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# The association between HLA genes and autoimmune liver disease

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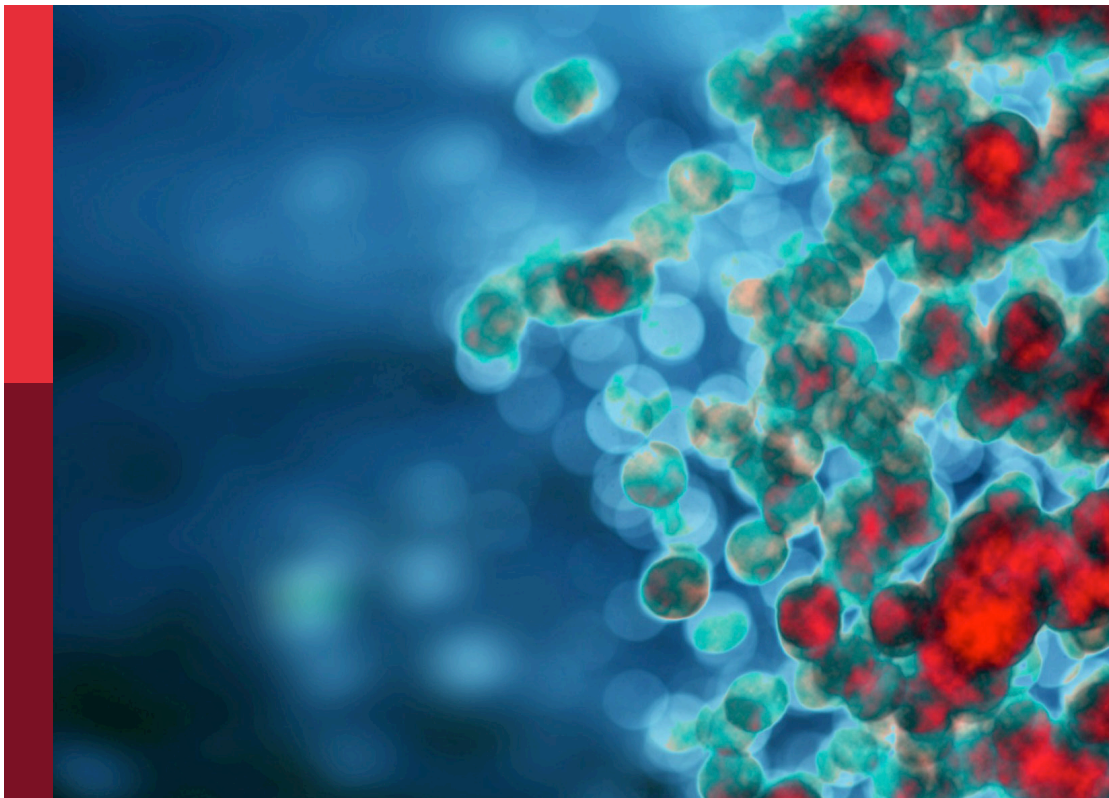

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# The association between HLA genes and autoimmune liver disease

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# Editorial: The association between HLA genes and autoimmune liver diseases

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Editorial on the Research Topic
The association between HLA genes and autoimmune liver disease

This Research Topic aims to establish the links between HLA and non-HLA genes in autoimmune liver disease (AILD) in a diverse population that is inclusive of different ages, races and ethnicities. This is translational with original research articles as well as state of the art mini-reviews summarizing current knowledge. In this collection, we included four laboratory-based investigations from Asia and Turkey, located Western Asia and southeastern Europe, Southwestern and Far East Asia; and 6 mini-reviews from United States, United Kingdom/Europe, South America and Canada. In a large cohort of patients with primary biliary cholangitis (PBC) from China, Huang et al., reported that four disease predisposition HLA genes, *DRB1*08:03, DRB1*07:01, DRB1*14:05, and DRB1*14:54* were present in high frequency and majority had already developed cirrhosis, further confirming the link between HLA genes and liver disease severity. In addition, they reported a link between HLA genes and Lactobacillus, indicating possible role of microbiota in disease progression. Mulinacci et al. have focused their work on studying the immunogenetics of PBC, both HLA associated and non-HLA associated in the pre-genome wide association studies (GWAS) and post GWAS eras. The role of environment in increasing the risk of developing PBC was elucidated by studies in North-east of England where there was a higher prevalence in urban areas with strong coal-mining heritage and in New York city clusters of PBC were found in patients living in zip codes that contained or were adjacent to toxic waste sites. They also discuss using Antigen specific immunotolerance – tolerogenic vaccines - as well as utilizing nanotechnology to develop tolerogenic immune modifying nanopeptides as a therapeutic strategy in PBC.

Using metabolomics, Yang et al., identified inosine as a metabolite with immunomodulatory effects and noted that it was highly altered between *DRB1*04:05* positive and negative Chinese patients with AIH. Administration of inosine in a murine model of Con-A induced acute AIH demonstrated its protective effect by attenuating hepatocyte apoptosis, preventing oxidative stress and inhibiting the activation and glycolysis of $CD4^{pos}$ T effector cells. This may have potential in the development of future therapy of acute AIH. Lapierre and Alvarez discussed how the main genetic

association in type 1 and type 2 AIH is with HLA class II genes and the HLA DR locus, and that a lesser association with non-HLA genes exists. Studies that implicate single nucleotide polymorphisms (SNPs) in genes including TNF-α, Vitamin D, and AIRE in increasing susceptibility to AIH have been included and they have also highlighted the use of animal models to study the effect of a particular genetic background or specific genetic mutation in the development of AIH. We learned from the investigation by Yuksel et al. that HLA *DRB*11* was most prominent in Turkish patients with AIH. They report that in children with AIH, immunosuppression could severely reduce the number of regulatory B cells (B regs, $CD20^{pos}CD24^{pos}CD38^{pos}$), which actually tended to be at a higher frequency than in healthy controls. On the other hand, immunoregulatory cells such as naïve and activated T regs ($CD4^{pos}FOXP3^{high}CD45RA^{neg}$), fluctuate over time. Tacrolimus was reported to be a B reg-sparing drug. Autoimmune B effectors played a complex role in the pathogenesis of AIH, one of the hallmarks of AIH is positivity for autoantibodies, ANA, SMA in type 1 AIH (AIH-1) and LKM1 in type 2 AIH (AIH-2). AIH-1 and AIH-2 have partially overlapping HLA gene profiling, HLA-DR3 for both subtypes of AIH, HLA-DR7 is unique for AIH-2. While most studies in AIH analyse susceptibility of HLA alleles according to AIH subtypes, Cancado et al. have given an interesting twist by exploring the relationship between genetic markers of susceptibility linked to HLA with individual serological markers of disease, using celiac disease as a prototype.

Although there is still no clear answer to the question whether HLA genes play a decisive role in disease development and severity, Ahuja et al. described an important role played by non-HLA genes in AIH patients, not previously reported from India. They reported association of HLA *DRB1*03* gene and the increased frequency of GG genotype of cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4) CT60 mutation in a cohort of North Indian patients who were anti-SLA positive and poor responders to immunosuppressive therapy. Their investigation has reiterated that both HLA and non-HLA genes play a role in the disease manifestation of AIH. In the review focused on pediatric autoimmune liver diseases, Mack reports data from the largest cohort of children with AIH and autoimmune sclerosing cholangitis (ASC) from Kings College Hospital that showed that the presence of HLA *DRB1*03* conferred the highest risk of AIH and ASC compared to healthy controls. Data from other large cohorts in Germany and South America are included as well as information on HLA genotypes that are protective from AILD. She has suggested several research initiatives including using modern technologies like HLA3D to isolate MHC-restricted autoantigens in AILD as well as designing novel therapies using nanotechnology.

The role of HLA and microbiome in the development of autoimmune liver disease is summarized succinctly by Beretta-Piccoli et al. and colleagues. The fact that 80% of the blood supply to the liver is from the splanchnic circulation, lends credence to the fact that gut microbiome and the many factors that influence it, be it changes in gut permeability, dysbiosis, translocation of microbial products into the circulation allowing molecular mimicry to trigger autoimmune inflammatory responses or simply changes in dietary intake, ethnicity or geography may have a profound influence in the development of autoimmune disease. This is also a fertile area for manipulation and drug discovery. There is a difference between the genetic risk and observed risk of autoimmune liver disease and in a beautifully written minireview illustrated with several tables and figures, Czaja has summarized existing knowledge on how epigenetics could influence not only the development of autoimmune liver diseases but also outcomes by altering key processes like DNA methylation and miRNA (microRNA). This has the potential to be another exciting area of new drug development. In conclusion, we hope that this collection of translational offerings will be of interest to both the clinician and the basic scientist and allow one to get updated on the current status of HLA and non-HLA in relation with AILD, with focus mainly on AIH and PBC, particularly from areas where such data was not previously available.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Epigenetic Aspects and Prospects in Autoimmune Hepatitis

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The observed risk of autoimmune hepatitis exceeds its genetic risk, and epigenetic factors that alter gene expression without changing nucleotide sequence may help explain the disparity. Key objectives of this review are to describe the epigenetic modifications that affect gene expression, discuss how they can affect autoimmune hepatitis, and indicate prospects for improved management. Multiple hypo-methylated genes have been described in the $CD4^+$ and $CD19^+$ T lymphocytes of patients with autoimmune hepatitis, and the circulating micro-ribonucleic acids, miR-21 and miR-122, have correlated with laboratory and histological features of liver inflammation. Both epigenetic agents have also correlated inversely with the stage of liver fibrosis. The reduced hepatic concentration of miR-122 in cirrhosis suggests that its deficiency may de-repress the pro-fibrotic *prolyl-4-hydroxylase subunit alpha-1 gene*. Conversely, miR-155 is over-expressed in the liver tissue of patients with autoimmune hepatitis, and it may signify active immune-mediated liver injury. Different epigenetic findings have been described in diverse autoimmune and non-autoimmune liver diseases, and these changes may have disease-specificity. They may also be responses to environmental cues or heritable adaptations that distinguish the diseases. Advances in epigenetic editing and methods for blocking micro-ribonucleic acids have improved opportunities to prove causality and develop site-specific, therapeutic interventions. In conclusion, the role of epigenetics in affecting the risk, clinical phenotype, and outcome of autoimmune hepatitis is under-evaluated. Full definition of the epigenome of autoimmune hepatitis promises to enhance understanding of pathogenic mechanisms and satisfy the unmet clinical need to improve therapy for refractory disease.

**Keywords: autoimmune, hepatitis, epigenome, chromatin modifications, micro-ribonucleic acids, treatment**

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## 1 INTRODUCTION

Autoimmune hepatitis has genetic risk factors within and outside the major histocompatibility complex (MHC) (1, 2). The genetic risk factors within the MHC affect mainly the predisposition for autoimmune hepatitis. The susceptibility alleles reside on the *HLA-DRB1 gene* where they can vary in association with ethnicity and age (3–9). The genetic risk factors outside the MHC are less established. They are mainly polymorphisms or point mutations that may affect individual pathways within the immune response (cytokine milieu, lymphocyte activation, and cell

migration) (1, 2, 10–18). The major risk-laden loci are present in approximately 50% of patients with autoimmune hepatitis (19), and they do not explain the observed risk of the disease (19–21).

Epigenetics is a burgeoning science that describes molecular modifications and mechanisms that can modulate gene activity without altering the nucleotide sequence of deoxyribonucleic acid (DNA) (22–26). The epigenetic changes have cell type specificity and stability through cell replication (27), and they have been heritable in diverse experimental models (25, 28). Key epigenetic modifications have been described in the nuclear chromatin that can affect gene transcription (29–31), and small non-coding ribonucleic acids are epigenetic agents that can affect translation of the gene product (32, 33). The epigenetic modifications may be induced by environmental cues (34–37), and they have a durability that may contribute to a transgenerational inheritance through the germline (25, 28, 37, 38). Furthermore, the epigenetic changes are modifiable, reversible, and amenable to therapeutic intervention (19, 39–43).

Epigenetics may explain the difference between the genetic risk and observed risk of autoimmune hepatitis, and it may account for individual variations in clinical phenotype and outcome that cannot be explained by the MHC, genetic polymorphisms, or point mutations (39, 44–47). Chromosomal regions may undergo structural adaptations in response to environmental cues that alter DNA transcription (22, 38), and non-coding ribonucleic acids, especially micro-ribonucleic acids (miRNAs), may induce degradation or translational repression of messenger ribonucleic acids (mRNAs) (48–53).

Salient epigenetic effects have already been identified in experimental models and patients with diverse liver diseases, including alcoholic steatohepatitis (54, 55), non-alcoholic fatty liver disease (NAFLD) (56–58), primary biliary cholangitis (PBC) (59–61), primary sclerosing cholangitis (PSC) (62–64), cholangiocarcinoma (62, 65–67), hepatocellular cancer (68, 69), and autoimmune hepatitis (21, 70, 71). They have also been implicated in various non-liver diseases, including systemic lupus erythematosus (SLE) (72, 73), rheumatoid arthritis (74, 75), systemic sclerosis (76, 77), diverse neurodegenerative diseases (78), and various cancers (79–82). Investigations of the epigenetic modifications affecting gene expression in autoimmune hepatitis may improve its management and satisfy an unmet clinical need for more effective therapy of refractory disease (83–85).

The goals of this review are to describe the epigenetic modifications that affect gene expression, examine transgenerational inheritance of epigenetic marks, present the key epigenetic changes in autoimmune hepatitis and other liver diseases, and indicate the prospects that epigenetics will enhance understanding of pathogenic pathways and treatment options in autoimmune hepatitis.

## 2 METHODS

Abstracts were identified in PubMed using the search words "Epigenetic changes in liver disease," "Epigenetic changes in autoimmune hepatitis", "microRNAs in liver disease", and "microRNAs in autoimmune hepatitis". Selected full-length articles constituted the primary bibliography. Selected references cited in the primary sources constituted a secondary bibliography, and a tertiary bibliography was developed from references cited in the secondary bibliography. Several hundred abstracts were reviewed, and the number of full-length articles that were examined was 205.

## 3 EPIGENETIC MODULATION OF GENE TRANSCRIPTION

The transcriptional activity of genes occurs within chromatin (86). Chromatin is composed of histones arranged in octamers and double-stranded DNA that makes 1.65 turns around each octamer (38, 39, 86, 87) (**Figure 1**). Two copies of four core histones (H2A, H2B, H3, and H4) comprise the octamer (38, 86–88), and each DNA-enwrapped octamer constitutes a nucleosome (89). The nucleosomes are linked by a short DNA sequence of 60 base pairs, and the beaded filament is condensed and packaged in the nucleus as chromatin (86). A histone linker molecule maintains proper packaging of the DNA by binding to the site of DNA entry and exit from each nucleosome (86, 87, 90).

### 3.1 Impact of DNA Methylation on Gene Transcription

The methylated state of the DNA (39, 91–93) influences transcriptional activity within the nucleosome. Modifications in the chromatin structure can alter access and binding of transcription factors to the enhancer/promoter sequences of the DNA that are pivotal for transcription (94, 95) (**Figure 1**). The inability of RNA polymerase (RNAP) to access the DNA binding site can prevent opening of the double-stranded DNA and copying of the nucleotide sequence (39, 92).

DNA methylation occurs at a site in which a cytosine nucleotide (C) is separated from a guanine nucleotide (G) by a phosphate molecule (p) (46) (**Table 1**). Methylation of the cytosine in the CpG dinucleotide to 5-methylcytosine is mediated by DNA methyltransferases (DNMTs), and the methylation inhibits the binding of transcription factors to the DNA (91, 92) (**Figure 1**). It can also alter chromatin structure by attracting proteins that bind to the methylated cytosine (46, 92). The net effect of DNA methylation is to repress transcriptional activity and silence gene expression (39).

Ten-eleven translocation methylcytosine dioxygenase (TET) enzymes mediate the oxidation of the methylated cytosine to 5-hydroxymethylcytosine (39, 96, 97, 118, 119) (**Table 1**). This product can then undergo additional processing and demethylation by thymine-DNA-glycosylase and excision repair (118, 120, 121). The restoration of cytosine to its unmodified state can de-repress transcriptional activity and promote gene expression (**Figure 1**). The counter effects of DNMTs and TET enzymes on DNA methylation constitute a homeostatic mechanism that can respond to diverse stimuli, be disrupted in disease states, and be manipulated by therapeutic interventions (39, 122).

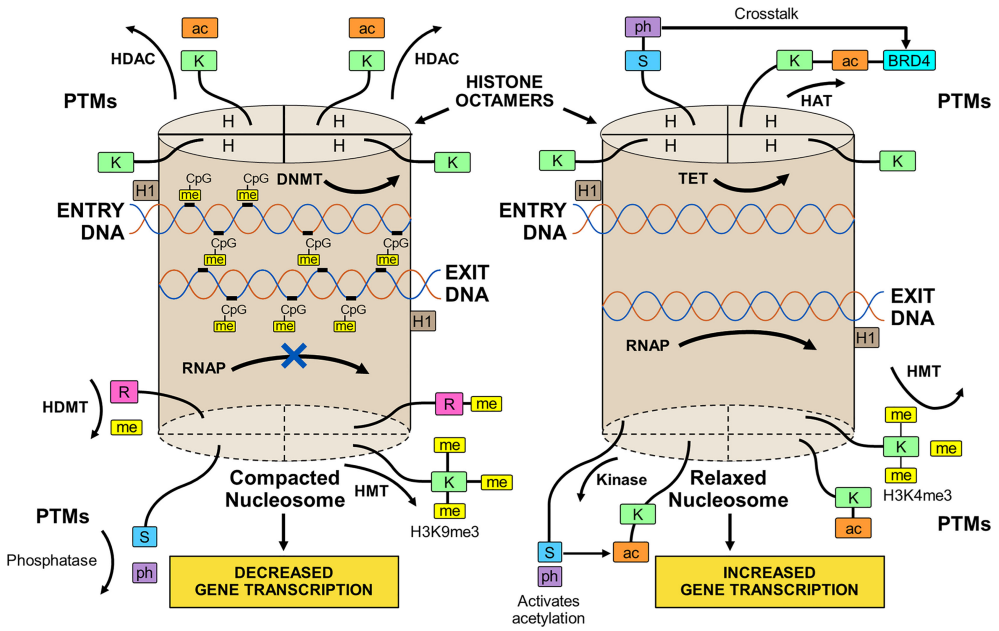


**FIGURE 1** | Compacted and relaxed nucleosomes. Nucleosomes consist of two copies of four different histones (H) arranged as a histone octamer and double-stranded deoxyribonucleic acid (DNA) wrapped 1.65 times around each octamer. The entry and exit of the DNA from the nucleosome is secured by a linker histone (H1). Each core histone within the octamer has an N-terminal tail that can undergo post-translational modifications (PTMs) by the attachment of methyl (me), acetyl (ac), or phosphate (ph) groups to a particular amino acid in the histone tail. Lysine (K), serine (S), or arginine (R) are among other amino acids that can serve as attachment sites. The PTMs are orchestrated by various enzymes. Methylation of the histone tail is catalyzed by histone methyltransferase (HMT); acetylation is catalyzed by histone acetyltransferase (HAT); and phosphorylation is catalyzed by kinases. The PTMs can be reversed by enzymes that dissociate the appended groups from the amino acid residues. Acetylation is reversed by histone deacetylase (HDAC); methylation is reversed by histone demethylase (HDMT); and phosphorylation is reversed by phosphatases. Histone acetylation relaxes the nucleosome and promotes gene transcription, and histone de-acetylation compacts the nucleosome (heterochromatin) and represses gene transcription. Histone methylation can decrease (H3K9me3) or increase (H3K4me3) transcription depending on the methylation site and other variables. Histone phosphorylation can recruit other molecules, such as bromo-domain-containing protein 4 (BRD4), to the acetylation site (crosstalk) and promote gene transcription. DNA can be methylated by DNA methyltransferase (DNMT) or de-methylated by ten-eleven translocation methylcytosine dioxygenase (TET). DNA methylation is restricted to sites in which cytosine (C) is separated from guanine (G) by a phosphate (p). Methylated DNA is compacted and transcription factors have limited access to transcription sites. Ribonucleic acid polymerase (RNAP) is prevented (X) from copying the nucleotide sequence, and gene transcription is decreased. De-methylated DNA is relaxed; RNAP can open the double-stranded DNA; and gene transcription is increased.

## 3.2 Impact of Histone Modifications on Gene Transcription

The N-terminal tail of the core histones can undergo multiple post-translational modifications (PTMs) that include acetylation, methylation, and phosphorylation (39, 86, 113, 123–126) (**Table 1**). The PTMs can alter the chemical structure, charge, and configuration of the histones, and the cumulative effect of multiple histone modifications can determine the transcriptional activity of the DNA (127) (**Figure 1**). PTMs also influence the cellular repair response to DNA injury (128). The modification of histones is a dynamic process that can preserve the integrity of the genome (129) and modulate transcriptional activity to maintain biological homeostasis (39, 86).

### 3.2.1 Histone Acetylation

The transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to a lysine residue on the histone tail constitutes histone acetylation, and the process is mediated by the histone acetyltransferases (HATs) (39, 78, 86, 98, 99) (**Table 1**). Histone acetylation can promote transcriptional activity by neutralizing differences in charge between the positively charged histones and the negatively charged DNA. The relaxed chromatin can promote transcriptional activity (39, 86) (**Figure 1**). Histone deacetylases (HDACs) can reverse the acetylation process by hydrolyzing the acetyl group on the lysine residue, compacting the chromatin into heterochromatin, and repressing transcriptional activity of the DNA (78, 86, 99, 100).

