) overall and according to possession of HLA-DR3/DR7/DR13 (middle panel), or A1-B8-DR3 (right panel). (B-C) Plasma IFN- γ and IL-17 levels in JAILD patients, FDRs and HCs overall and according to HLA profiles. (D) Correlation between IFN- γ and PD-1 in JAILD patients (n=27), FDRs (n=25) and HCs (n=24).

Figure 6. Circulatory immune regulatory cytokines and their relative ratios compared to IFN- γ and IL-17. (A-C) Plasma levels of IL-4, IL-10 and IL-2 in JAILD patients (n=27), FDRs (n= 25) and HCs (n= 24) overall and in relation to possession of HLA-DR3/DR7/DR13 or A1-B8-DR3. (D and E) Ratios of IFN- γ /IL-10 and IFN- γ /IL-2 in JAILD patients (n=27), FDRs (n= 25) and HCs (n= 24).

Figure 7. Regulatory T-cell frequency in family members with (n=7) or without (n=25) autoimmune disease (AID) compared to HCs (n=34).