### 3.2.2 Histone Methylation

The transfer of methyl groups from S-adenosylmethionine (SAM) to lysine or arginine residues on the histone tail constitutes histone methylation (101–105), and the methylation process is mediated by histone methyltransferases (HMTs) (86, 104, 128, 130) (**Table 1**). The impact of histone methylation on DNA transcription is less predictable than histone acetylation, and it varies by methylation site (lysine versus arginine), number of methylations (mono-, di-, or tri-methylation) and pattern of methylation (symmetric versus asymmetric) (101, 103, 104, 128). Trimethylation of histone

**TABLE 1** | Epigenetic properties and effects on gene transcription.

| Epigenetic Mark | Epigenetic Properties | Epigenetic Effects on Transcription |
|---|---|---|
| DNA methylation | CpG methylated at cytosine (46) | Transcriptional activity repressed (39) |
| | DNMT catalyzes 5-methylcytosine (91) | |
| | Attached proteins alter chromatin (92) | |
| | Transcription factors denied access (94) | |
| | RNAP unable to copy DNA (39, 92) | |
| DNA de-methylation | Cytosine demethylation by TETs (96, 97) | Transcriptional activity increased (39) |
| Histone acetylation | Lysine on histone tail acetylated (39, 86) | Transcriptional activity increased (86) |
| | Acetyl group from acetyl-CoA (98, 99) | |
| | HATs mediate acetyl group transfer (78) | |
| | Histone-DNA charges less (86) | |
| | Chromatin structure relaxed (39, 86) | |
| Histone de-acetylation | HDACs hydrolyze acetyl group (86) | Transcriptional activity repressed (86) |
| | Heterochromatin formed (100) | |
| Histone methylation | Methyl groups from SAM (101–105) | Unpredictable transcriptional effect (86) |
| | Added to lysine or arginine (101, 103, 105) | Varies by site, number, pattern (86, 101) |
| | HMTs catalyze methyl transfer (86, 104) | H3K4me3 activates transcription (111) |
| | No effect on charge of histone tail (86) | H3K9me3 silences transcription (106) |
| | Recruited molecules affect gene (106–110) | |
| Histone de-methylation | HDMTs remove methyl groups (104, 112) | Unpredictable transcriptional effect (86) |
| | Counterbalances HMTs (86) | Responds to changing conditions (86) |
| Histone phosphorylation | Phosphates from ATP by kinases (113) | Variable, context-related effects (114) |
| | Affects serine, threonine, tyrosine (113) | DNA damage response (114, 116, 117) |
| | Adds negative charge to histone (113) | Interacts with acetylated residues (114) |
| | Compacts or relaxes chromatin (114, 115) | |
| | Reversed by phosphatases (113) | |

*ATP, adenosine triphosphate; CpG, cytosine-phosphate-guanine dinucleotide; DNA, deoxyribonucleic acid; DNMTs, DNA methyltransferases; H3K4me3, trimethylation of histone H3 at lysine 4; H3K9me3, trimethylation of H3 at lysine 9; HATs, histone acetyltransferases; HDACs, histone deacetylases; HDMTs, histone demethylases; HMTs, histone methyltransferases; RNAP, RNA polymerase; SAM, S-adenosylmethionine; TETs, ten-eleven translocation enzymes. Numbers in parentheses are references.*

H3 at lysine 4 (H3K4me3) is the start site of transcription for most active genes (111, 128, 131, 132) (**Figure 1**), whereas trimethylation of H3 at lysine 9 (H3K9me3) is associated with heterochromatin and gene silencing (106, 128, 132).

Histone methylation does not affect the charge of the histone tail, and the impact of histone methylation on transcriptional activity relates mainly to the effects of molecules recruited to the methylated state and the sequence of adjacent amino acids (86, 107). Lysine methylation attracts diverse proteins mainly with chromo-domains that can modify chromatin structure and affect DNA transcription (108–110). Histone demethylases (HDMTs) can reverse the methylated PTM by removing methyl groups from the histone tails (86, 104, 112). The balance between HMTs and HDMTs is another homeostatic mechanism by which the genome can respond to changing conditions.

### 3.2.3 Histone Phosphorylation

Histone phosphorylation is a dynamic process affecting serine, threonine, and tyrosine residues in the N-terminal tail of the core histones (113) (**Table 1**). Kinases transfer phosphate groups from adenosine triphosphate (ATP) to the amino acid residues. Phosphorylation adds a negative charge to the histone, and the change in charge can remodel the chromatin. The phosphorylation process can be reversed by phosphatases that catalyze the hydrolysis and removal of the phosphate group (113) (**Figure 1**).

Histone phosphorylation occurs rapidly after DNA damage, and it is involved in the DNA damage response (DDR) (114, 116, 117) (**Table 1**). Phosphorylated histone residues are also associated with gene expression, including proto-oncogenes (133–135), and they can interrelate with histone residues that are acetylated to activate DNA transcription (114, 136, 137) (**Figure 1**). Phosphorylation of serine 10 at histone H3 (H3S10ph) activates DNA transcription by triggering acetylation of lysine 16 at histone 4 (H4K16ac) (138). The crosstalk between histone PTMs recruits bromo-domain-containing protein 4 (BRD4) to the nucleosome where it can bind to the acetylated lysine residue and promote DNA transcription (138–140).

The phosphorylation of histone is a rapidly changing process that can have contradictory effects depending on the context of the microenvironment (114). Histone phosphorylation is associated with chromatin compaction during mitosis and meiosis, but it can also be associated with chromatin relaxation under other circumstances (114, 115, 141). Therapeutic efforts to modulate histone phosphorylation must recognize the dynamic, interactive, labile, and context-dependent nature of the PTM.

## 4 EPIGENETIC MODULATION OF GENE TRANSLATION

MiRNAs are a subgroup of non-coding RNAs that by definition do not encode protein (142) (**Table 2**). They constitute a functional minority of non-coding RNAs (143), and they are members of a class that includes small interfering RNAs

**TABLE 2** | Epigenetic Properties and Effects of Micro-Ribonucleic Acids.

| miRNA Properties | miRNA Actions | miRNA Effects |
|---|---|---|
| Non-coding RNA (142–146)<br>•Small (21-25 nt) (48, 50)<br>•Derived from genome (48)<br>•Present in organelles (147) | Prevents mRNA translation (48)<br>Regulates cell processes (148) | Maintains cell homeostasis (147)<br>Responds to changing context (147) |
| Circulatory component (149)<br>•Vesicular transport (150, 151) | Cell-to-cell communication (149)<br>May affect other cell function (152) | Correlates with inflammation (70) |
| Diverse cell origins (153)<br>•>500 in humans (154)<br>•Multiple cell origins (153)<br>•One targets many genes (155)<br>•Many target same gene (156) | Critical physiological effects (157)<br>Context-cell dependent (157, 158)<br>Affects protein-encoding genes (50)<br>Specific for certain cell lines (159) | Variable disease specificity (153)<br>Many are disease-irrelevant (153)<br>May have distinctive patterns (70)<br>Associated with diseases (71, 160, 161) |
| Complex biogenesis (149, 162)<br>•Nuclear origin (149)<br>•Enzymatic processing (163)<br>•Exported to cytoplasm (164)<br>•Processed in RISC (51)<br>•Guide strand selected (165)<br>•Incorporated into RLC (165)<br>•Non-canonical pathways (166–168) | Guide strand seeks mRNA (155)<br>Binds 3' UTR of mRNA (51, 155) | Depends on complementarity (169)<br>Promotes mRNA degradation (169)<br>Represses mRNA translation (149)<br>Gene silencing (169) |

*mRNA, messenger ribonucleic acid; miRNA, micro-ribonucleic acid; nt, nucleotides; RNA, ribonucleic acid; RISC, RNA-induced silencing complex; RLC, RISC-loading complex; 3' UTR, 3' untranslated region of mRNA. Numbers in parentheses are references.*

(siRNAs) (144) and Piwi-interacting RNAs (piRNAs) (145, 146). MiRNAs are small (21-25 nucleotides), natural, genomic products that have multiple functions within their cell of origin (48, 50). They are present in the nucleus, nucleolus, and mitochondria where they can influence the intracellular processes of DNA transcription, repair, and splicing (147, 148, 170, 171). They can also silence the expression of genes that encode protein by preventing the translation of mRNA into a

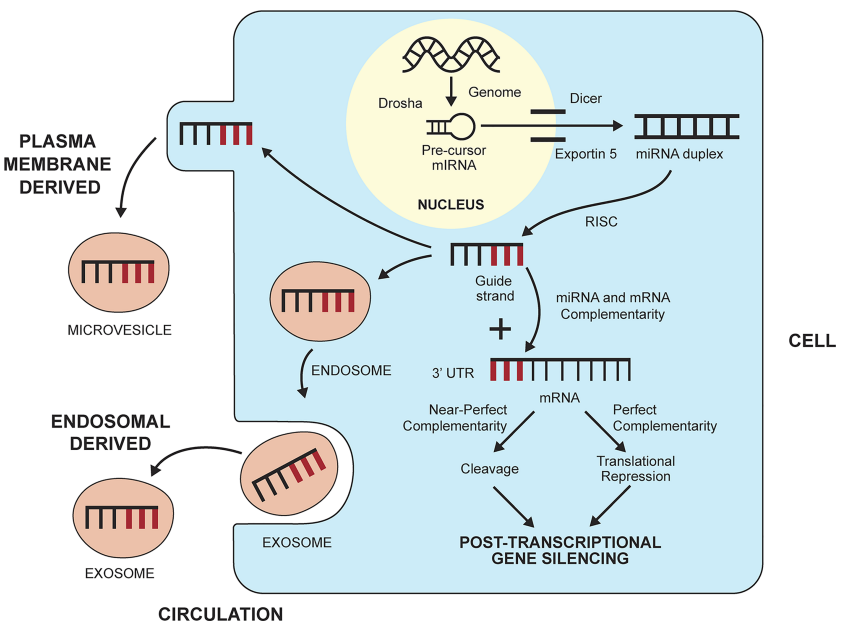


**FIGURE 2** | Biogenesis and gene silencing action of micro-ribonucleic acids (miRNAs). MiRNAs are derived from the cell genome and processed within the nucleus by the ribonuclease III enzyme, Drosha, into pre-cursor miRNA. The precursor miRNA is transported to the cytoplasm by exportin 5 and processed further by the ribonuclease II enzyme, Dicer, to a miRNA duplex. The duplex is processed in a RNA-induced silencing complex (RISC), and the strand with less stable 5' end is selected as the guide strand. The guide strand probes for complementary base pairs (bold lines) in the 3' untranslated region (3'UTR) of messenger RNA (mRNA). The degree of complementarity between the guide strand and the mRNA determines if the mRNA will undergo cleavage by endonucleases (perfect complementarity) or translational repression (near perfect complementarity). Either fate induces post-transcriptional gene silencing. MiRNAs can leave the cell and enter the circulation by forming a plasma membrane-derived microvesicle or an endosomal-derived exosome.

protein product (48, 50, 51) (**Figure 2**). MiRNAs are key epigenetic agents that act primarily outside the chromatin to degrade mRNA within the cytoplasm or otherwise repress its translation.

MiRNAs can enter the circulation within vesicles that develop from the endosomal compartment (exosomes) or separate from the plasma membrane (microvesicles, apoptotic bodies) (150, 151) (**Figure 2**). Circulating miRNAs have the potential to engage in cell-to-cell communication and affect the function of other cells, albeit their role in this capacity remains obscure (149, 152) (**Table 2**). Despite this limitation, circulating levels of miRNAs have been measured and correlated with the inflammatory activity of diverse diseases, including autoimmune hepatitis (70).

The number of miRNAs in humans has been estimated by a manually curated miRNA database as over 500 (154) (**Table 2**). Multiple miRNAs can regulate the expression of a single gene, and a single miRNA can influence multiple genes (155, 156). Diverse cell types produce miRNAs, and tissue and disease specificity can be difficult to demonstrate (153). Critical physiological and pathological effects have been ascribed to single miRNA-mRNA interactions that are context-dependent (157, 158), and certain miRNAs have been highly specific for individual cell lines (159). Preferential expression of particular miRNAs has been recognized in diverse diseases, including chronic liver disease (70, 71, 160, 161).

### 4.1 Biogenesis and Regulatory Actions of MiRNAs

MiRNAs originate in the nucleus as double-stranded RNA molecules that are encoded by the genome as primary miRNAs (149, 162) (**Table 2**). The primary miRNAs are then modified in the nucleus by a microprocessor complex containing the ribonuclease III enzyme, Drosha, to precursor miRNAs (149, 163, 172, 173) (**Figure 2**). The precursor miRNAs are exported to the cytoplasm by exportin 5 where the ribonuclease II enzyme, Dicer, modifies the precursor molecules further to form mature miRNA duplexes (164, 174). The duplexes are processed in a RNA-induced silencing complex (RISC) within the cytoplasm (51), and the strand with the less stable 5' end is selected for incorporation in the RISC-loading complex (RLC) as the guide strand (165). The other strand (passenger strand) is degraded by endonucleases (175).

The guide strand probes for complementary base pairs in the 3' untranslated region (3' UTR) of mRNAs in the cytoplasm (**Figure 2**). The "seed region" that identifies complementarity in the mRNA may consist of only 2-7 bases (51, 155). Near perfect complementarity between the miRNA and the mRNA triggers degradation of the mRNA by endonucleases and complete gene silencing (169) (**Table 2**). More commonly, the complementarity is less complete, and the miRNA mainly disrupts the translation of mRNA without triggering its degradation (translational repression) (51, 149). MiRNAs can also develop along non-canonical pathways that do not involve Drosha or Dicer (166–168). The biological functions of these miRNAs are uncertain in humans.

## 5 TRANSGENERATIONAL INHERITANCE OF EPIGENETIC MARKS

The DNA sequence and the epigenome are replicated during cell mitosis (25, 27), and DNA methylation (176), histone PTMs (38), and miRNAs (177) can be transmitted in the germline of mammals. Extensive re-programming of the epigenetic information occurs during gametogenesis and after fertilization, and transgenerational inheritance requires re-assembly or reconstruction of the epigenetic marks. DNA methylation and histone modifications can be re-assembled after mitosis (replicative transmission) or the epigenetic changes can be reconstructed in the germline by another inherited signal (reconstructive transmission) (28). Non-coding RNAs are templates that are pivotal to the reconstructive process, and they can be transmitted to the next generation in oocytes and sperm (28, 178, 179). Transgenerational inheritance requires proof that the original epigenetic signal is successfully transmitted and that heritability extends beyond the second generation.

The transmitted epigenetic changes may reflect environmental adaptations made by the parent and transmitted to the offspring through the germline (28, 35, 180, 181). The offspring of male mice who have been fed a low-protein diet inherit epigenetic marks that affect the *peroxisome proliferator-activated receptor alpha (PPARA) gene* which regulates lipid and cholesterol metabolism (180). The heritable epigenetic changes may also re-program responses to disease (182). Two generations of offspring from male rats with a history of liver fibrosis have inherited a resistance to hepatic fibrosis manifested by impaired differentiation of hepatic myofibroblasts, increased expression of the anti-fibrotic peroxisome proliferator-activated receptor-gamma (PPAR-γ) protein, and decreased production of the pro-fibrotic transforming growth factor beta 1 (TGF-β1) cytokine (182).

The demonstration of heritable epigenetic marks has been difficult to establish in humans because of confounding genetic, cultural, and environmental factors (37, 183), and heritability has been eliminated from the definition of epigenetics (22). Epigenetic changes within an individual may be acquired by external pressures (diet, lifestyle, toxic exposures) (184–187) or by intrinsic instability of the epigenome through successive cell divisions ("epigenetic drift") (188–192). Shared changes in the somatic epigenome of individuals in the same environment does not connote heritability unless expressed in the germline (sperm or egg) (25). Furthermore, the epigenetic marks in individuals with genetic identity cannot be assumed to be inherited. Genetically identical monozygotic twins may acquire epigenetic changes that are distributed throughout the genome and related to the commonality or diversity of their environment (187).

Family studies assessing discordant and concordant phenotypes have demonstrated the complexity of distinguishing inherited and acquired determinants. Fatty liver occurs in 17% of siblings and 37% of parents of overweight children (193). The severity of hepatic steatosis in the family members strongly correlates with body mass index (BMI) (193). Complete hereditability for fatty liver is evident after adjustments for age, gender, race, and BMI, but the phenotypic expression of the inherited risk probably relates to family attitudes

about diet and exercise (193). Similarly, heritable miRNAs for NAFLD (miR-331-3p and miR-30c) have been demonstrated in monozygotic and dizygotic twins, but most of the 21 miRNAs that have distinguished the twins with and without NAFLD have not been inherited (194). Although transgenerational inheritance of epigenetic marks has been demonstrated in experimental models (28, 180, 182) and humans (26, 37, 194), its impact on the occurrence of an individual disease is unsettled (35). Large longitudinal studies over several generations are necessary to establish the heritability of epigenetic changes in particular human diseases, and they would require concurrent analyses of the genome and epigenome (37, 183, 195).

# 6 EPIGENETIC CHANGES IN AUTOIMMUNE HEPATITIS

Investigations of the epigenetic changes in patients with autoimmune hepatitis have been limited, and they have focused mainly on DNA methylation patterns in circulating and liver-infiltrating lymphocytes (21) and on the profile of circulating miRNAs (70).

## 6.1 DNA Methylation Patterns

Most genes in the circulating $CD4^+$ and $CD19^+$ T lymphocytes of untreated patients with autoimmune hepatitis have been hypo-methylated, and this pattern has contrasted with the hyper-methylated pattern in PBC (21) (**Table 3**). The predominant hypo-methylated pattern has also been recognized in liver-infiltrating, periportal lymphocytes, and it has been reversible after glucocorticoid-induced, laboratory remission (21). The shift in the pre-treatment pattern of DNA hypo-methylation to the post-treatment pattern of DNA hyper-methylation has occurred in most genes, and it suggests that DNA hypo-methylation promotes disease activity by broadly enhancing the transcriptional activity of multiple genes. The cues that trigger the hypo-methylated state, the hypo-methylated genes that account for active disease, and the glucocorticoid actions that shift the methylation status and achieve remission are unclear.

## 6.2 MiRNA Profiles

Circulating levels of miR-21 and miR-122 have been increased in untreated patients with type 1 autoimmune hepatitis (70, 71, 196), and miR-155 has been increased in hepatic tissue (71, 196, 199) (**Table 3**). The serum miR-21 and miR-122 levels have correlated with serum alanine aminotransferase (ALT) levels, and the serum miR-21 level has correlated with the histological grade of liver inflammation (70). The histological expression of miR-21 in liver tissue has also correlated with serum ALT levels (196).

In contrast, the serum levels of both miR-21 and miR-122 have correlated inversely with the stage of hepatic fibrosis (70), and reduced hepatic concentrations of miR-122 have been associated with cirrhosis (196) (**Table 3**). MiR-122 markedly attenuates the expression of the gene for prolyl-4-hydroxylase subunit alpha-1 (*P4HA1*) in hepatic stellate cells (197), thereby preventing the hydroxylation and maturation of stable collagen (198). The findings in autoimmune hepatitis suggest that serum miR-21 and miR-122 levels are biomarkers of inflammatory activity (206) and that a pathological deficiency of miR-122 may promote hepatic fibrosis by de-repressing *P4HA1* (196, 197).

**TABLE 3** | Epigenetic marks in autoimmune hepatitis and other autoimmune liver diseases.

| Autoimmune Liver Disease | Epigenetic Marks | Epigenetic Effects |
|---|---|---|
| Autoimmune hepatitis | Hypo-methylated genes in $CD4^+$ T cells (21) | Contrasts with PBC (21) |
| | Mainly in periportal lymphocytes (21) | May increase gene transcription (21) |
| | Shifts to hyper-methylated with steroids (21) | May promote disease activity (21) |
| | | Reversible with steroid treatment (21) |
| | Serum miR-21 and miR-122 increased (70, 71) | Correlates with inflammation (70) |
| | | Inversely correlates with fibrosis (70) |
| | Hepatic miR-122 reduced in cirrhosis (196) | Deficiency promotes fibrosis (196) |
| | | miR-122 inhibits *P4HA1* in HSCs (197) |
| | | Deficiency favors collagen formation (198) |
| | Circulating miR-155 levels low (199) | Contrasts with ALD and NASH (199) |
| | miR-155 increased in liver tissue (199) | May indicate autoimmune process (199) |
| PBC | Preferential silencing of X chromosome (200) | Affects female predisposition (200, 201) |
| | Excessive silencing Y chromosome (201) | |
| | De-methylation of gene for CXCR3 (59) | Affects hepatic migration of T cells (59) |
| | H4 acetylation of pro-inflammatory genes (202) | Influences inflammatory activity (202) |
| | Hypo-methylation of gene for CD40L (203) | Promotes B cells, IgM production (203) |
| | miR-122, miR-141, miR-26 panel (160) | High diagnostic accuracy for PBC (160) |
| | Down-regulation of miR-223 and miR-21 (204) | Signals histological progression (204) |
| PSC | H3K4me3 of *CDKN2A* (63, 205) | Increases cholangiocyte senescence (63) |
| | | Possible disease progression (63) |
| | H3K27ac of *BCL2-like 1* (64) | Increases anti-apoptotic BCL-xL (64) |
| | | Promotes survival of senescent cells (64) |

*ALD, alcoholic liver disease; BCL2-like 1, B-cell lymphoma 2-like 1 gene; BCL-xL, B-cell lymphoma-extra large; CD40L, CD40 ligand; CDKN2A, cyclin-dependent kinase inhibitor 2A gene; CXCR3, C-X-C chemokine receptor 3; H3K4me3; trimethylation of H3 at lysine 4; H3K27ac, acetylation of H3 at lysine 27; H4, histone 4; HSCs, hepatic stellate cells; IgM, immunoglobulin M; NASH, non-alcoholic steatohepatitis; P4HA1, prolyl-4-hydroxylase subunit alpha-1 gene, PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis. Numbers in parentheses are references.*

Circulating levels of miR-155 have been significantly lower in patients with autoimmune hepatitis regardless of glucocorticoid therapy than in normal individuals (199) (**Table 3**). In contrast, miR-155 concentrations in liver tissue from patients with autoimmune hepatitis have been 7.6 ± 5.6-fold higher than in liver tissue obtained from normal control subjects ($P< 0.01$) and significantly higher than in liver tissue from patients with alcoholic liver disease or non-alcoholic steatohepatitis (NASH) (199). The findings suggest that the hepatic expression of miR-155 in autoimmune hepatitis is particularly associated with immune-mediated liver injury. This possibility is supported by the implication of miR-155 in the pathogenesis of other autoimmune diseases (207). The discrepancy between serum and tissue levels may reflect active mobilization of miR-155 from the circulation to the liver.

### 6.3 Familial Occurrence

The heritability of autoimmune hepatitis through epigenetic traits is unexplored. The familial occurrence of autoimmune hepatitis in Sweden has been mainly among siblings (208, 209) and spouses (208). Among 6269 patients with autoimmune hepatitis in a Swedish database, only siblings have had a significantly increased risk [standardized incidence ratio (SIR), 3.83, 95% confidence interval (CI), 2.09-6.45] (208). Furthermore, the risk for autoimmune hepatitis has been greater among spouses than among siblings (SIR for husbands, 5.91, 95% CI, 2.53-11.7; SIR for wives, 6.07 (95% CI, 2.59-12.02) (208). The risk of autoimmune hepatitis among siblings and spouses in Sweden suggests that epigenetic changes induced by environmental factors may be contributory.

The SIR of autoimmune hepatitis among first-degree relatives has been 4.9 (95% confidence interval [CI], 1.8-10.7) in a Danish database, and the 10-year cumulative risk of autoimmune hepatitis in this group has been 0.10% (95% CI, 0.04-0.23) (210). Among second-degree relatives, there has been no increased risk, whereas among monozygotic twins, the concordance rate for autoimmune hepatitis has been 8.7% (95% CI, 1.1-28) (210). In the composite experience of 32 medical centers in the Netherlands, familial occurrence has been recognized in 0.3% of 564 patients with autoimmune hepatitis, and the disease has occurred in monozygotic twins, the mother of a patient, and the cousin of another patient (211). In each of these experiences, the overall risk of autoimmune hepatitis in family members has been low; heritability has rarely extended beyond the first generation; shared environmental exposures have not been assessed; and the contribution of shared genetic factors has not been evaluated. Differences in the community occurrence of autoimmune hepatitis might also be valuable in assessing non-genetic factors for the disease.

## 7 EPIGENETIC CHANGES IN OTHER AUTOIMMUNE LIVER DISEASES

Histone modifications, DNA methylation status, and miRNAs in blood and liver tissue have been evaluated in experimental models and patients with diverse autoimmune and non-autoimmune liver diseases (19, 39, 86). The investigations have been driven by efforts to catalogue the disease-associated findings and identify associations with pivotal pathogenic mechanisms. Key insights have been derived from studies of PBC (212–214) and PSC (63, 64, 212), and they may prompt and direct future investigations of autoimmune hepatitis (19, 21, 70). The epigenetic changes have not been evaluated for disease-specificity nor have they been fully translated into clinical phenotypes.

### 7.1 Epigenetic Findings in PBC

The epigenetic changes described in PBC have been discovered mainly by assessing factors influencing its clinical phenotype (212–214). The importance of epigenetic changes has been demonstrated in monozygotic twins concordant (215) and discordant (216) for PBC, and the female predisposition for PBC has guided investigations of the epigenetic influence on the X-chromosome. A preferential, parent-specific, silencing of the X chromosome has been described in women with PBC (200), and an excessive epigenetic silencing of alleles of the Y chromosome has been demonstrated in men with PBC (201). Furthermore, an aberrant DNA methylation pattern of the promoter region of *CXCR3* on the X chromosome of $CD4^+$, $CD8^+$, and $CD14^+$ T cells may affect their differentiation and hepatic migration (59) (**Table 3**). The acetylation of histone 4 in the promoter region of diverse pro-inflammatory genes can enhance their expression in PBC (202), and DNA hypo-methylation of the gene expressing the CD40 ligand (CD40L, also called CD154) in $CD4^+$ T cells may promote B cell maturation and immunoglobulin class switching. The epigenetic effect may contribute to the increased serum levels of immunoglobulin M (IgM) in PBC (203).

A panel of miRNAs, including miR-122-5p, miR-141-3p, and miR-26b-5p, has had high diagnostic accuracy for PBC and a sensitivity that has exceeded that of the serum alkaline phosphatase level (160) (**Table 3**). Step-down expression of miR-223-3p and miR-21-5p in peripheral blood B cells has signaled histological progression of PBC from stage I to stage III (204), and decreased levels of the molecules involved in the biogenesis of miRNAs (prolyl 4-hydroxylase subunit alpha 1 and Argonaute 2) have suggested a widespread disruption of the homeostatic network in a murine model of PBC (61). This hypothesis has been supported by experimental evidence that non-selective stimulation of miRNA biogenesis with enoxacin can up-regulate miRNA production in $CD8^+$ T cells, decrease T cell proliferation, and reduce interferon-gamma (IFN-γ) production (61).

### 7.2 Epigenetic Findings in PSC

The epigenetic factors contributing to the progression of PSC have focused mainly on factors influencing the phenotype of the cholangiocytes. Senescent cholangiocytes, defined as cells that have been irreversibly arrested in the G1 or G2 phase of the cell cycle (217, 218), are abundant in the liver of patients with PSC (205) (**Table 3**). The cholangiocytes exhibit features of a senescence-associated secretory phenotype (SASP) that is characterized by the hypersecretion of pro-inflammatory

cytokines, chemokines and growth factors (205, 219). The *cyclin-dependent kinase inhibitor 2A (CDKN2A) gene* has been associated with cholangiocyte senescence (205), and histone methylation (H3K4me3) increases its transcriptional activity and the possibility of disease progression (63). Histone acetylation (H3K27ac) of the promoter of the *B-cell lymphoma 2-like 1 gene (BCL2-like 1)* increases expression of the anti-apoptotic protein, B-cell lymphoma-extra large (BCL-xL). This epigenetic change may promote the resistance of senescent cholangiocytes to apoptosis and prolong their survival (64). Both sites of histone modification have been proposed as potential therapeutic targets (63, 64).

The studies in PBC and PSC affirm the strong association of epigenetic modifications in immune-mediated chronic liver disease, and they suggest that the epigenetic modifications can impact on the clinical phenotype, reflect disease-specificity, aid in diagnosis, and direct future therapeutic interventions. They also identify key areas in autoimmune hepatitis that have been unassessed or under-evaluated. Investigations of the epigenetic effects on the X and Y chromosomes, familial predisposition, and heritability of autoimmune hepatitis are wanting.

# 8 EPIGENETIC FINDINGS IN NON-AUTOIMMUNE LIVER DISEASES

Studies in alcoholic liver disease (54, 55) and non-alcoholic fatty liver disease (NAFLD) (220–229) have emphasized the pervasive, interactive, and composite effects of epigenetic modifications in each disease. They have also indicated the needs to associate changes in disease expression to clinically relevant features and to explore the heritable and adaptive nature of the epigenetic modifications. These insights are foundational for future studies in autoimmune hepatitis since they may clarify the mechanisms of occurrence, recurrence and progression.

## 8.1 Epigenetic Findings in Alcoholic Liver Disease

A plethora of epigenetic changes involving DNA methylation, histone modification, and circulating miRNA levels have been described in experimental models and patients with alcoholic liver disease and alcoholic steatohepatitis (54, 55). Epigenetic modifications have been demonstrated in genes that may influence the metabolism of ethanol (230–234), the activity of enzymes that mediate histone acetylation (HATs, sirtuins) (55, 235), the vigor of the inflammatory response (236), and the generation of hepatic fibrosis (224, 237) (**Table 4**). Furthermore, increased circulating levels of several miRNAs have been described that may be biomarkers of alcohol-related liver injury (miR-155) (55, 238, 241, 242), indicators of a disrupted intestinal mucosal barrier (miR-212) (239), or mediators of lipid and cholesterol metabolism (miR-122) (240). The abundance of epigenetic changes and interactions has indicated a complexity that must be edited for clinical relevance. A similar complexity of epigenetic interactions can be anticipated in autoimmune hepatitis, and future investigations must be directed by the pivotal clinical needs to understand and control the severity, progression, and recurrence of the disease.

## 8.2 Epigenetic Findings in NAFLD

Hypermethylation of CpG99 in the regulatory region of the *patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene* and hypomethylation of CpG26 in the regulatory region of the *parvin beta 1 (PARVB1) gene* have

**TABLE 4** | Epigenetic marks in non-autoimmune liver diseases.

| Non-Autoimmune Liver Disease | Epigenetic Marks | Epigenetic Effects |
|---|---|---|
| Alcoholic liver disease | DNA methylation changes (54, 55) | Modulation of ethanol metabolism (234) |
| | Histone PTMs (54, 55) | Mediation of inflammation (236) |
| | Multiple genes affected (54, 55) | Progression of liver fibrosis (224, 237) |
| | | Activity of histone acetylation (55, 235) |
| | Increased circulating miR-155 (238) | Biomarker of alcohol injury (55, 241, 242) |
| | Increased circulating miR-212 (239) | Denotes disrupted intestinal barrier (239) |
| | Increased circulating miR-122 (240) | Mediates lipid metabolism (240) |
| NAFLD | Hyper-methylated *PNPLA3* (220) | Hepatic steatosis and inflammation (221, 222) |
| | Hypo-methylated *PARVB1* (220) | Hepatic fibrosis, steatosis, activity score (223) |
| | Variably DNA methylated genes (224) | Severe hepatic fibrosis (224) |
| | Histone acetylation of *TNFA* (225) | Increased inflammation (225) |
| | Histone acetylation of *FASN* (226) | Up-regulated lipogenesis in hepatocytes (226) |
| | Increased circulating miR-122, miR-34a, and miR-16 (227) | miR-122, miR-34a associated with lipid levels, fibrosis stage, and inflammation (227) |
| | High serum miR-122 levels (228) | Increased serum ALT activity (228) |
| | Low liver miR-122 levels (228, 229) | Associated with NASH (228, 229) |
| | mir-331-3p and miR-30c strongly associated with each other (194) | Lipid and metabolic pathways (194) |
| | Present in twins with NAFLD (194) | |
| | May be heritable (194) | |

*ALT, alanine aminotransferase; DNA, deoxyribonucleic acid; FASN, fatty acid synthase gene; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PARVB1, parvin beta 1 gene; PNPLA3, patatin-like phospholipase domain-containing protein 3 gene; PTMs, post-translational modifications; TNFA, tumor necrosis factor alpha gene. Numbers in parentheses are references.*

been associated with advanced hepatic fibrosis in patients with NAFLD (220) (**Table 4**). The rs738409 (G-allele) polymorphism of *PNPLAS* has been associated with hepatic steatosis and inflammation in patients with NAFLD (221, 222), and the *PARVB* variant has been associated with steatosis grade, NAFLD activity score, and hepatic fibrosis (223). Differential methylation of CpG sites within other genes known to affect hepatic fibrosis have also distinguished patients with severe fibrosis (224).

Histone acetylation of the pro-inflammatory genes, *tumor necrosis factor alpha* (*TNFA*) and *monocyte chemotactic protein 1* (*MCP1*; also called *CD2*) have been up-regulated in a murine model of obesity (225), and the histone acetylation of the gene stimulating transcription of fatty acid synthase (*FASN*) has been associated with *de novo* lipogenesis in human hepatocytes (226) (**Table 4**). Serum levels of miR-122, miR-34a and miR-16 have also been increased in patients with NAFLD compared to patients with chronic hepatitis C, and the serum levels of miR-122 and miR-34a have correlated with biochemical tests and histological assessments of fibrosis stage and inflammatory activity (227).

Serum levels of miR-122 have also been 7.2-fold higher in patients with non-alcoholic steatohepatitis (NASH) than in healthy control subjects and 3.1-fold higher in patients with NASH than in patients with simple steatosis (228) (**Table 4**). Hepatic expression of miR-122 has been down-regulated in NASH compared to patients with simple steatosis (228) or normal liver (229), and the hepatic expression of miR-122 has been mostly near lipid-laden hepatocytes (228). The physiological significance of miR-122 in the development of NASH has been postulated, but not evident in all investigations (243) or validated as a pivotal pathogenic factor (194).

Studies of monozygotic and dizygotic twins have demonstrated that discordance for NAFLD has been associated with 21 miRNAs, including miR-122 ($P$=0.002) and miR-34a ($P$=0.04) (194) (**Table 4**). MiR-331-3p ($P$=0.0007) and miR-30c ($P$=0.011) have been preferentially expressed in the twins with NAFLD, and the strong correlation of miR-331-3p and miR-30c with each other (R=0.90, $P$=2.2 x $10^{-16}$) has suggested their shared involvement in NAFLD (194). This hypothesis has been supported by evidence that the seven gene targets shared by miR-331-3p and miR-30c have included genes affecting lipid and metabolic pathways (194).

The multiplicity of epigenetic changes associated with NAFLD may reflect differences in environmental cues (lifestyle, diet, age-related exposures, surgeries) (184, 187, 193) and transgenerational inheritance of gene modifiers (25, 38, 194). The profiling of the epigenome of sperm from lean and obese men has disclosed marked differences in the expression of small non-coding RNA and DNA methylation patterns which may have reflected inherited and acquired changes (184) (**Table 4**). The rapid remodeling of DNA methylation in the sperm of morbidly obese men who have undergone bariatric surgery has indicated the dynamic plasticity of epigenetic changes under environmental pressure (184). The challenge has been to identify the key factor or combination of factors that can be moderated in a particular clinical situation. The plasticity of the epigenetic changes in response to environmental cues or therapeutic intervention and the expression of these epigenetic responses in the germline are key features that warrant investigation in autoimmune hepatitis.

# 9 EPIGENETIC MANIPULATIONS

Therapeutic manipulation of disease-associated epigenetic changes is possible by interventions that affect the enzymes that modify the chromatin structure, the targets recognized by circulating miRNAs, and the environmental factors that promote instability of the epigenome (39, 86, 244). Interventions that affect enzymatic modulation of the chromatin structure have been directed at DNA methylation (245), histone methylation (246, 247), and histone acetylation (248–250). Interventions that affect target recognition by pivotal miRNAs have involved engineered molecules that mask the chosen gene product or substitute a decoy (251–256). Interventions that stabilize the epigenome have included risk-reduction, lifestyle modifications (19, 34, 257) and dietary supplementation with S-adenosylmethionine (258, 259), methyl group donors (260, 261), vitamin C (122), or vitamin D (262–267). The major concerns have been the lack of target selectivity and the uncertain risk of deleterious off-target consequences (19, 43).

## 9.1 Therapeutic Modulation of Chromatin Structure

DNMT inhibitors, HDAC inhibitors, HDAC activators, and HMT inhibitors have been the principal interventions directed at the enzymatic bases for disease-associated epigenetic changes in chromatin. These interventions have been studied mainly in experimental models of liver disease and patients with malignancy (39, 86, 244) (**Table 5**).

### 9.1.1 DNA Methyltransferase Inhibition

DNA hyper-methylation has been a strong feature of hepatocellular carcinoma (HCC), and guadecitabine (also called SGI-110) is a DNMT inhibitor. Guadecitabine has sensitized HCC cells to oxaliplatin by inhibiting signaling pathways that have promoted HCC growth in mice (245).

### 9.1.2 Histone Deacetylase Inhibition

HDACs have been highly expressed in patients with HCC related to chronic hepatitis B virus infection, and they have been a prognostic biomarker associated with tumor growth and reduced survival (268). HDAC inhibition has suppressed proliferation of HCC cells *in vitro* (268), and the pan-HDCA inhibitor, panobinostat, has been effective in experimental models of HCC when combined with sorafenib (249). HDAC inhibitors have been well-tolerated in clinical protocols, and trials have been extended to non-tumorous diseases, including

**TABLE 5** | Therapeutic manipulations of epigenome.

| Enzymes | Rationale | Experimental and Clinical Experience |
|---|---|---|
| DNMT inhibitors | DNA hyper-methylation in HCC (245) | Guadecitabine limits HCC in mice (245) |
| HDAC inhibitors | HDACs high in HBV-related HCC (268) | HDAC inhibitors limit HCC *in vitro* (268) |
| | HDAC inhibitors tolerated in trials (269) | Panobinostat effective in HCC model (249) |
| HDAC activators | Deficient sirtuin 1 in NAFLD model (270) | Resveratrol limits rodent NAFLD (272) |
| | Resveratrol activates sirtuin 1 (271–274) | Resveratrol no effect in humans (250) |
| HMT inhibitors | EZH2 catalyzes histone methylation (275) | DZNep inhibits liver fibrosis in mice (247) |
| | H3K27me3 represses *PPARG* (276) | |
| | *PPARG* inhibition increases fibrosis (277) | |
| **miRNA targets** | | |
| Anti-sense oligonucleotides | Limits miRNA binding to mRNA (278) | Clinical trials in diverse diseases (280–282) |
| | Preserves mRNA (71, 279) | |
| Decoy mRNA targets | Decoy mRNA binds miRNA (254–256) | Experimental models (254–256) |
| | "Sponge effect" depletes miRNA (255) | |
| Drugs | General miRNA deficiency possible (61) | Enoxacin down-regulated CTLs in murine PBC (61) |
| | Drugs can enhance miRNA biogenesis (61) | |
| **Supplements** | | |
| SAM | Methyl groups improve methylation (259) | Less demethylase activity in cell lines (258) |
| | Dietary methyl groups helped in rats (261) | Preserved DNA methylation (259) |
| Vitamin C | Supports activity of TET enzymes (122) | De-methylated DNA in mouse cells (122) |
| Vitamin D | Limits transcription of TGF-β, TIMP (266) | Prevents experimental fibrosis (262–265) |

*CTLs, cytotoxic $CD8^+$ T cells; DNA, deoxyribonucleic acid; DNMT, DNA methyltransferase; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC, histone deacetylases; HMT, histone methyltransferase; mRNA, messenger ribonucleic acid; miRNA, micro-ribonucleic acid; NAFLD, non-alcoholic fatty liver disease; PPARG, peroxisome proliferator-activated receptor gamma gene; PBC, primary biliary cholangitis; SAM, S-adenosylmethionine; TGF-β, transforming growth factor-beta; TET, ten-eleven translocation enzyme; TIMP, tissue inhibitors of metalloproteinases. Numbers in parentheses are references.*

neurodegenerative diseases and inflammatory disorders (269) (**Table 5**).

### 9.1.3 Histone Deacetylase Activation

Sirtuin 1 (SIRT1) promotes the deacetylation of histones and regulates glucose and fat metabolism (270, 283). Deficient hepatic expression of SIRT1 has been accompanied by metabolic dysfunction in a murine model (270). The polyphenol, resveratrol, activates the deacetylase, SIRT1 (248, 284, 285), and it has improved the survival of mice on a high calorie diet (286) (**Table 5**). Resveratrol has also protected rodents from diet-induced steatohepatitis through a variety of signaling pathways (271–274). Resveratrol has not had a therapeutic benefit in overweight and obese men with established NAFLD (250), and the role of HDAC activation as a protective or therapeutic intervention for NAFLD remains unclear in humans.

### 9.1.4 Histone Methyltransferase Inhibition

Epigenetic modifications of chromatin have been implicated in the trans-differentiation of hepatic stellate cells into myofibroblasts (39), and the enzymes that regulate the methylation of DNA (287, 288) and histone (246) have been prime therapeutic targets (247, 277). Hepatic fibrosis is regulated by a series of epigenetic relays that include down-regulation of miR-132, binding of the methyl-CpG binding protein 2 (MeCP2) to the 5' end of the PPAR-γ-producing gene (*PPARG*), and activation of the enhancer of zeste homolog 2 (EZH2) (277) (**Table 5**).

EZH2 is an epigenetic regulator that represses gene transcription by catalyzing the trimethylation of histone 3 at lysine 27 (H3K27me3) (275, 289, 290). The formation of H3K27me3 in the 3' exon of *PPARG* represses the anti-fibrotic effect of this gene (276) and promotes hepatic fibrosis (277) (**Table 5**). Therapeutic disruption of the pro-fibrotic epigenetic pathway is possible at multiple sites, but the pivotal epigenetic step for myofibroblast differentiation is trimethylation of *PPARG* at H3K27 (276). 3-Deazaneplanocin A (DZNep) is a pan-inhibitor of histone methyltransferase, and its use in a murine model of toxin-induced liver injury has inhibited the histological progression of hepatic fibrosis (247).

## 9.2 Therapeutic Modulation of MiRNAs

MiRNAs are prime targets for therapeutic manipulation because circulating miRNA levels have distinguished certain diseases and the gene silencing action of miRNAs can disrupt pivotal homeostatic pathways that regulate immune and inflammatory responses (40, 42, 43). The principal method of targeting miRNAs in experimental models and patients in clinical trials has been the use of anti-sense oligonucleotides (antimirs) (71, 278, 279) (**Table 5**). These molecules are engineered to block the binding of a selected miRNA to its targeted mRNA, and they prevent the miRNA from silencing the gene product. The binding affinity, stability, and potency of antimirs can be enhanced by diverse modifications of the core molecule. The modified molecules have been designated antagomirs (252, 253, 291). Anti-sense obligonucleotides have been evaluated in clinical treatment trials for Alport syndrome (280), chronic hepatitis C (281), and chronic lymphocytic leukemia (282).

RNA transcripts have also been designed to mimic the selected natural mRNA and protect it from degradation or

translational repression by miRNAs. The decoy mRNA binds with the natural miRNA and prevents it from silencing the natural mRNA (254–256) (**Table 5**). Drugs have also been used to non-selectively stimulate the biogenesis of miRNAs (61). Widespread deficiency of miRNAs may allow the expression of genes that promote disease activity, and drug-induced, non-selective stimulation of miRNA biogenesis may silence the expression of these deleterious genes (61). These interventions await rigorous preclinical evaluations and clarification of their safety profile (43).

### 9.3 Therapeutic Modulation of Environmental Factors and Use of Dietary Supplements

Multiple environmental factors have been associated with diverse epigenetic changes, and lifestyle modifications may reduce the risk of disease-provoking epigenetic changes (19, 34, 58, 257, 292, 293) (**Table 5**). Medications (procainamide, hydralazine, and 5-azacytidine) (294–297), pollutants (tobacco smoke, aerosolized contaminants, and heavy metals) (298–301), and infection (302) have been associated with changes in DNA methylation that may affect gene expression. Furthermore, environmentally-induced epigenetic changes have been associated with the occurrence or progression of diverse immune-mediated diseases (rheumatoid arthritis, PBC, and SLE) (72, 298, 303–305). Epigenetic changes that are potentially deleterious and heritable have also been associated with nutritional deficiencies, stress, ultraviolet light, radiation, and trauma (19, 34, 257). Lifestyle modifications that avoid excessive, high risk exposures may protect against deleterious epigenetic effects, but their efficacy has been difficult to establish.

Dietary supplements have also been described in experimental animals that enhance the supply of methyl groups (S-adenosylmethionine, diverse methyl donors) (258–261), activate the TET enzymes that de-methylate DNA (vitamin C) (122), and alter the transcription of mRNAs that promote hepatic fibrosis (vitamin D) (262–267) (**Table 5**). S-adenosylmethionine has inhibited demethylase activity and preserved DNA methylation in cell lines (258, 259). Dietary supplementation with methyl groups has promoted DNA hyper-methylation and prevented transgenerational amplification of obesity in a mouse model (260). It has also modified the methylation profile of the gene expressing fatty acid synthase and reduced hepatic triglyceride accumulation in rats fed a high fat, high sucrose diet (261). Vitamin C has supported the activity of TET enzymes, and it has promoted the de-methylation of DNA in the embryonic stem cells of mice (122). 1, 25-dihydroxyvitamin D has repressed the transcription of mRNAs for TGF-β and tissue inhibitors of metalloproteinases (TIMP). It has also up-regulated the transcription of metalloproteinases and prevented progressive hepatic fibrosis (262–267). These promising pre-clinical experiences await validation in randomized clinical trials that define their utility in specific diseases (259).

## 10 EPIGENETIC PROSPECTS IN AUTOIMMUNE HEPATITIS

Findings that have already been made in diverse autoimmune (63, 64, 212–214) and non-autoimmune (220, 223–226, 232) liver diseases support the prospect that multiple, clinically-relevant, epigenetic marks will be identified in autoimmune hepatitis. They also support the prospect that pivotal genes affecting critical pathogenic pathways will be recognized and that interventions will be assessed to modify an aberrant gene expression or pattern (247, 250, 260, 261, 271, 281). The success of these projections in changing the management of autoimmune hepatitis will depend on proofs of causality, confident identification of critical gene expressions or patterns, and precise editing of the epigenetic landscape.

### 10.1 Proofs of Causality

Progress toward targeted epigenetic management of autoimmune hepatitis ideally requires proof of causality for each epigenetic mark and a hierarchy of candidates based on measured consequences. Methods that disrupt and restore the epigenetic mark in experimental models or cell systems can establish and quantify causality. The clustered, regularly interspaced, short palindromic repeats (CRISPR) of base sequences in segmental DNA and the CRISPR-associated protein 9 (Cas9) system consists of a guide RNA that matches the DNA target site and an endonuclease (Cas9) that performs site-specific DNA cleavage (306, 307) (**Table 6**). This system has been re-purposed for epigenetic editing by engineering a "deactivated" Cas9 protein (dCas9) that lacks nuclease activity (309–312, 320, 321). The CRISPR-dCas9 system can target specific DNA loci without changing the DNA sequence, and dCas9 can deliver sequence-specific motifs to a desired location in the epigenome (308). Site-specific epigenetic editing that can block or restore gene expression in experimental models or cell lines can prove causality and develop a hierarchy of candidates for therapeutic targeting.

### 10.2 Identification of Critical Gene Targets or Patterns

A distinctive profile of circulating miRNAs (70, 71, 196, 199) and the hypo-methylation of multiple genes (21) have already been described in autoimmune hepatitis. Future investigations must identify the genes whose expressions are affected by these miRNAs (miR-21, miR-122, and miR-155) and the hypo-methylation (**Table 6**). The hypo-methylated *forkhead box p3 (Foxp3) gene* stabilizes the expression of Foxp3 on regulatory T cells (Tregs) and maintains their integrity (319). Preservation of this hypo-methylated state may constitute a mechanism by which to achieve and maintain quiescent disease (322, 323). Hypo-methylation may also stimulate genes with deleterious actions, and treatments that hyper-methylate genes non-selectively may compromise Treg function (322). Clarification of the genes implicated in autoimmune hepatitis by the circulating miRNAs and their hypo-methylated status will be essential in understanding the complexity and interactivity of potential epigenetic targets.

**TABLE 6** | Epigenetic prospects in autoimmune hepatitis.

| Epigenetic Prospects | Rationale | Expectations |
|---|---|---|
| Proofs of causality | Epigenetic marks lack proofs of causality (308)<br>CRISPR-dCas9 allows epigenetic editing (309)<br>Site-specific editing can prove causality (308)<br>Hierarchy of targets possible (310–312) | Prime therapeutic target(s) selected (308) |
| Identification of gene targets or patterns | miR-21 is cue to affected genes (70) | Key gene prospects of miR-21 assessed:<br>• *programmed cell death protein 4* (313)<br>• *TNF-α-induced protein 8-like 2* (314) |
| | miR-122 is cue to affected genes (70, 196) | Key gene prospects of miR-122 assessed:<br>• *hypoxia inducible factor 1-α* (315)<br>• *prolyl-4-hydroxylase subunit α-1* (197) |
| | miR-155 is cue to affected genes (199, 207) | Key gene prospects for miR-155 assessed:<br>• *suppressor of cytokine signaling* (316)<br>• *c-musculoaponeurotic fibrosarcoma* (317)<br>• *Src homology 2-containing inositol-5'-phosphatase 1* (318) |
| | Hypo-methylated genes already recognized (21) | Key hypo-methylated prospect assessed:<br>• *forkhead box p3 (Foxp3)* (319) |
| | Multiple genes can have composite effect (21)<br>Gene patterns can affect outcome (194) | Epigenetic network recognized (19) |
| Therapeutic epigenetic editing | CRISPR-dCas9 edits precisely (309–312, 320, 321) | Individual and multiple edits possible (312)<br>Elimination of non-selective enzymes (308)<br>Site-specific enzyme delivery (308, 312)<br>Correction of miRNA deficiencies<br>Modulation of miRNA gene expression<br>Homeostasis of stimulatory/inhibitory genes |
| | Uncertain off-target effects (43) | Highly selective, precise edits (308, 312)<br>Rigorous safety assessments<br>Monitoring protocols |

*CRISPR, clustered, regularly interspaced, short palindromic repeats; dCas9, deactivated CRISPR-associated protein 9; miRNA, micro-ribonucleic acid; TNF, tumor necrosis factor. Numbers in parentheses are references.*

Additional characterization of the epigenome of autoimmune hepatitis can be anticipated, and it may identify multiple up- and down-regulated genes that have a composite effect. Multiple gene expressions have distinguished patients with NAFLD (194), and multiple hypo-methylated genes have been described in autoimmune hepatitis (21). The multiplicity of implicated genes may reveal a pattern that distinguishes autoimmune hepatitis and influences its phenotype and outcome. The pattern may also reveal a common basis for autoimmunity or have disease-specificity.

### 10.3 Editing the Epigenetic Landscape

The CRISPR-dCas9 system promises to replace the use of enzymes that non-selectively alter DNA methylation and PTMs (309–312, 320, 321) (**Table 6**). It may also limit or eliminate the need to target miRNAs with anti-sense oligonucleotides (71, 252, 278, 279) or mRNA mimics (254–256). HATs, acetyl groups, DNMTs, and TET enzymes can be tethered to the dCas9 protein and delivered to the chosen epigenetic site by the CRISPR-dCas9 system (312, 320). The effectiveness of the CRISPR-dCas9 system in editing the epigenome of autoimmune diseases is unknown, but future investigations should evaluate its ability to edit multiple epigenetic marks, restore homeostatic balance between immune stimulatory and inhibitory genes, and modulate the genes that generate particular miRNAs. The major safety concern is the uncertainty of unintended off-target effects (34, 43).

## 11 CONCLUSIONS

The epigenome is a largely unstudied domain in autoimmune hepatitis, and its rigorous evaluation may yield results that complement, complete, or change the current knowledge base. The epigenome is dynamic, reactive, adaptable, reversible, and potentially heritable. The epigenetic landscape could influence the predisposition, phenotype, pathogenesis, and outcome of autoimmune hepatitis, and it could reflect environmental factors that can be modified or avoided. The epigenetic landscape could also have diagnostic and prognostic implications that could help direct management. Methods that allow highly selective editing of the epigenome promise to expand treatment options by modulating the expression of pivotal genes or the composite effect of multiple genes. The key challenges are to determine the pivotal epigenetic changes or patterns associated with autoimmune hepatitis, understand the interactive network of genes with opposing actions that promote the disease, and develop interventions that restore homeostatic balance with minimal risk of unintended off-target consequences.

## AUTHOR CONTRIBUTIONS

AC researched, designed, and wrote this article. The tables and figure are original, constructed by the author, and developed solely for this review.

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# Metabolic heterogeneity caused by HLA-DRB1*04:05 and protective effect of inosine on autoimmune hepatitis

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Autoimmune hepatitis (AIH) is an autoimmune disease caused by disruption of liver immune homeostasis. Genetic studies have revealed the predisposition of AIH with the human leukocyte antigen (HLA) region. Recently, metabolomics integrated with genomics has identified many genetic loci of biomedical interest. However, there is no related report in AIH. In the present study, we found that HLA-DRB1*04:05 was linked to the clinical features and prognosis of AIH in Chinese patients. Furthermore, our patients were divided into DRB1*04:05 positive and DRB1*04:05 negative groups and the metabolic profiling was done by HPLC/MS. We chose inosine, one of the highly altered metabolites, to explore the effect on an acute severe hepatitis murine model. The results showed that inosine treatment attenuated hepatocyte apoptosis, enhanced antioxidant ability and inhibited the activation and glycolysis of $CD4^+$ T cell. We propose that inosine participates in the regulation of AIH through its protective effect on hepatocytes and inhibition of overactivated immune cells, which might provide a potential novel approach in treating acute form of AIH.

KEYWORDS

**autoimmune hepatitis, HLA-DRB1*04:05, metabolomics, inosine, $CD4^+$ T cell**

## Introduction

Autoimmune hepatitis (AIH) is a rare chronic progressive liver disease characterized by antibodies, hypergammaglobulinemia, and interface hepatitis. Although the etiology and pathogenesis remain unclear, the genetic and environmental factors play an important role in the development of the disease. Genetic studies have found that genetic predisposition to AIH is associated with the human leukocyte antigen (HLA) region (1). Increasing research has identified HLA-DR3 or -DR4 as the most convincing disease susceptibility locus (2). HLA-DRB1*03:01 and DRB1*04:01 are associated with

AIH in European populations (3). HLA-DRB1*04:05 is considered to increase susceptibility to AIH in Japanese and Korean populations (4–6). The results showed that DRB1*04:05 was significantly associated with elevated serum IgG and anti-smooth muscle antibody positivity, which established the role of HLA in the progression of AIH. However, the precise mechanisms have not been sufficiently revealed.

Metabolomics has developed rapidly as a new omics technique after genomics, transcriptomics, and proteomics. Metabolites are intermediates and final products of cellular activities, and their levels can be considered the result of the interaction between the genome, transcriptome, and proteome (7). To date, many disease-related metabolomics have been performed to define the different metabolites between each group and facilitate the characterization of different pathological conditions (8–11). Li et al. identified several cirrhosis-associated metabolites in AIH and revealed the potential of drug regulation metabolism in the treatment of AIH (10). Recently, the integration of genetics and metabolomics has identified many genetic loci of biomedical interest (12). Kirchberg et al. conducted a prospective study to investigate the association between the metabolic profile and HLA-risk in celiac disease patients (11). However, there is no report on the difference in metabolites of AIH patients related to the HLA allele.

In this study, we performed HLA-DRB1 genotyping on Chinese AIH patients and divided the patients into DRB1*04:05 positive and negative groups. HPLC/MS was used to detect the difference in metabolic profiles between the two groups. Inosine, reported as a metabolite with immunomodulatory effects (13, 14), was found to be highly altered between the DRB1*04:05 positive and negative groups. There are few reports on inosine in AIH. To explore the effect of inosine, we used a concanavalin A (Con A)-induced murine model. Inosine treatment ameliorated liver damage by attenuating hepatocyte apoptosis and increasing antioxidant ability. Furthermore, inosine inhibited the activation and glycolysis of $CD4^+$ T cells to impede the inflammatory response (Figure 1). The current study revealed HLA DRB1*04:05 related metabolic heterogeneity and provided the potential function of inosine in acute form of AIH.

# Materials and methods

## Patients and healthy volunteers

In the present study, a total of 74 peripheral blood samples were collected from patients with AIH between January 2019 and November 2021 at the West China Hospital of Sichuan University. The diagnostic criteria adhered to the International Autoimmune Hepatitis Group (1999) guidelines (15, 16). Among all patients, 72 patients had AIH-1 and 2 patients had AIH-2. Peripheral whole blood was available for HLA genotyping (Weihe Biotechnology Inc. Nanjing, China). The clinical characteristics of these patients were listed in Supplementary Table S1. In addition, 48 peripheral blood samples were randomly selected to detect metabolic profiles and their clinical characteristics were listed in Supplementary Table S2. The whole study was approved by the Independent Ethics Committee of West China Hospital and conducted in accordance with the relevant principles.

## HPLC–MS/MS analysis

Serum from patients was prepared by centrifugation of whole blood at 2000 g for 10 min., placed in the Eppendorf tubes and resuspended in prechilled 80% methanol by well vortexing. Then the samples were incubated on ice for 5 min and centrifuged at 15000 g and 4°C for 20 min. Subsequently, the samples were transferred to a fresh Eppendorf tube, centrifuged at 15000 g for 20 min, and injected into the LC-MS/MS system for analysis. LC–MS/MS analyses were performed using an ExionLC™ AD system (SCIEX) coupled with a QTRAP® 6500+ mass spectrometer (SCIEX) in Novogene Co., Ltd. (Beijing, China). MRM (multiple reaction monitoring) was used to detect the experimental samples. These metabolites were annotated using the KEGG database (http://www.genome.jp/kegg/), HMDB database (http://www.hmdb.ca/) and Lipidmaps database (http://www.lipidmaps.org/). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed at metaX. The metabolites with VIP > 1.0 and P value < 0.05 and fold change > 1.2 or < 0.833 were regarded as differential metabolites. The functions of these metabolites and metabolic pathways were studied using the KEGG database.

## Animals

Female C57BL/6J mice (aged 8-10 weeks; weighing 20-22 g) were obtained from the Experimental Animal Center of Sichuan University (Chengdu, China). All animal experiments were approved by the Animal Ethics Committee of West China Hospital, Sichuan University. The ConA-induced murine model was established according to the previous study (17). After 7 days of adaptive feeding, all mice were randomly assigned to three groups as follows: (1) NC group: mice were given an intravenous injection of sterile saline as a control; (2) Con A group: mice were administrated Con A (Sigma–Aldrich, St. Louis, MO, United States) intravenously at a dose of 10 mg/kg body weight; (3) Con A + Inoine (Ino) group: Mice were injected with 300mg/kg body weight inosine (MedChemExpress, Monmouth Junction, NJ, USA) and administered with an equal volume of Con A. Inosine was dissolved in sterile saline. All mice were sacrificed at 24 h after injection for sample collection.

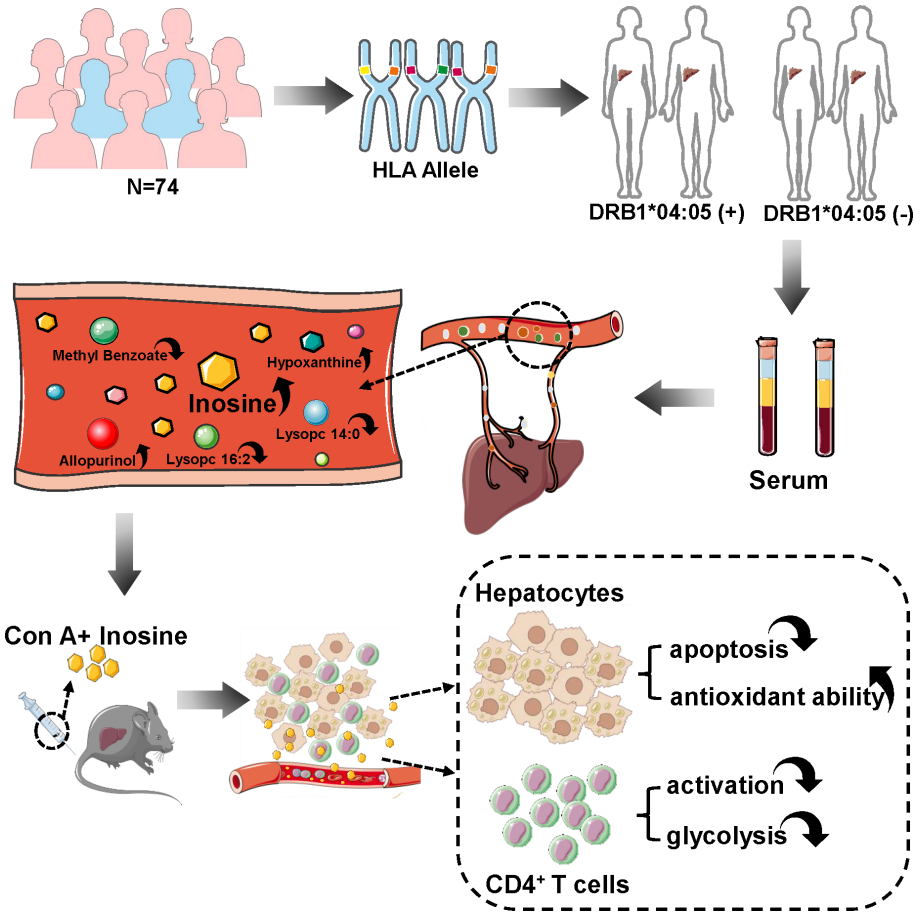


FIGURE 1
Overview of this study. 74 patients were recruited and their blood was collected for HLA genotyping. These patients were divided into DRB1*04:05 positive and negative groups to detect serum metabolic profiles. Inosine is a highly alerted metabolite. Con A-induced hepatitis murine model was used to explore the effect of inosine. After injection, inosine treatment was found to attenuate hepatocyte apoptosis, enhance antioxidant ability, and inhibit the activation and glycolysis of $CD4^+$ T cells.

## Cell culture

Splenic mononuclear cells were isolated from mice by mouse lymphocyte separation medium (Dakewei, Shenzhen, China) as previously described (17). Single-cell suspensions were separated by mechanical disruption of mouse spleens through 70-μm cell strainers and were harvested by gradient centrifugation at 800 g for 30 min, and washed in PBS twice for the experiment. Purified $CD4^+$ T cells were negatively selected using the MojosortTM Mouse $CD4^+$ T Cell Isolation kit (Biolegend, San Diego, CA, USA). The purity of $CD4^+$ T cell was > 95% (Supplementary Figure S1A), and the cells were cultured in RMPI 1640 medium with recombinant murine IL-2 (20 IU/ml, Novoprotein, Shanghai, China). The cells were stimulated with plate-bound anti-CD3 (2.5 μg/ml, LEAF™ Purified anti-mouse CD3ε, Biolegend, San Diego, USA) and soluble anti-CD28 (2.5 μg/ml, LEAF™ Purified anti-mouse CD28, Biolegend, San Diego, USA), and the cells were incubated for 24 h for further experiments.

## Flow cytometric analysis

The liver mononuclear cells were collected and suspended in PBS as described previously (18). In general, the liver cell suspension was centrifuged at 30 g for 5 min. Supernatants were collected, washed in PBS, and resuspended in 40% Percoll (Solarbio, Beijing, China). Furthermore, the cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 800 g. Liver mononuclear cells were collected from the interphase, washed twice in PBS, and resuspended in PBS for FACS analysis. The expression of cell surface molecules was detected by staining with antibodies against CD3 (APC-Cy7,

Biolegend, San Diego, USA), CD4 (FITC, Biolegend, San Diego, USA), CD8 (Percp-Cy5.5, Biolegend, San Diego, USA), CD25 (PE, Biolegend, San Diego, USA), CD69 (APC, Biolegend, San Diego, USA), and DAPI (Biolegend, San Diego, USA), diluting each antibody according to the manufacture's instruction.

## Liver function assay and cytokine assay

Retroorbital blood samples were collected from mice in each group and centrifuged at 1000 g for 10 min. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were detected by an automatic dry biochemical analyzer (Hitachi, Tokyo, Japan). The levels of IFN-γ and TNF-α were measured using mouse ELISA detection kits according to the manufacturer's recommendations (Dakewei, Shenzhen, China). The final density values were measured at 450 and 570 nm by a microplate reader (BioTek, Winooski, VT, United States). In addition, the supernatant of the cell culture was collected by centrifugation at 300 g for 10 min. The levels of cytokines were analyzed by ELISA kits according to the manufacturer's instructions (MultiSciences, Hangzhou, China).

## Histopathology assay and tunnel staining

Liver tissues were fixed in 4% formalin and embedded in paraffin. The samples were sliced into 3-4 μm sections, followed by dewaxing and rehydration. Furthermore, the sections were stained with hematoxylin and eosin (H&E) to observe the level of tissue damage by light microscopy (Servicebio, Wuhan, China). Six optional fields per section were selected to calculate the histological score. The injury score (Suzuki's criteria) is recognized as the degree of liver injury with three indicators, including sinusoidal congestion, hepatocyte necrosis and ballooning degeneration (19). TUNEL staining was carried out with a TUNEL BrightRed Apoptosis Detection Kit (Biossci, Wuhan, China) and observed under a fluorescence microscope. The average number of $TUNEL^+$ cells in six fields of each section was counted and used to calculate the ratio of apoptotic cells. All the slides were evaluated by at least three professional researchers in a double-blind assessment.

## Measurement of oxidative and antioxidant biomarkers

The serum concentration of malondialdehyde (MDA) was measured by an MDA assay kit (Beyotime Biotech, Shanghai, China) according to the manufacturer's instruction. The serum level of $Fe^{2+}$ was determined by a spectrophotometric method and quantified at a wavelength of 586 nm. The ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) was detected by a commercial GSSG/GSH quantification kit (DOJINDO, Tokyo, Japan). The luminescence of all above was detected by the luminometer (PerkinElmer, MA, United States).

## Cell proliferation

Cell proliferation was measured using the CCK-8 assay. $CD4^+$ T cells were seeded in 96-well plates at 500,000 cells/well and stimulated with CD3/CD28. After 24 stimulations, 20 ul CCK-8 reagent (Beyotime Biotech, Shanghai, China) was added to each well and incubated for 4 h at 37°C away from light. The optical density (OD) values were determined using a microplate reader at 450 nm. Each assay was performed in triplicate.

## Lactic acid assay and ATP concentration

The lactic acid level from each sample was measured using a commercial kit (Jiancheng, Nanjing, China) according to the manufacture's instruction. The ATP concentration was detected with an ATP assay kit (Beyotime Biotech, Shanghai, China). The luminescence of each sample was detected by the luminometer (PerkinElmer, MA, United States).

## Glucose uptake assay

$CD4^+$ T cells were stimulated for 24 hours in the presence or absence of inosine. Moreover, the cells were washed twice in PBS and incubated at 37 °C for 20 minutes in PBS containing 2-NBDG (0.1-0.3 mM) before analysis using flow cytometry.

## Quantitative Real-Time PCR

Total RNA was extracted from liver tissues and cells in each group using TRIzol reagent (Tiangen, Beijing, China). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit Fluorometer (Invitrogen) were used to assess RNA quality. cDNA was synthesized using a PrimeScrip RT reagent kit (Takara, Shiga, Japan), and all primers were obtained from Tsingke (Beijing, China). RT–qPCR was performed using SYBR Green Supermix on a CFX96 RT–qPCR detection system (BioRad, Hercules, CA, United States). The expression levels of ncRNAs were normalized to actin expression level.

## Western blot analysis

Protein extraction from each sample was performed using radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. The protein

concentration was determined by a BCA kit (Beyotime,Biotech, Shanghai, China). Equivalent amounts of total protein were separated on an SDS–PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% nonfat milk at RT for 1h. Then, the PVDF membranes were incubated with NLRP3 (Adipogen, Inc), Caspase 3 (Abcam, Inc.), GPX4 (Abcam, Inc), PFKFB3 (Abcam, Inc.), HK2 (Abcam, Inc), GLUT1 (Huabio,Inc) and β-actin (Cell Signaling Technology, Inc) overnight at 4°C. The blots were washed and incubated with an HRP-conjugated secondary antibody at 37°C for 1 hour. Finally, the membranes were washed twice and detected by a chemiluminescence kit. Protein quantification was carried out using ImageJ software (NIH) and relative to β-actin expression.

## Statistical analysis

All data are presented as the mean ± SD or median. and were statistically analyzed with GraphPad Prim 9 (GraphPad Software Inc., San Diego, CA, United States) and SPSS 28.0 software (SPSS Inc., Chicago, IL, United States). Student's t-test, one-way ANOVA, the Mann–Whitney *U* test, and the chi-square test were performed to compare the differences between two groups or intergroup differences. Statistical significance was labeled according to the p value as *p value < 0.05, **p value < 0.01, and ***p value < 0.001.

# Results

## HLA-DRB1*04:05 allele associated with the clinical features of AIH patients

Genetic susceptibility to AIH is strongly associated with HLA genes and HLA-DRB1*04:05 was considered to increase the susceptibility of AIH in Asian populations (2, 20). To explore the relationship between the HLA-DRB1*04:05 allele and disease course in Chinese AIH patients, we performed HLA-DRB1 genotyping on 74 adult AIH patients. The allele frequency of HLA-DRB1*04:05 was 14.9%, and 22 patients were HLA-DRB1*04:05 positive (Table 1). Furthermore, we divided the patients into HLA-DRB1*04:05 positive group and HLA-DRB1*04:05 negative group. The clinical characteristics between the two groups are compared in Table 2. The age and proportion of females were similar between the two groups. For laboratory examination, no significant difference was found in ALT, AST, ALP, GGT, ALB, or IgM. Higher serum levels of IgG and GLB were seen in the HLA-DRB1*04:05 positive group than in the negative group. The positive rate of ANA was higher in DRB1*04:05 positive patients than in DRB1*04:05 negative patients, but the difference did not reach statistical significance (P=0.061). The positive rate of SLA was higher in the DRB1*04:05 positive group than in the DRB1*04:05 negative group (P=0.045), which is associated with disease severity (21). For histological examination (inflammation grade (G) and fibrosis stage (S)), there was no significant difference between the two groups. However, the IAIHG score was higher in DRB1*04:05 positive patients than in DRB1*04:05 negative patients(P=0.046). Although the rate of patients achieving complete biochemical remission within 6 months was similar between the two groups, the time to remission was longer in the DRB1*04:05 positive group than in the DRB1*04:05 negative group (6.3 (2.0, 23.6) vs. 4.0 (0.2, 16.7), P=0.046). This may suggest that HLA-DRB1*04:05 was associated with response to treatment. Moreover, the frequency of other extrahepatic autoimmune diseases, such as Sjogren's syndrome (SS) and rheumatoid arthritis (RA), was higher in DRB1*04:05 positive patients than in DRB1*04:05 negative patients (36.4% vs. 7.7%, P=0.007). Some studies revealed an association between the progression of joint destruction and HLA-DRB1*04:05 in RA (22). Recently, increasing research has focused on the effect of

TABLE 1 Frequencies of HLA-DRB1 alleles in autoimmune hepatitis.

| DRB1 alleles | AIH patients n = 74 | DRB1 alleles | AIH patients n = 74 |
|---|---|---|---|
| 01:01 | 2% (3) | 12:01 | 2.7% (4) |
| 03:01 | 16.9% (24) | 12:02 | 7.4% (10) |
| 04:01 | 2% (3) | 13:02 | 1.4% (2) |
| 04:03 | 0.7% (1) | 13:12 | 2.7% (4) |
| 04:05 | 14.9% (22) | 14:01 | 4.1% (6) |
| 04:06 | 0.7% (1) | 14:04 | 1.4% (2) |
| 04:11 | 0.7% (1) | 14:05 | 2.0% (3) |
| 07:01 | 2.7% (4) | 14:54 | 0.7% (1) |
| 08:03 | 6.8% (10) | 15:01 | 8.1% (11) |
| 09:01 | 12.2% (18) | 15:02 | 2.0% (3) |
| 10:01 | 2% (3) | 16:02 | 4.1% (6) |
| 11:01 | 2% (3) | | |

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TABLE 2 Clinical characteristics of HLA-DRB1*04:05 positive and HLA-DRB1*04:05 negative AIH patients at diagnosis.

| Features | DRB1*04:05 (+) n = 22 | DRB1*04:05 (-) n = 52 | P value |
|---|---|---|---|
| Age (years) | 56 (25, 74) | 52 (26, 75) | 0.574 |
| Gender (M:F) | 5:17 | 7:45 | 0.520 |
| ALT (IU/L) | 210 (23, 1904) | 201 (22, 1543) | 0.808 |
| AST (IU/L) | 261 (36, 1585) | 235 (26, 1751) | 0.896 |
| ALP (IU/L) | 148 (80, 256) | 150 (59, 598) | 0.428 |
| GGT (IU/L) | 138 (42, 764) | 121 (17, 1003) | 0.948 |
| ALB (g/L) | 35.8 ± 5.3 | 37.7 ± 5.8 | 0.183 |
| GLB (g/L) | 44.6 ± 11.4 | 38.5 ± 7.5 | 0.029 |
| IgG (g/L) | 29.3 ± 11.0 | 23.3 ± 7.3 | 0.025 |
| IgM (mg/L) | 1680 (251, 5670) | 1470 (411, 8400) | 0.696 |
| ANA | | | 0.061 |
| <1:100 | 1 | 14 | |
| ≥1:100 | 21 | 38 | |
| SLA | | | 0.045 |
| – | 17 | 47 | |
| + | 5 | 2 | |
| G | | | 0.136 |
| 0~2 | 6 | 23 | |
| 3~4 | 16 | 27 | |
| S | | | 0.261 |
| 0~2 | 15 | 27 | |
| 3~4 | 7 | 23 | |
| Pretreatment IAIHG score | 18 (10, 24) | 14 (10, 21) | 0.040 |
| Complete biochemical remission within 6 months n (%) | 9 (40.9%) | 27 (51.9%) | 0.386 |
| Time to achieve biochemical remission (months) | 6.3 (2.0, 23.6) | 4.0 (0.2, 16.7) | 0.046 |
| Extrahepatic autoimmune disorders | 8 (36.4%) | 4 (7.7%) | 0.007 |

HLA, human leukocyte antigen; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; ALB, albumin; GLB, globulin; IgG, immunoglobulin G; IgM, immunoglobulin M; ANA, antinuclear antibody; SLA, soluble liver antigen; G, inflammation grade; S, fibrosis stage; IAIHG, international autoimmune hepatitis group.

genetic variations on the metabolic pathways (11). However, there have been few reports on AIH. Therefore, we divided the patients into groups according to the DRB1*04:05 allele to determine the serum metabolite profile.

## Patients with positive and negative DRB1*04:05 expression showed differences in metabolic profiles

To investigate the influence of the DRB1*04:05 allele on metabolic changes, we collected serum from DRB1*04:05 positive and DRB1*04:05 negative patients and performed metabolic profiling by LC/MS. As shown in Figure 2A, principal component analysis (PCA) revealed a trend of discrimination between DRB1*04:05 positive and negative patients. The partial least squares discriminant analysis (PLS-DA) score plot indicated the distinct separation of the two groups. Moreover, no obvious overfitting was observed in the permutation test (Figure 2B). Metabolites with VIP > 1.0 and P value < 0.05 and fold change > 1.2 or < 0.833 were regarded as differential metabolites. We first identified 70 metabolites with significant differences between the DRB1*04:05 positive and DRB1*04:05 negative group (Figure 2C). A heatmap was constructed based on the differential metabolites between the two groups and revealed clearing clustering (Figure 2D). Additionally, the functions of all metabolites and metabolic pathways were studied using the KEGG database. The main pathways involved were confirmed to be metabolism, including lipid metabolism, amino acid metabolism, carbohydrate metabolism and nucleotide metabolism (Figure 2E). The KEGG enrichment scatterplot showed the top 20 pathways based on the differentially altered metabolites (Figure 2F). According to Table 3, the top 10 most highly altered metabolites were mainly in nucleotides and their derivatives, including inosine, hypoxanthine-9-beta-D-Arabinofuranoside, hypoxanthine and allopurinol. Increasing evidence demonstrates that inosine possesses a wide range of anti-inflammatory and immunomodulatory properties (14, 23). Thus, we further explored the effect of inosine on the Con A-induced murine model.

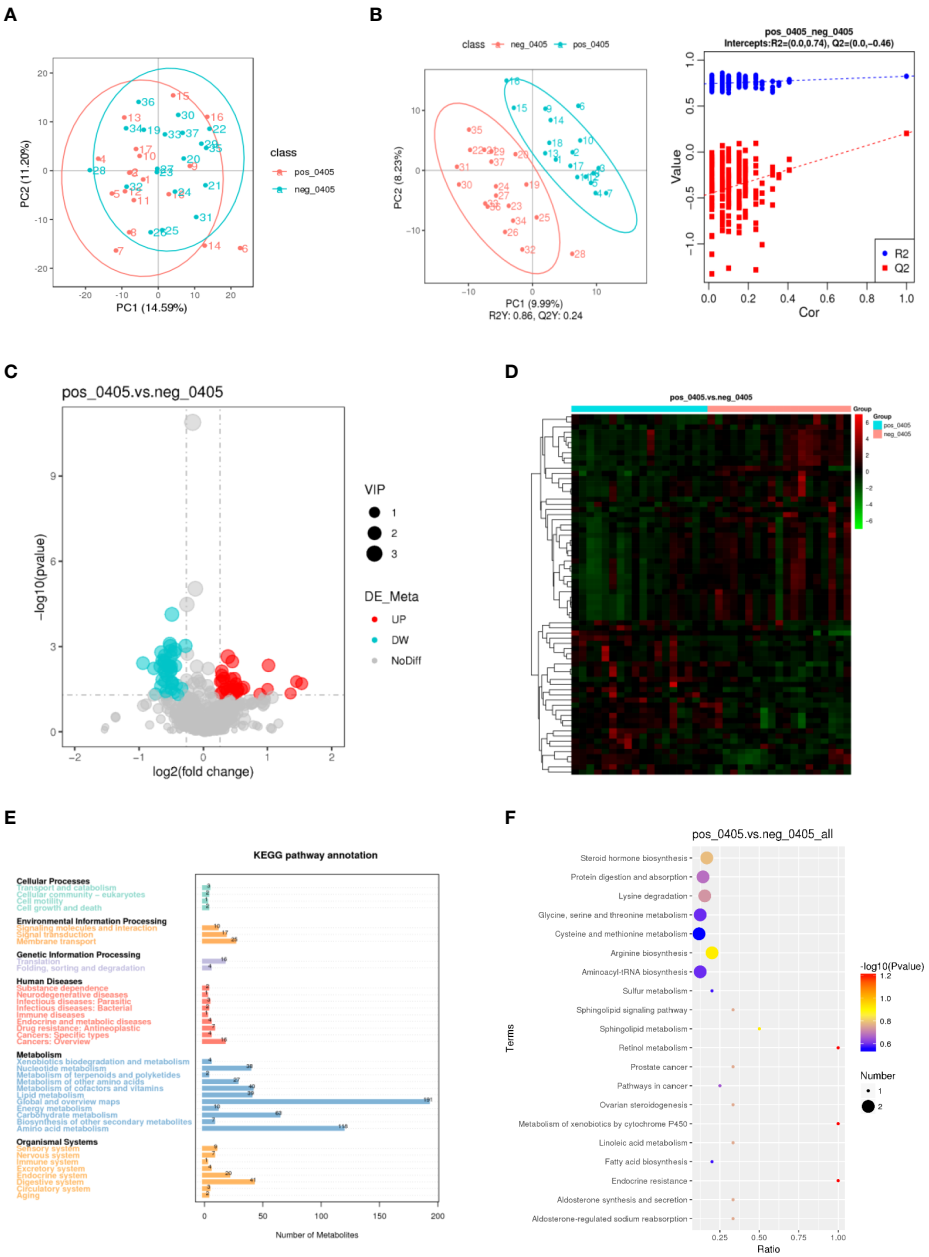


FIGURE 2
Metabolic profiles of DRB1*04:05-positive and -negative patients. **(A)** PCA score scatter plot of metabolites obtained from LC/MS. **(B)** PLS-DA score plots and validation plots showing separation of metabolites of the two groups. **(C, D)** Volcano plot and heatmap showing differences in metabolites between the two groups. **(E)** KEGG pathway annotations. **(F)** KEGG enrichment scatterplot showing the top 20 metabolic pathways.

## Inosine treatment attenuated hepatocyte apoptosis and increased antioxidant ability in Con A-induced murine model

Con A-induced hepatitis is characterized by lymphocyte-induced liver damage (24). To investigate the effect of inosine treatment on the Con A-induced murine model, all mice were sacrificed at 24 h after Con A injection (Figure 3A). The serum ALT and AST levels in the mice were measured. Compared to the NC group, the serum ALT and AST levels were dramatically elevated in the Con A group, but inosine treatment significantly decreased the AST and ALT levels (Figure 3B). In addition, H&E staining and TUNEL assays were performed. As shown in Figure 3C, there were more massive necrotic areas, severe

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TABLE 3 The top 10 metabolites with the most obvious changes.

| Name | Class | log2FC | P value | VIP |
|---|---|---|---|---|
| Glucarate O-Phosphoric Acid | Carbohydrates And Its Derivatives | 1.532529963 | 0.020129482 | 1.626484086 |
| Inosine | Nucleotide And Its Derivates | 1.451125765 | 0.016981841 | 1.570893577 |
| Hypoxanthine-9-beta-D-Arabinofuranoside | Nucleotide And Its Derivates | 1.358837416 | 0.044542092 | 1.342088144 |
| Hypoxanthine | Nucleotide And Its Derivates | 1.01556013 | 0.004595258 | 1.877520914 |
| Allopurinol | Nucleotide And Its Derivates | 1.000665369 | 0.031138467 | 1.434797824 |
| Lysopc 16:2 | Phospholipid | -0.940409983 | 0.003823747 | 1.968538981 |
| Dulcitol | Carbohydrates And Its Derivatives | 0.880385746 | 0.046832344 | 1.546999895 |
| Lysopc 20:0 | Phospholipid | -0.771378043 | 0.005859601 | 1.862457189 |
| Lysopc 14:0 | Phospholipid | -0.742518756 | 0.0464681 | 1.700699346 |
| Methyl Benzoate | Benzoic Acid And Its Derivatives | -0.669830793 | 0.004902561 | 1.980387329 |

sinusoidal congestion and increased inflammation were observed in the Con A group than in the NC group. However, inosine treatment clearly ameliorated these pathological symptoms. Moreover, the proportion of TUNEL$^+$ cells in the Con A group was significantly larger than that in the NC group. However, treatment with inosine significantly reduced the proportion of apoptotic cells (Figure 3D). Apoptosis and inflammation-related proteins were also analyzed in the three groups. Compared to that in the NC group, the expression of Caspase-3 and the nod-like receptor pyrin domain 3 (NLRP3) inflammasome were increased in the Con A group but decreased in the Con A + Inosine group (Figure 3I). These results showed that inosine treatment clearly attenuated hepatocyte apoptosis.

To further assess the antioxidant effect of inosine treatment on Con A-induced hepatitis, the serum levels of MDA, $Fe^{2+}$ and the GSH/GSSG ratio were measured. In contrast to the NC group, the MDA, and $Fe^{2+}$ contents were increased in the Con A group. Inosine treatment significantly decreased the levels of MDA and $Fe^{2+}$ (Figures 3E, F). Furthermore, compared to the NC group, the GSH/GSSG ratio was decreased in the Con A group and was clearly reversed by inosine treatment (Figure 3G). Moreover, the expression levels of mRNAs related to lipid peroxidation were analyzed. As shown in Figure 3H, the hepatic mRNAs expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and acyl-CoA synthetase long-chain family member 4 (ACSL4) were upregulated in the Con A group compared to the NC group, while treatment with inosine dramatically attenuated these adverse changes. Furthermore, inosine treatment increased the expression level of ferritin heavy chain 1 (FTH1). Although the mRNA expression level of glutathione peroxidase 4 (GPX4) in the inosine group had an increasing trend but without significance, treatment with inosine recovered GPX4 protein expression (Figure 3I). All these data suggested that inosine alleviated lipid peroxidation and increased antioxidant ability in Con A-treated mice.

## Treatment with inosine inhibited immune activation and prevented the inflammatory response in Con A-induced hepatitis

Con A-induced hepatitis is a T-cell mediated murine model, accompanied by the activation of immune cells and the release of many cytokines. To investigate the effect of inosine treatment on immune cells, we detected the frequency of activated T cells, as well as macrophages and neutrophils in the livers of Con A-induced mice by flow cytometry. The results showed that the frequencies of hepatic CD4$^+$CD25$^+$, CD4$^+$CD69$^+$, CD8$^+$CD25$^+$ and CD8$^+$CD69$^+$ T cells were significantly increased after Con A injection when compare to normal control, along with the frequencies of CD11b$^+$F4/80$^+$ cells and CD11b$^+$Ly6g$^+$ cells (Figures 4A–D). The gating strategy was shown in Supplementary Figure S1A. Treatment with inosine greatly decreased the frequencies of CD4$^+$CD25$^+$ T cells, CD8$^+$CD25$^+$ T cells, CD4$^+$CD69$^+$ T cells and CD8$^+$CD69$^+$ T cells (Figures 4A, B). However, there were no significant differences observed for the frequencies of CD11b$^+$F4/80$^+$ cells and CD11b$^+$Ly6g$^+$ cells between the Con A group and the Con A + inosine group (Figures 4C, D). These results suggested that inosine might ameliorate Con A-induced hepatitis by suppressing the activation of CD4$^+$ T cells and CD8$^+$ T cells. Furthermore, the serum IFN-γ and TNF-α levels were measured to assess the inflammatory response in Con A-induced mouse model. Compared to the NC group, there was a dramatic elevation in the serum IFN-γ and TNF-α levels in the Con A group. However, treatment with inosine clearly suppressed this change in IFN-γ and TNF-α levels (Figure 4E). The expression levels of mRNAs related to inflammation were determined. As shown in Figure 4F, the hepatic mRNAs expression of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were upregulated in the Con A group, while inosine treatment clearly attenuated these changes. However, the expression level of TNF-α and IL-4 did not vary significantly between the Con A group and the Con A+ Inosine group. These results implied that treatment with inosine prevented the inflammatory response.

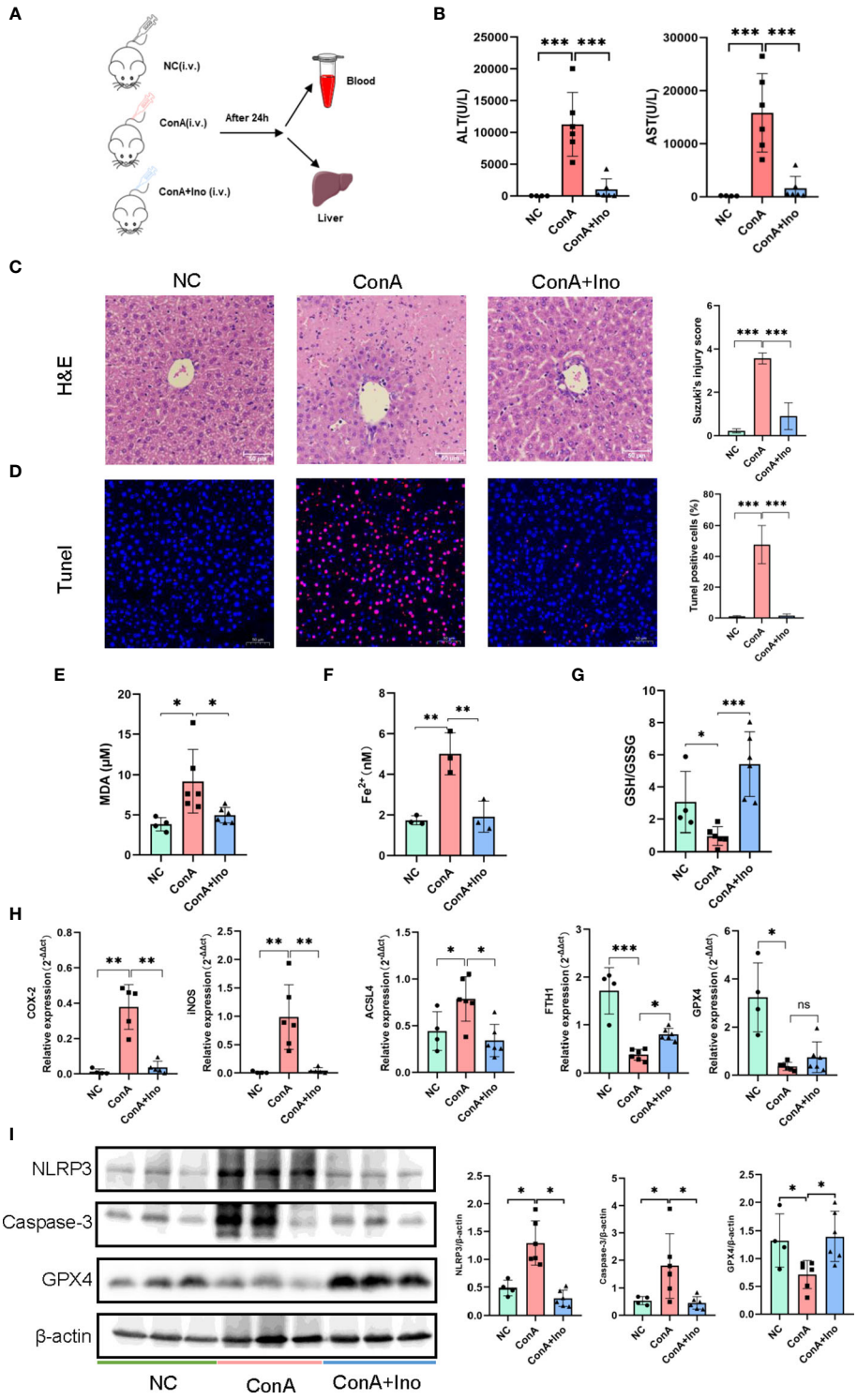


FIGURE 3
The effect of inosine on Con A-induced murine model. **(A)** Animal experimental design (N = 4-6). **(B)** ALT and AST levels. **(C)**. H&E staining. **(D)** TUNEL assays. **(E)** Serum MDA levels. **(F)** Serum $Fe^{2+}$ levels. **(G)** The ratio of GSH/GSSG. **(H)** The gene expression of COX2, iNOS, ACSL4, FTH1 and GPX4, normalized to β-actin. **(I)** the protein expression of NLRP3, Caspase-3 and GPX4, relative to β-actin expression. The figure only shows some of the samples, but protein quantification was calculated for all samples in each group. *p value < 0.05, **p value < 0.01, and ***p value < 0.001.

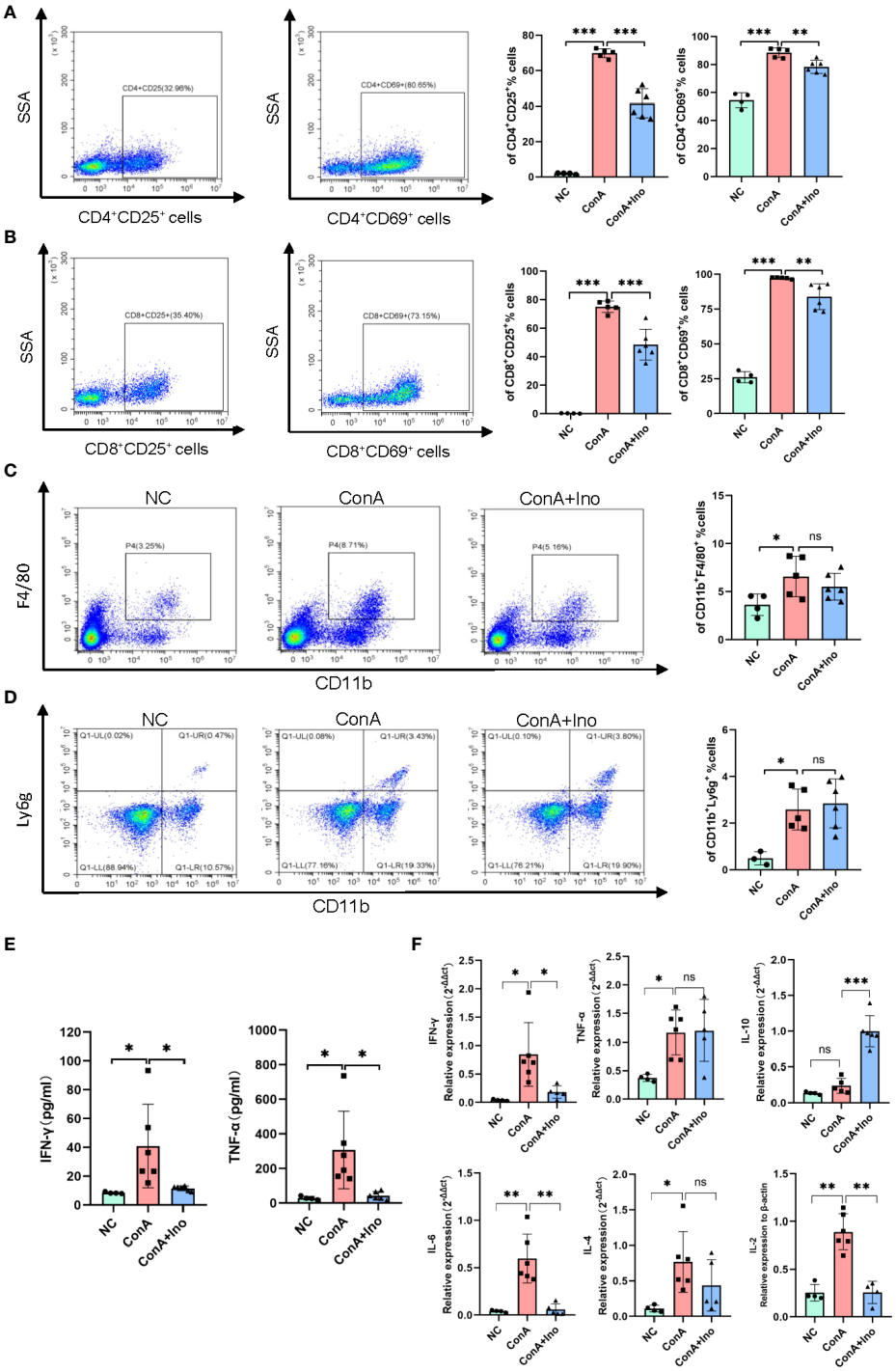


FIGURE 4
Effect of inosine on the inflammatory response. **(A-D)**. Flow cytometric analysis of the frequency of $CD4^+CD25^+$ T cells, $CD8^+CD25^+$ T cells, $CD4^+CD69^+$ T cells, $CD8^+CD69^+$ T cells, $CD11b^+F4/80^+$ cells and $CD11b^+Ly6g^+$ cells. **(E)** Serum levels of IFN-γ and TNF-α. **(F)** The gene expression of IFN-γ, TNF-α, IL-10, IL-6, IL-4, and IL-2, normalized to β-actin. *p value < 0.05, **p value < 0.01, and ***p value < 0.001.

### Inosine regulated the activation and glucose metabolism of CD4$^+$ T cells *in vitro*

Con A is a T-cell mitogen that can activate CD4$^+$ T cells, followed by an inflammatory reaction. To better explore the effect of inosine on CD4$^+$ T cells, CD4$^+$ T cells were isolated from mouse spleen by immunomagnetic cell sorting. The purity of CD4$^+$ T cells was approximately 99% (Supplementary Figure S1B). Then the CD4$^+$ T cells were stimulated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb for 24 h to examine the effects of inosine. The frequency of CD4$^+$CD25$^+$ cells dramatically increased after TCR simulation (Supplementary Figure S1C). Then, we examined the dose dependent effect of inosine on CD25 expression. Treatment with 0.4-2 mM inosine significantly reduced the frequency of CD25$^+$ cells and did not exhibit cytotoxicity (Supplementary Figures S1D, E). Since CD25 expression was suppressed by approximately 50% by 2 mM inosine without cytotoxicity, we used this concentration for further studies of the suppression of T cell activation (Figure 5A). A CCK8 assay was performed to measure the effect of inosine on the proliferation of CD4$^+$ T cells. The results showed that inosine treatment significantly suppressed cell proliferation (Figure 5B). Next, the IFN-γ and TNF-α levels in the supernatants of cell cultures were determined by ELISA. The results showed that treatment with inosine reduced the release of IFN-γ and TNF-α (Figure 5C). To investigate the effect of inosine on cytokine production at the transcriptional level, we detected the mRNA expression levels of IFN-γ, TNF-α, IL-10 and IL-2 by RT-qPCR. As shown in Figure 5D, inosine significantly suppressed the IFN-γ, TNF-α and IL-2 expression levels and increased the IL-10 expression level. These data supported that inosine had anti-inflammatory effects on CD4$^+$ T cells.

During activation, T cells require various metabolic pathways to carry out their functions. Previous studies have demonstrated enhanced glycolysis in T cells following TLR stimulation. Therefore, we detected the lactic acid level and ATP concentration in the culture supernatants. The results showed that inosine treatment suppressed lactic acid secretion and prompted ATP production (Figures 5E, F). Moreover, CD4$^+$T cells treated with inosine had significantly lower glucose uptake compared than untreated cells, as indicated by the significant decrease in 2-NBDG (Figure 5G). To further investigate glycolysis alterations in CD4$^+$ T cells, the expression of glycolytic-related genes and proteins was examined. Compared to the control, inosine treated CD4$^+$T cells expressed significantly lower mRNA levels of glucose transporter 1 (GLUT1), hexokinase 2 (HK2) and 6-phosphofructo-2-kinase (PFKFB3) except pyruvate kinase-M2 (PK-M2), all of which are involved in glycolysis (Figure 5H). However, lactate dehydrogenase (LDH) mRNA expression levels did not vary significantly between the two groups. Furthermore, the protein expression levels of GLUT1 and HK2 were also significantly downregulated in inosine-treated CD4$^+$T cells compared to control cells. The PFKFB3 expression level in the inosine group tended to be similar with that in the control group (Figure 5I). In general, these results demonstrated that inosine treatment inhibited glycolysis in CD4$^+$ T cells.

## Discussion

Several studies have demonstrated that AIH is related to HLA-DRB1*03:01 and DRB1*04:01 in European populations (3) and DRB1*04:05 in Japanese populations (4–6). In the present study, we showed an association between clinical features of AIH in Chinese patients and HLA-DRB1*04:05, which accounted for 14.9% of the allele frequency in our patients. The specific demographic features of Chinese AIH patients were compared between the DRB1*04:05 positive and DRB1*04:05 negative groups. The serum level of IgG, the positive rate of SLA and the IAIHG score were higher in the HLA-DRB1*04:05 positive group than in the negative group, as previously described (5, 25). Moreover, the time to remission was longer in the HLA-DRB1*04:05 positive group than in the negative group, which may suggest that HLA-DRB1*04:05 was associated with the response to treatment. However, the allele frequency of DRB1*03:01 was 16.9%, which was inconsistent with the previous study (4). This may be due to our limited number of patients. We also compared the clinical characteristics between DRB1*0301 positive and DRB1*0301 negative patients. The results showed that there was almost no association between clinical features of AIH in Chinese patients and HLA-DRB1*03:01 (Supplementary Table S3). In addition, there were no healthy controls for HLA haplotyping. Thus, the results could not reveal the predisposition of AIH in Chinese patients with DRB1*03:01, which was also one of the limitations of this study. Although HLA alleles are considered to confer the risk for various autoimmune diseases, the precise mechanism remains unclear. Some studies have demonstrated that autoantigens encoded by HLA genes are expressed on the cell surface and presented to immune cells, which activates the downstream immune process (26). Thus, Chinese AIH patients with DRB1*04:05 have typical clinical traits, probably because of the proteins presented by HLA-DRB1*04:05.

Metabolomics is used to detect small-molecule metabolite profiles to characterize different pathological conditions such as fibrosis and cirrhosis. Most studies have focused on the metabolic fingerprint to find noninvasive biomarkers for the diagnosis and management of autoimmune liver diseases, mainly compared to healthy controls (27). To our knowledge, there have been no previous studies on the associations of serum metabolites and HLA alleles in AIH. Thus, we divided our patients into two groups (DRB1*04:05

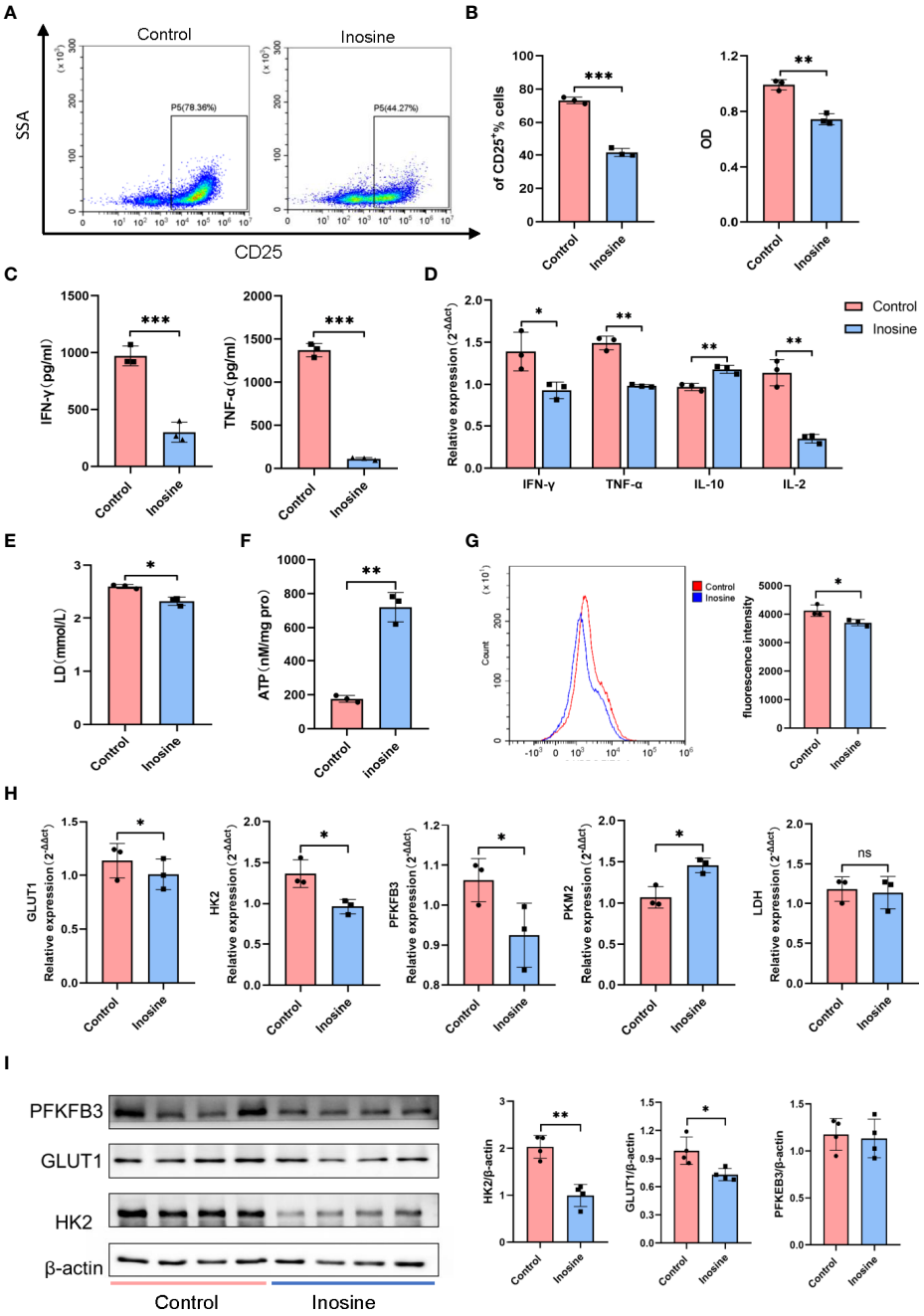


FIGURE 5
The effect of inosine on CD4$^+$ T cells. **(A, B)** Suppression of the activation and proliferation of CD4$^+$ T cells by inosine (2 mM): flow cytometric analysis and CCK8 assay. **(C)** Levels of IFN-γ and TNF-α in the culture supernatants. **(D)** The mRNA expression of IFN-γ, TNF-α, IL-10 and IL-2. **(E, F)** Lactic acid level and ATP concentration in the culture supernatant. **(G)** Glucose uptake of CD4$^+$ T cells (2-NBDG). **(H)** the mRNA expression of GLUT1, HK2, PK-M2, PFKFB3 and LDH, and relative to β-actin expression. **(I)** Protein expression of PFKFB3, GLUT1 and HK2, and relative to β-actin expression. *p value < 0.05, **p value < 0.01, and ***p value < 0.001.

positive and negative) and detected serum metabolite profiles. Our results identified 70 metabolites with significant differences between the DRB1*04:05 positive and DRB1*04:05 negative groups. The top 10 most highly altered metabolites between two groups were mainly in nucleotides and their derivates, including inosine, hypoxanthine-9-beta-D-Arabinofuranoside, hypoxanthine and allopurinol. The main pathways involved were the

metabolism related such as lipid metabolism, amino acid metabolism, carbohydrate metabolism and nucleotide metabolism. These results revealed the metabolic heterogeneity caused by HLA-DRB1*04:05.

Inosine, as a highly changed metabolite between the two groups, is a natural purine nucleoside composed of hypoxanthine and D-ribose. Previous studies found that inosine is not only a physiological metabolite but also possesses anti-inflammatory and immunoregulatory functions (28, 29). Con A-induced hepatitis is a common experimental acute hepatitis murine model. Consequently, the Con A-induced mouse model was used to detect the effect of inosine treatment. The results demonstrated the protective effect of inosine on Con A-induced liver damage. Inosine treatment obviously reduced the serum ALT and AST levels, which are considered the most informative biochemical markers for diagnosing liver injury. Moreover, treatment with inosine clearly attenuated hepatocyte apoptosis and prevented oxidative stress. H&E staining and TUNEL assays showed that inosine significantly ameliorated the pathological symptoms and reduced the apoptotic cells. Caspase-3, as the final executor of apoptosis, was decreased by inosine treatment, as was NLRP3. MDA, a secondary metabolite produced by free-radical attack, is widely used to reflect the extent of cellular injury (30). Inosine treatment obviously suppressed the elevation of serum MDA levels induced by Con A. Glutathione is a tripeptide that is involved in antioxidant and drug metabolism. The levels of GSH and GSSG are regarded as important indicators of oxidative stress. Treatment with inosine clearly reversed the GSH/GSSG ratio. The expression of oxidative stress genes such as COX-2, iNOS and ACSL4 was suppressed by inosine treatment. Furthermore, the expression of antioxidative stress genes and proteins associated with ferroptosis such as FTH1 and GPX4, was significantly reversed. All these results showed that inosine treatment attenuated hepatocyte apoptosis and increased antioxidant ability in acute severe hepatitis mice.

Con A-induced hepatitis is characterized by the activation of T cells and the release of many cytokines. Our results showed that the frequencies of hepatic $CD4^+CD25^+$ T cells, $CD8^+CD25^+$ T cells, $CD4^+CD69^+$ T cells and $CD8^+CD69^+$ T cells were reduced by inosine treatment. CD25 and CD69 are common activation markers of T cells. In addition, the serum level of and gene expression of proinflammatory cytokines such as IFN-γ, TNF-α, IL-6 and IL-2 were all reduced by the treatment with inosine, while the anti-inflammatory factors such as IL-10 increased. IFN-γ and TNF-α are associated with many inflammatory diseases, including rheumatoid arthritis and systemic lupus erythematosus. IL-6 and IL-2 are related to local or systemic inflammation (31, 32). Thus, the results revealed the inhibitory effect of inosine on immune cells.

$CD4^+$ T cells play a crucial role in maintaining immune homeostasis, but their overactivity has been related to the development of many immune-mediated inflammatory diseases, including AIH. Several studies have demonstrated that $CD4^+$ T cells are part of the presence in the inflammatory infiltrate in AIH (33). AIH patients often present an impaired T cell number and function (3). Thus, the $CD4^+$ T cells were isolated from mouse spleens and stimulated with CD3/CD28. The results showed that inosine treatment suppressed the activation and proliferation of $CD4^+$T cells *in vitro*, which is consistent with the *in vivo* results. Additionally, inosine inhibited the release of proinflammatory cytokines, such as IFN-γ, TNF-α and IL-2, from the stimulated $CD4^+$ T cells and reduced the related gene expression. Recent advances have shown that T-cell activation and proliferation are supported by metabolic reprogramming (34, 35). Many findings have revealed that aerobic glycolysis is linked to the T cell activation (36, 37). Metabolic inhibitors, especially glycolysis inhibitors, are used to target autoreactive T cells for the treatment of autoimmune diseases (38). Our data newly suggested that inosine could significantly reduce glycolytic genes expression, glucose uptake and the expression of glucose transporters, ultimately leading to a less glycolytic phenotype. This is the first study to reveal alerted glycolysis in $CD4^+$ T cells treated with inosine.

This study also has some limitations. The first limitation of our study is that there were no healthy controls for HLA haplotyping, which may not reveal the predisposition of AIH in Chinese patients with HLA-DRB1*04:05. However, the results showed an association between HLA and clinical features of AIH patients. Second, we did not choose the top metabolite to study, because the highly altered metabolites were mainly in nucleotides and their derivates. Inosine, as the top alerted metabolite of nucleotides, has been reported to possess immunomodulatory properties (23). In the future studies, we will explore the role of other metabolites in AIH. In addition, there are some deficiencies in the Con A-induced murine model to completely mimic the pathogenesis of AIH patients. However, Con A-induced liver injury, as a lymphocyte-induced hepatitis murine model, is commonly used to study the pathological mechanisms of autoimmune liver diseases (24).

In conclusion, HLA-DRB1*04:05 is linked to the clinical features of Chinese AIH patients and causes metabolic heterogeneity. Inosine, as a highly altered metabolite, might be involved in the development of AIH through its protective effect on hepatocytes and inhibition of overactivated immune cells, which provide a potential approach for acute form of AIH treatment.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Ethics statement

The studies involving human participants were reviewed and approved by the Independent Ethics Committee of West China Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Ethics Committee of the West China Hospital, Sichuan University.

## Author contributions

LY and XF designed the experiments. FY and LZ performed the experiments and wrote the manuscript. YS and SZ assisted with the experiments. YZ and RM checked the English grammar and polished the English language in the manuscript. All the authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.982186/full#supplementary-material

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# HLA, gut microbiome and hepatic autoimmunity

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Genetic susceptibility to autoimmune liver diseases is conferred mainly by polymorphisms of genes encoding for the human leukocyte antigens (HLA). The strongest predisposition to autoimmune hepatitis type 1 (AIH-1) is linked to the allele *DRB1*03:01*, possession of which is associated with earlier disease onset and more severe course. In populations where this allele is very rare, such as in Asia, and in *DRB1*03*-negative patients, risk of AIH-1 is conferred by *DRB1*04*, which is associated with later disease onset and milder phenotype. AIH type 2 (AIH-2) is associated with *DRB1*07*. The pediatric condition referred to as autoimmune sclerosing cholangitis (ASC), is associated with the *DRB1*13* in populations of Northern European ancestry. *DRB1*1501* is protective from AIH-1, AIH-2 and ASC in Northern European populations. Possession of the *DRB1*08* allele is associated with an increased risk of primary biliary cholangitis (PBC) across different populations. *DRB1*03:01* and *B*08:01* confer susceptibility to primary sclerosing cholangitis (PSC), as well as *DRB1*13* and *DRB1*15* in Europe. The hepatic blood supply is largely derived from the splanchnic circulation, suggesting a pathophysiological role of the gut microbiome. AIH appears to be associated with dysbiosis, increased gut permeability, and translocation of intestinal microbial products into the circulation; molecular mimicry between microbial and host antigens may trigger an autoaggressive response in genetically-predisposed individuals. In PBC an altered enteric microbiome may affect intestinal motility, immunological function and bile secretion. Patients with PSC have a gut microbial profile different from health as well as from patients with inflammatory bowel disease without PSC.

KEYWORDS

**autoimmune hepatitis, AIH, primary biliary cholangitis (PBC), primary sclerosing cholangites (PSC), gut microbiome, human leucocyte antigen (HLA)**

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# Introduction

Autoimmune liver diseases, including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are believed to be triggered by as yet poorly characterized environmental factors that lead to loss of tolerance to self-antigens in genetically predisposed individuals. Among genetic factors, the most relevant lie within the human leukocyte antigen (HLA) region on chromosome 6. Among environmental factors, infections, drug/toxins and microbiome have been suggested to be involved. A recent animal model of AIH, using transgenic mice on the non-obese diabetic (NOD) background carrying human HLA-DR3 and immunized with a DNA plasmid coding for a fusion protein of human CYP2D6/FTCD – the autoantigens targeted in AIH type 2 -, showed

that not only did the human HLA-DR3 predispose the animals to AIH, but also influenced the microbiome composition compared to the HLA-DR3 negative controls (1), suggesting a direct link between HLA and microbiome. The present short review recapitulates current knowledge on HLA associations and microbiome changes in autoimmune liver disease.

# The human leukocyte antigen

HLA are proteins expressed on the surface of a variety of cell types involved in antigen recognition by T cells. HLA molecules physiologically create a groove which embeds a short antigenic peptide to be presented to the T cell receptor. The genes encoding for the HLA proteins, located on the short arm of chromosome 6, are highly polymorphic, ensuring that different individuals are able to recognize and respond to a wide range of different antigenic peptides. Genetic predisposition to autoimmune diseases is often conferred by HLA alleles, and autoimmune liver diseases are no exception. Class I HLA proteins, expressed on all nucleated cells and recognized by CD8 T cells, display peptides derived from cytosolic proteins; class II HLA molecules, expressed on B cells, dendritic cells and macrophages, and recognized by CD4 T cells, display peptides derived from extracellular proteins, upon endocytosis. The HLA genes are characterized also by linkage disequilibrium extending over a large number of genes, meaning that certain alleles are inherited together with higher frequency than expected by random recombination during meiosis. This feature may make it difficult to assess the impact of a specific allele on the pathophysiology of diseases associated with HLA alleles. Interestingly, the so called 8.1 ancestral genotype is a very long haplotype containing alleles predisposing to AIH and PSC, as well as to a host of autoimmune diseases in Caucasian populations, including myositis syndromes, Sjögren syndrome, myasthenia gravis, and celiac disease (2, 3). The clinical observation of the frequent coexistence of hepatic and extrahepatic autoimmunity is possibly linked to a shared genetic predisposition involving different autoantigens.

## Autoimmune hepatitis

AIH is a rare chronic inflammatory liver disease affecting all ages, characterized by positive autoantibodies, elevated immunoglobulin G (IgG) serum levels, interface hepatitis at liver histology, and swift response to immunosuppressive treatment (4). Some 10-20% of AIH patients respond unsatisfactorily to pharmacologic treatment and progress to liver failure, requiring liver transplantation (5). AIH is subdivided into type 1 (AIH-1) and type 2 (AIH-2) according to autoimmune serology, AIH-1 being associated with positive anti-nuclear (ANA) and/or anti-smooth muscle (SMA) antibodies, and AIH-2 being associated with anti-liver kidney microsomal (LKM) and/or anti-liver cytosol type 1 (LC-1) antibodies (5). While AIH-1 affects all ages, AIH-2 affects mainly children and adolescents. Autoimmune sclerosing cholangitis (ASC) is a pediatric condition defined by AIH, almost universally AIH-1, coexisting at diagnosis with abnormal cholangiogram (6). It reportedly affects 40-50% of AIH-1 pediatric patients, and has a more aggressive course, requiring liver transplantation more frequently than classical AIH (6, 7).

Although the aetiology of AIH and ASC remains unknown, genetic and environmental risk factors have been described, the latter including exposure to drugs and viruses (5). While non-HLA polymorphisms have been reported to increase the risk of AIH, the strongest genetic predisposition is conferred by possession of certain HLA alleles: the high diagnostic value of the predisposing HLA alleles is underscored by the inclusion of HLA in the revised AIH diagnostic criteria (5, 8). The HLA predisposing role to AIH, recognized already in 1977 by Opelz et al. and in 1980 by Mackay and Tait, was confirmed in 1991 in a landmark study from King's College Hospital, London, UK, which identified a significantly higher frequency of the haplotype *A1-B8-DR3* in a cohort including 96 Northern European AIH adolescent and adult patients (three anti-LKM-positive) as compared to healthy controls (9). Possession of this haplotype was also associated to younger age at diagnosis, more frequent relapse and need for liver transplantation (9) (Table 1). In DR3-negative subjects, AIH susceptibility was conferred by HLA DR4, which was associated with later disease onset and milder phenotype. Recently, a large paediatric study from the same centre confirmed the association of the *A1-B8-DR3* haplotype with all types of paediatric autoimmune liver diseases, DR3 homozygosity conferring the highest risk, being also associated with fibrosis at disease onset (22). Possession of the *HLA-DRB1*03:01* allele and of the *A1-B8-DRB1*0301* haplotype is associated with treatment failure and increased risk of cirrhosis, suggesting a more aggressive phenotype in *DRB1*03*-positive patients (10). Interestingly, this haplotype is linked also to a polymorphism of the *TNF-A* gene, which leads to

TABLE 1 Main HLA alleles and haplotypes predisposing to autoimmune hepatitis.

| HLA allele or haplotype | Ancestry | Disease | Population age | Reference |
|---|---|---|---|---|
| *A1-B8-DR3* | Northern Europe | AIH-1 and AIH-2 | Adolescent and adults | (5) |
| *HLA DR4* | Northern Europe, Japan, China, South Korea, India, Iraq, Iran | AIH-1 | Adults and children* | (5, 9–17) |
| *A1-B8-DRB1*03:01* | Northern Europe | AIH-1, AIH-2 and ASC | Children | (6) |
| *HLA-DRB1*03:01*<br>*HLA-DRB1*03* | Northern Europe, Thailand, Iraq, Iran, Brazil | AIH-1 | Adults and children | (5, 6, 9, 16–19) |
| *HLA-DRB1*07* | Northern Europe, Iraq | AIH-2 | Children | (6, 16) |
| *HLA-DRB1*08* | India, Iran | AIH-1 | Adults | (15, 20, 21) |
| *HLA B* | China | AIH-1 | Adults | (22) |
| *HLA-DQA1*01:01* | Thailand | AIH-1 | Adults | (18) |
| *HLA-DRB1*13* | India, Iraq, Northern Europe, Brazil, Iran | AIH-1 and ASC | Adults** and children | (6, 15–17, 19) |
| *HLA-DRB1*13:01* | Argentina | AIH-1 | Children | (23) |
| *HLA-DRB1*14* | India | AIH-2 | Adults and children | (15) |

*in India, Japan and Iraq.
**in Iran.
AIH-1, type 1 autoimmune hepatitis; AIH-2, type 2 autoimmune hepatitis; ASC, autoimmune sclerosing cholangitis.

a higher production of tumour necrosis factor alpha (TNF-α) (10). *HLA-DRB1*03* and *DRB1*04* encode the amino acid sequences LLEQKR and LLEQRR at positions 67-72 of the HLA β chain (11). *HLA-DRB1*1501*, which is protective from AIH-1, AIH-2 and ASC in Northern European populations, encodes alanine at position 71, implying that the amino acid at this position is essential to confer susceptibility (22) (Table 2).

The strong association of AIH-1 with HLA polymorphisms has been confirmed by the two genome-wide association studies (GWAS) performed so far in AIH, both including only AIH-1 adult patients (12, 13). The first GWAS, carried out in a Northern European population, identified the class II *HLA-DRB1*0301* as the allele with the strongest association with AIH-1, and *HLA-DRB1*0401* as the second most strongly AIH-1- associated allele. The second GWAS study, carried out in a Chinese population, identified a single nucletotide polymorphism in the class I HLA-B locus as the strongest association with AIH-1. The study also identified two non-HLA loci associated with AIH-1, i.e. the CD28-CTLA4-ICOS and the SYNPR loci, the first coding for proteins involved in T cell activation, and the latter coding for a protein expressed on synaptic membranes (13).

There are geographical and ethnic differences in HLA allele frequencies, mirrored by differences in HLA associated to AIH in different populations. In Japan, China, South Korea and Taiwan, adult AIH-1 is associated with *DRB1*04*, *DRB1*03* being very rare in the general population, thus potentially explaining the different AIH phenotype in East Asia, which is characterised by later onset and milder disease (14–16, 18, 23) (Table 1). However, in Thailand AIH-1 is associated with *HLA-DRB1*03:01*, and *HLA-DQA1*01:01* (19). In India, a study including both children and adults, identified *DRB1*04* and *DRB1*08* as risk alleles for AIH-1, and *HLA-DRB1*04* as risk factor for paediatric AIH, in contrast to its protective role in paediatric Northern European populations, and in line with Japanese data (16, 20). Similarly, a small paediatric study from Iraq, not distinguishing between AIH and ASC, reported *DRB1*03*, *DRB1*04* and *DRB1*13* being all associated to AIH-1 (17). *DRB1*13* was also found to be associated with paediatric AIH-1 in the above-mentioned study from India, which also found an association of AIH-2 with *HLA-DRB1*14* (20). Interestingly, a large study from Argentina found that *DRB1*13:01* confers susceptibility for paediatric AIH-1, whereas *DRB1*13:02*, which differs only in one amino acid, confers protection (24) (Table 1).

Genetic studies in AIH-2 have been limited by the rarity of the disease. The largest cohort investigated so far is the above-mentioned study by the King's College Hospital including only

TABLE 2 HLA alleles protective from autoimmune hepatitis.

| HLA allele or haplotype | Ancestry | Disease | Population age | Reference |
|---|---|---|---|---|
| *HLA-DRB1*11* | Iraq, Iran | AIH-1 | Adults | (16, 17) |
| *HLA-DRB1*13:02* | Argentina | AIH-1 | Children | (23) |
| *HLA DRB1*15:01* | Northern Europe, Iraq, Iran | AIH-1, AIH-2 and ASC | Children | (6, 16, 17) |
| *HLA DRB1*04* | Northern Europe | AIH-1, AIH-2 and ASC | Children | (6) |
| *HLA DRB5* | Iran | AIH-1 | Adults | (17) |

AIH-1, type 1 autoimmune hepatitis; AIH-2, type 2 autoimmune hepatitis; ASC, autoimmune sclerosing cholangitis.

children of European ancestry, which reported an association of AIH-2 with *HLA-DRB1*07*, whereas *HLA-DRB1*15* is protective, as it is for AIH-1 and ASC (22). These results have been confirmed by a smaller study from Iraq (17). The predisposing role of *DRB1*07* for AIH-2 has been shown also in Brazil (25). The literature on genetic associations of ASC is also scant, since the differentiation of AIH-1 from ASC is not systematically done in paediatric studies. The association of ASC with *DRB1*13* reported in the King's College Hospital study was confirmed by a small study from Brazil (26); this allele was found to be associated to AIH-2 in Iran (27).

A Japanese study reported a role for class I HLA and killer cell immunoglobulin like receptors (KIR) in association in the predisposition to AIH-1 (15). Natural killer (NK) cells express KIR inhibitory receptors on their surface, which recognize self MHC class I molecules, expressed on all nucleated cells: engagement of these inhibitory receptors protects healthy cells from destruction by NK cells. The investigators, besides confirming that *HLA-DRB1*04* confers an increased risk of AIH-1 in Japanese adults, found a strong protective role of the combination KIR3DL1/HLA-B Bw4-80Thr, suggesting a role for NK cells in the pathophysiology of AIH-1 (15).

Attempts have been made to investigate the link between HLA alleles and AIH-1 sub-phenotypes, besides the mentioned association of *DRB3* with more severe disease. According to a retrospective Japanese study, the frequency of *DRB1*04* is not different in adult AIH-1 patients presenting with acute severe disease as compared to those presenting with chronic AIH (14). Similar results have been reported by a small Greek study (28). A large German study reported that absence of HLA B8 and presence of HLA DR7 are associated with an increased risk of AIH-induced acute liver failure (29).

## Primary biliary cholangitis

PBC, previously referred to as primary biliary cirrhosis, is an uncommon autoimmune liver disease characterized by inflammation and destruction of the small- and medium-sized intrahepatic bile ducts (21). It affects mainly middle-aged women and is associated with anti-mitochondrial antibody and/or PBC-specific ANA, i.e. ANA displaying a rim-like or a multiple dots nuclear staining pattern on HEp2 cells. Similarly to AIH, despite unknown aetiology, predisposing genetic background and environmental triggers have been reported (21, 30). The important predisposition conferred by genetics is reflected by the high PBC concordance rate of 63% in identical twins, being the highest among a host of autoimmune diseases (31). The unique genealogic database of Iceland offered the opportunity to investigate the familial risk of PBC: Ornolfsson et al. not only did confirm an increased risk in first-degree relatives, but also showed that the risk is increased up to fifth-degree relatives (32). Owing to the strong autoimmune features of PBC, HLA polymorphisms have been investigated, leading to the demonstration that *DRB1*08* confers predisposition across different populations (33, 34). In a study including a population from the UK and one from Northern Italy, *DRB1*08:01*, was associated with increased PBC risk, whereas *DRB1*13* was protective; *DRB1*11 was* protective only in the Italian population (35, 36). In China and Japan, the strongest predisposition has been linked to possession of the *DRB1*08:03*, which is only one amino acid different from *DRB1*08:01* and may have a similar role in antigen presentation (37–39). *DRB1*11* (in Italy and Japan but not in the UK) and *DRB1*13* (in the UK, Northern Italy and Japan) are protective (35, 38, 40). In Japan, *HLA-DQB1*06:04* and *HLA-DQB1*03:01* have been reported to be protective, the latter being protective also in China, Europe and North America (37, 39, 41, 42). Due to linkage disequilibrium with *HLA-DRB1*08:03*, *DQA1*01:03* and *DQB1*06:01* have also been associated to PBC in Japan and China (38, 39). A distinctive HLA association with the haplotype *DRB1*0301-DQB1*02:01* has been reported in Sardinia, known for its genetic isolation (43). Associations have been reported also for HLA DP alleles, including HLA *DPB1*03:01* in a German study and *DPB1*17:01* in a Chinese study (37, 44).

The first GWAS in PBC published in 2009 and performed in a Nort American population, identified *HLA-DQB1* has having the strongest association (45). All subsequent GWAS confirmed the prominent association of HLA with PBC (42, 46–54).

## Primary sclerosing cholangitis

PSC is a rare cholestatic liver disease leading to fibrosis and strictures of the bile ducts, associated with inflammatory bowel disease (IBD) in 80% of patients of Northern European origin, and showing a male preponderance (55). IBD comorbidity is lower in Southern European and Asiatic subjects (55). Affected patients are at increased risk of cholangiocarcinoma, colorectal cancer, gallbladder carcinoma and hepatocellular carcinoma (56).

Although the cause of PSC remains unknown, there is a genetic predisposition, mirrored by a nearly 10-times higher disease risk in first-degree relatives of affected patients (55). Similarly to AIH and PBC, the strongest genetic predisposition is conferred by HLA alleles, i.e. *DRB1*03:01* and *B*08:01*, both being part of the above-mentioned 8.1 ancestral phenotype, which is associated with a host of autoimmune diseases, including AIH and ASC (2, 57–59). A European study, later confirmed by a study including European Americans, Hispanics and African Americans, identified also *DRB1*13* as a risk allele, which is included in the haplotype *HLA-DRB1*13:01-DQA1*01:03-DQB1*06:03* (60, 61). Since *HLA-DRB1*03:01* is also associated to AIH

and ASC, and *HLA-DRB1*13* is also associated to ASC, these alleles predispose to AIH, ASC and PSC: whether PSC represents a late stage phenotype of ASC, remains an open question; of note, both diseases lack female preponderance (62). However, the *HLA-DRB1*15:01*, reported to represent a risk allele in PSC (60), was found to be protective to all forms of paediatric autoimmune diseases in an ethnically similar population, suggesting that HLA polymorphisms are only a component of the complex etiopathogenesis of AIH, ASC and PSC.

The PSC-associated haplotypes *A*01-C*07-B*08-DRB1*0301-DQB1*0201* and *A*03-C*07-B*07 -DRB1*1501-DQB1*0602* share class I antigens with the same binding properties to KIR: these haplotypes do not carry the *HLA-Bw4* and *HLA-C2* alleles, which are ligands for the inhibitory KIRs 3DL1 and 2DL1 (63). This observation suggests a role for NK cells in the pathophysiology of PSC (57).

The GWAS studies carried out so far in PSC confirm that HLA has the strongest association with PSC (55, 56). This observation supports the notion that PSC is an autoimmune disease, despite the paradox of lack of response to immunosuppression, a feature shared with the other cholestatic autoimmune liver disease, namely PBC.

Main HLA alleles and haplotypes predisposing to or protecting from PBC and PSC are summarized in Table 3.

## Potential treatment implications

Though the predisposition conferred by HLA points towards a prominent pathophysiological role of the adaptive immune system in autoimmune liver diseases, the target antigens of the autoimmune attack in PSC, AIH-1 and ASC remain largely unknown. A precise knowledge of the antigenic peptides binding to the HLA proteins of an individual patient would pave the way to peptide-based therapies, by using modified peptides which block autoreactive T cells upon binding to the HLA groove, forming an HLA peptide inhibitory complex. Therefore, future research should focus on identifying autoantigenic proteins and their immunodominant epitopes.

Whether HLA can be included in an interactome-based approach to identify new drugs needs to be investigated (54, 64).

# Gut microbiome

The human microbiome has been implicated in the occurrence of several immune-mediated diseases, ranging from rheumatoid arthritis, type 1 diabetes, IBD, and multiple sclerosis (65). Intestinal microbiome may be involved also in the pathogenesis of autoimmune liver disease (66, 67). The blood

TABLE 3 Main HLA alleles and haplotypes predisposing to or protecting from primary biliary cholangitis and primary sclerosing cholangitis.

| Predisposing HLA allele or haplotype | Ancestry | Disease | Reference |
|---|---|---|---|
| *HLA-DRB1*08* | Italy | PBC | (34) |
| *HLA-DRB1*08:01* | UK, Italy | PBC | (35) |
| *HLA-DRB1*08:03* | China, Japan | PBC | (37, 38) |
| *HLA-DQB1*06:01* | China, Japan | PBC | (39, 40) |
| *HLA-DRB1*08:03- HLA-DQB1*06:01* | Japan | PBC | (40) |
| HLA-DRB1*03 | Europe | PSC | (58, 60) |
| HLA-B*08:01 | Europe | PSC | (59) |
| *HLA-DRB1*13* | Europe, European Americans, African Americans, Hispanics | PSC | (60, 61) |
| *HLA-DRB1*15:01* | Europe | PSC | (60) |
| *HLA-DQB1:02* | Europe | PSC | (60) |
| *HLA-DQA1*05:01* | Europe | PSC | (60) |
| **Protective HLA alleles** | | | |
| *HLA-DRB1*13* | UK, Italy, Japan | PBC | (35) |
| *HLA-DRB1*11* | Italy, Japan | PBC | (35, 40) |
| *HLA-DQB1*03:01* | Japan, China, Europe, North America | PBC | (35, 39, 40) |
| *HLA-DQB1*06:04* | Japan | PBC | (40) |
| *DRB1*13:02-DQB1*06:04* | Japan | PBC | (40) |
| *HLA-DRB1*04* | Europe, European Americans, African Americans, Hispanics | PSC | (60, 61) |
| *HLA-DQB1*03:02* | Europe | PSC | (60) |

PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

supply to the liver derives in large amount from the splanchnic circulation, making it exposed to bacteria and bacterial products from the intestinal microbiome. Alterations in the intestinal microbiome might lead to liver damage.

## Autoimmune hepatitis

Changes in the intestinal microbiome have been documented in an experimental model of AIH (1) employing HLA-DR3 transgenic mice on the non-obese diabetic (NOD) background. AIH was induced by immunization with a DNA plasmid coding for a fusion protein of human CYP2D6/FTCD, the autoantigens targeted in AIH-2. HLA-DR3, as mentioned above, is strongly linked to AIH. Not only the HLA-DR3 positive mice that were immunized did develop chronic liver injury recapitulating AIH, but they also had a different microbiome composition compared to the HLA-DR3 negative mice. Mice that developed AIH displayed a reduced diversity of their microbiota. Another experimental model using $Tet2^{\Delta DVAV}$ mice, which are deficient in the epigenetic regulator Tet methylcytosine dioxygenase 2 (TET2), shows a key role of microbiome and cytotoxic T lymphocytes in inducing a liver disease closely resembling AIH (68). Moreover, a compound of 15 probiotics has been reported to decrease hepatic inflammation, serum transaminase levels, Th1 and Th17 cells, and increase the number of regulatory T cells in a murine model of AIH, while protecting intestinal barrier integrity, blocking lipopolysaccharide (LPS) translocation, inhibiting the toll-like receptor 4/nuclear factor κB (TLR4/NF-κB) pathway activation and the production of inflammatory cytokines in both liver and ileum (69).

Also in humans, AIH appears to be associated with dysbiosis, increased gut permeability, and translocation of intestinal microbial products into the systemic circulation (70). Changes in the microbiome in association to a leakier mucosal barrier might permit translocation of bacteria and their products promoting an inflammatory response. Moreover, molecular mimicry between microbial and host antigens may trigger or intensify an autoaggressive response in genetically-predisposed individuals (71, 72).

Zona occludens 1 and occludin, structural proteins that function in the binding of intestinal epithelial cells, are decreased in patients with AIH compared to health (70), and this decrease worsens with progression of liver disease. Moreover, AIH patients display a reduced number of anaerobes (*bifidobacterium and lactobacillus species*) within the enteric microbiome. The concept of bacterial translocation is supported by the observed increase in plasma levels of lipopolysaccharide (LPS) in AIH (70), which worsens with more advanced liver damage. Plasma LPS produced from bacterial translocation can reach the liver through the portal vein. LPS binds to toll-like receptors (TLR) on liver cells, including hepatocytes, stellate cells, Kupffer cells, and sinusoidal epithelial cells, leading to their activation and resulting in an inflammatory milieu predisposing to autoimmunity and fibrosis. The bacterial colonization may also favour the secretion of IL-10 by regulatory T cells (73, 74).

Breech of the intestinal mucosal barrier allows migration of gut-derived bacteria and bacterial products from the intestinal lumen to extra-intestinal sites including the liver. Once the translocated bacterial products, including LPS and unmethylated cytosine–phosphate–guanine (CpG), potent stimulators of the innate immune system, reach the liver they activate TLRs and non-obese diabetes (NOD)-like receptors (NLRs). Migration of activated T lymphocytes from the gut to the liver, including those expressing gut-specific homing receptor alpha4beta7-integrin, has also been implicated in the pathogenesis of autoimmune liver damage within a 'gut/liver axis' framework (75), though they can also be found in other forms of liver disease (76).

## Primary biliary cholangitis

In PBC an altered enteric microbiome may affect intestinal motility, immunological function and bile secretion (77–79). PBC patients have fewer *Acidobacteria, Lachnobacterium, Bacteroides*, and *Ruminococcus species* than healthy individuals and a higher number of opportunistic microbes, such as *Y-Proteobacteria, Enterobacteriaceae, Neisseriaceae, Spirochaetaceae, Veillonella, Streptococcus, Klebsiella*, and *Actinol species*. In addition, PBC patients have elevated plasma levels of a variety of cytokines including IL-1β, IL-6,IL-8, IL-18, IL-16, IP-10, MIG, IL-2RA, TNF-α, and macrophage migration inhibitory factor; the lower the levels of microbes normally present in health the higher the levels of inflammatory cytokines (80).

These findings suggest that alterations within the microbiome in PBC have an impact on immune function. PBC has also been associated with abnormal accumulation of LPS in biliary epithelial cells (81, 82), which might activate host immune response. Polymorphisms in the gene coding for TLR 4, which binds to LPS, have been demonstrated in PBC and might favour LPS accumulation in biliary epithelial cells (BEC) triggering host immune responses, further supporting a link between microbial products, the immune system, and PBC (83–85). In addition to LPS, other intestinal microbial components, such as flagellin and cytosine-phosphorothioate-guanine oligonucleotide, concur in eliciting BEC-destructive immune responses (65).

Several studies have investigated the role of infectious agents as triggers of the immune attack on BEC in PBC (86). Anti-mitochondrial antibodies exhibit cross-reactivity with antigens of *Escherichia coli* and *Novosphingobium aromaticivorans* (87, 88). *Escherichia coli* or *N. aromaticivorans* infection might

provoke, in genetically susceptible individuals, the production of anti-mitochondrial antibodies that target pyruvate dehydrogenase complex-E2 (PDC-E2) through a process of molecular mimicry (83, 87, 89–92). This theory is supported by an animal model where mice inoculated with *N. Aromaticivorans* develop PBC-like disease (93), and by the observation that PDC-E2 shares sequence homologies with intestinal *Escherichia coli* (83, 87, 89–92). Recently, it has been shown that exposure to *Escherichia coli* elicits specific antibody to *Escherichia coli* PDC-E2 that leads to epitope spreading and production of the classical human anti-PDC-E2 autoantibody, possibly being the first step to development of human PBC (30).

In addition to *Escherichia coli, Klebsiella species* have been implicated in the development of PBC through a mechanism of molecular mimicry (94).

## Primary sclerosing cholangitis

The frequent association of PSC with IBD strengthen the notion of a link between the gut microbiome and the liver. Owing to intestinal inflammation allowing translocation of enteric pathogens through the mucosal barrier, enteric microbial components may reach the liver through the portal circulation and provoke damage to cholangiocytes (95). Alternatively, gut activated T cells may home on to the liver and trigger immune-mediated damage (75, 96). However, as mentioned above, presence of gut-derived activated T cells is not confined to PSC and other autoimmune liver diseases (76).

Patients with PSC have a gut microbial profile different from health as well as from patients with IBD without PSC (97–99). PSC patients have a reduction of intestinal bacterial diversity, with an increased presence of *Veillonella, Rothia, Enterococcus, Streptococcus, Clostridium, Lactobacillus, Fusobacterium and Haemophilus species*. In particular, *Enterococcus, Lactobacillus*, and *Fusobacterium species* are associated with PSC independently of IBD, probiotic use, cirrhosis, liver transplantation, ursodeoxycholic acid or antibiotic treatment (97–99).

The use of antibiotics, including vancomycin, metronidazole, tetracycline, sulfasalazine, azithromycin, and minocycline has been reported to improve laboratory indices in PSC, but not the progression of liver disease (100). The improvement observed with the use of antibiotics may derive from modifications in the microbiome that results in a decreased production of inflammatory molecules.

Faecal microbiota transplantation (FMT), which alters the host microbiome, has been attempted as a therapeutic measure for PSC in a small pilot study. Microbial diversity increased and the alkaline phosphatase levels decreased in treated patients, though it is unclear whether this was associated with a clinical benefit (101). In this pilot study FMT was applied through the colonic route, but a small bowel route might be more appropriate based on animal studies showing that small bowel, but not colonic, dysbiosis is associated with hepatobiliary inflammation, and that small bowel bacterial overgrowth results in a PSC-like disease (102). Larger clinical trials are needed to investigate the role of FMT in PSC.

Both PSC and, to a lesser extent, AIH are associated with positivity for the autoantibody referred to as atypical peripheral anti-neutrophil cytoplasmic antibody (pANCA), also known as peripheral anti-nuclear neutrophil antibody (pANNA). This antibody is reportedly directed to Beta-tubulin and cross-reacts with a bacterial antigen, FtsZ (103), an evolutionary precursor protein of Beta-tubulin present in almost all intestinal bacteria, suggesting that molecular mimicry to bacterial products may also play a role in the development of PSC and AIH.

# Conclusions

The strongest genetic predisposition to all autoimmune liver diseases is conferred by HLA alleles, suggesting a preponderant role of adaptive immunity and particularly of T cells in their pathophysiology. Therefore, knowledge of the autoantigens recognized by T cells is key to advance our understanding of the immunopathogenesis of these diseases. Even in the diseases where the autoantigens are known (AIH-2 and PBC), little is known about the T cells autoantigenic peptides, knowledge of which could pave the way to novel therapeutic approaches. The role of the intestinal microbiome in shaping the phenotype of autoimmune liver disease is currently under investigation. Though most microbiome studies were conducted in wealthy countries in North America and Europe, skewing our understanding of human-microbe interactions, preliminary findings highlight the potential importance of its manipulation in therapeutic interventions.

# Author contributions

BTB-P drafting the first part of the manuscript, critical review and approval of final version. GM-V, DV: drafting the second part of the manuscript, critical review and approval of final version. All authors contributed to the article and approved the submitted version.

# Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# New insights on the role of human leukocyte antigen complex in primary biliary cholangitis

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Primary Biliary Cholangitis (PBC) is a rare autoimmune cholangiopathy. Genetic studies have shown that the strongest statistical association with PBC has been mapped in the human leukocyte antigen (HLA) locus, a highly polymorphic area that mostly contribute to the genetic variance of the disease. Furthermore, PBC presents high variability throughout different population groups, which may explain the different geoepidemiology of the disease. A major role in defining HLA genetic contribution has been given by genome-wide association studies (GWAS) studies; more recently, new technologies have been developed to allow a deeper understanding. The study of the altered peptides transcribed by genetic alterations also allowed the development of novel therapeutic strategies in the context of immunotolerance. This review summarizes what is known about the immunogenetics of PBC with a focus on the HLA locus, the different distribution of HLA alleles worldwide, and how HLA modifications are associated with the pathogenesis of PBC. Novel therapeutic strategies are also outlined.

KEYWORDS

**primary biliary cholangitis, human leukocyte antigens complex, HLA haplotypes, genetics, immunotolerance**

# Introduction

Primary biliary cholangitis (PBC) is a rare pathology that can evolve to cirrhosis and liver failure. Although the etiology has not been completely explained, there is evidence supporting that autoimmunity targeting intrahepatic biliary ducts plays a central role in the pathogenesis (1).

PBC presents features that makes it a typical autoimmune condition. Firstly, it is characterized by the presence of specific autoantibodies, i.e. antimitochondrial autoantibodies (AMAs), which have a central role in diagnosis; secondly, the intense infiltrations of lymphocytes damaging bile ducts is a typical histological finding; finally, PBC patients have a high prevalence of concurrent autoimmune disorders (2). Like other autoimmune diseases, environmental and genetic factors have been called in action to explain its pathogenesis. PBC onset is thought to follow the interaction of an environmental trigger(s) with a predisposing genetic background. Concerning environmental factors, recurrent urinary tract infections, cigarette smoking, hair dyes, and the use of hormone replacement therapies have all been associated with increased risk of PBC (3). Furthermore, a growing number of studies have reported a higher prevalence in urban and polluted areas (4).

Genetic predisposition has a significant role in the onset of PBC too (5). The observation of clustering of cases within families and the high concordance rate between monozygotic twins support this claim. Örnolfsson et al. used a nationwide genealogical database and defined the relative risk for first-, second-, and third-degree relatives of PBC patients as 9.13, 3.16, and 2.59, respectively (6). The odds ratio for PBC of an individual with a sibling affected by the same condition is 10.5, in line with other autoimmune disorders (7). Further, the concordance rate for PBC in monozygotic twins is estimated to be 63%, among the highest reported in autoimmune conditions (3). From a genetic perspective, PBC is a complex trait, defined by Lander et al. as "any phenotype that does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus" (8). This means that multiple gene variants increase or decrease the genetic risk with a relatively small effect size (9). A strong contribution in the advancement of the knowledge of PBC and its genetics has been given by the genome-wide association studies (GWAS). GWAS employ a large scanning of the entire genome for specific genetic variants (typically single nucleotide polymorphisms, SNPs) to identify alleles statistically associated with the disease (10). The strongest statistical association with PBC has been mapped in the human leukocyte antigen (HLA) locus. The HLA region is a highly polymorphic area of the genome located on chromosome 6p21 covering 6.7 Mb (11), and encoding hundreds of loci related to adaptive immune response, including histone and tRNA genes, several key immune response genes, as well as those of the major histocompatibility complex (MHC). The extended MHC region can be subdivided into three classes: class I (extended and classical, respectively, containing the *A, B, C* as well as *MICA* and *MICB* loci); class II (extended and classical, respectively, containing the *DPA1/DPB* and *DQA1/DQB1* loci); class III (containing the *DRA1/DRB1* loci).

Apart from the HLA region, a significant number of non-HLA genes have been identified, and many of the alleles involved genes implicated in innate immunity. In particular, studies have found alterations in genes and pathways involved in antigen presentation and production of interleukin (IL)-12 (*IRF5, SOCS1, TNFAIP3, NF-κB*, and *IL-12A*), activation of T cells, and interferon γ (IFN-γ) production (*TNFSF15, IL12R, TYK2, STAT4, SOCS1, NF-κB*, and *TNFAIP3*), as well as activation of B cells and production of immunoglobulins (*POU2AF1, SPIB, PRKCB, IKZF3*, and *ARID3A*) (Table 1) (12–20).

This review outlines the current evidence about the genetic association between HLA variants and PBC, the link between HLA haplotypes and clinical manifestations in various populations worldwide, the alleged role in disease pathogenesis, and novel therapeutic strategies.

# Genetic studies for HLA variants

From a methodological point of view, it is possible to separate genetics studies regarding HLA in PBC by identifying pre-GWA-, GWA- and post-GWA-studies. A summary of the most significant HLA variants associated with PBC can be found in Table 2.

## Pre-GWAS era

Before the advent of the GWAS era (approximately from 1992 up to 2009), several candidate studies pointing to the HLA region were performed. The analysis of the HLA region identified a relatively small number of alleles associated with PBC in several independent studies (21–26); these results were more reliable than similar studies on non-HLA alleles, that were mostly underpowered and resulted in several signals not validated in following GWAS (27).

First studies on HLA in PBC were conducted in the early '90 on UK population, revealing the presence of *DR8* as predisposing factor for disease onset (21). Donaldson and Invernizzi associated the HLA loci *DRB1*08* with an increased risk of PBC in British and Italian individuals respectively, and *DRB1*11* and *B1*13* with a protective effect in Italians (28, 29). Despite these consistent findings, we should bear in mind that these studies suffered from relatively weak statistical power, a strong potential for type 1 statistical error, and were criticized for their *a priori* approach.

TABLE 1 List of PBC-related non-HLA associations.

| *Chr* | *Locus* | *Candidate gene(s)* | *SNP* | *OR* | *Country* | *Reference* |
|---|---|---|---|---|---|---|
| *1* | 1p13 | CD58 | rs2300747 | 1.30 | Japan/China | *Qui et al.* |
| | 1p31 | IL12RB2 | rs72678531 | 1.51 | Europe/North America | *Liu et al.* |
| | 1p36 | MMEL1 | rs3748816 | 1.33 | Europe/North America | *Hirschfield et al.* |
| | 1q31 | DENND1B | rs12134279 | 1.34 | Europe/North America | *Mells et al.* |
| *2* | 2q12 | IL1RL1, IL1RL2 | rs12712133 | 1.14 | Europe/North America | *Cordell et al.* |
| | 2q32 | STAT4, STAT1 | rs10931468 | 1.50 | Europe/North America | *Mells et al.* |
| | 2q32 | STAT4, STAT1 | rs10168266 | 1.31 | Japan/China | *Qui et al.* |
| | 2q33 | CD28, CRLA4, ICOS | rs4675369 | 1.37 | Japan/China | *Qui et al.* |
| | 2q33 | CD28, CRLA4, ICOS | rs7599230 | 1.37 | Japan/China | *Qui et al.* |
| | 2q36 | CCL20 | rs4973341 | 1.22 | Europe/North America | *Cordell et al.* |
| *3* | 3p24 | PLCL2 | rs1372072 | 1.20 | Europe/North America | *Mells et al.* |
| | 3q13 | CD80 | rs2293370 | 1.39 | Europe/North America/Japan/China | *Liu et al.* |
| | 3q25 | IL12A | rs2366643 | 1.35 | Europe/North America/Japan/China | *Liu et al.* |
| *4* | 4p16 | DGKQ | rs11724804 | 1.22 | Europe/North America | *Cordell et al.* |
| | 4q24 | NFkB1 | rs7665090 | 1.26 | Europe/North America/Japan/China | *Mells et al.* |
| *5* | 5p13 | IL7R | rs6871748 | 1.30 | Europe/North America/Japan/China | *Liu et al.* |
| | 5p13 | IL7R | rs6897932 | 1.52 | Europe/North America/Japan/China | *Kawashima et al.* |
| | 5q21 | C5orf30 | rs526231 | 1.15 | Europe/North America | *Cordell et al.* |
| | 5q33 | IL12B, LOC285626 | rs2546890 | 1.15 | Europe/North America | *Cordell et al.* |
| *6* | 6q23 | OLIG3, TNFAIP3 | rs6933404 | 1.18 | Europe/North America | *Cordell et al.* |
| *7* | 7p14 | ELMO1 | rs6974491 | 1.25 | Europe/North America | *Mells et al.* |
| | 7q32 | IRF5 | rs35188261 | 1.52 | Europe/North America | *Liu et al.* |
| *9* | 9p32 | TNFSF15 | rs4979462 | 1.57 | Japan/China | *Nakamura et al., Kawashima et al.* |
| | 9p32 | TNFSF15, TNFSF8 | rs4979467 | 1.40 | Japan/China | *Qui et al.* |
| *11* | 11q13 | RPS6KA4 | rs538147 | 1.23 | Europe/North America | *Mells et al.* |
| | 11q23 | POU2AF1 | rs4938534 | 1.38 | Japan/China | *Nakamura et al., Kawashima et al.* |
| | 11q23 | CXCR5, DDX6 | rs77871618 | 1.55 | Japan/China | *Qui et al.* |
| | 11q23 | CXCR5, DDX6 | rs80065107 | 1.39 | Europe/North America | *Liu et al.* |
| | 11q24 | ETS1 | rs12574073 | 1.33 | Japan/China | *Kawashima et al.* |
| *12* | 12p13 | TNFRSF1A, LTBR | rs1800693 | 1.27 | Japan/China | *Liu et al.* |
| | 12q24 | SH2B3 | rs11065979 | 1.20 | Europe/North America | *Liu et al.* |
| *13* | 13q14 | TNFSF11 | rs3862738 | 1.33 | Europe/North America | *Juran et al., Liu et al.* |
| *14* | 14q24 | RAD51B | rs911263 | 1.26 | Europe/North America | *Liu et al.* |
| | 14q32 | TNFAIP2 | rs8017161 | 1.22 | Europe/North America | *Mells et al.* |
| *16* | 16p12 | PRKCB | rs3785396 | 1.35 | Japan/China | *Kawashima et al.* |
| | 16p13 | CLEC16yA, SOCS1 | rs12708715 | 1.29 | Europe/North America | *Liu et al.* |
| | 16q24 | IRF8 | rs11117432 | 1.31 | Europe/North America | *Mells et al.* |
| *17* | 17q12 | IKZF3 | rs17564829 | 1.26 | Europe/North America | *Liu et al.* |
| | 17q12 | IKZF3 | rs9303277 | 1.43 | Japan/China | *Kawashima et al.* |
| | 17q12 | Multiple genes | rs9635726 | 1.38 | Japan/China | *Qui et al.* |
| | 17q21 | MAPT | rs17564829 | 1.25 | Europe/North America | *Liu et al.* |
| *19* | 19p12 | TYK2 | rs34536443 | 1.91 | Europe/North America | *Liu et al.* |
| | 19q13 | SPIB | rs3745516 | 1.46 | Europe/North America | *Liu et al.* |
| *22* | 22q13 | SYNGR1 | rs2267407 | 1.29 | Japan/China | *Liu et al.* |

Chr, chromosome; SNP, single-nucleotide polymorphism; OR, odds ratio. Studies are ordered by chromosome.

## GWAS era

In GWAS, the frequency of genetic variants (SNPs) is compared between cases and controls. If there is a statistical association sufficiently divergent from the distribution seen in a control group (typically a p-value < $5*10^{-8}$), variants are considered associated with the phenotype (30). The strength of the association is typically evaluated by the Odds Ratio (OR), calculated based on

TABLE 2 List of PBC-related HLA associations.

| *Reference* | *Year* | *Total subjects (cases+controls)* | *Country* | *HLA associations* |
|---|---|---|---|---|
| *Uderhill et al.* | 1992 | 321 | UK | **DR8** |
| *Morling et al.* | 1992 | 1227 | Denmark | **DR3** |
| *Gregory et al.* | 1993 | 493 | UK | **DR8** |
| *Onishi et al.* | 1994 | 492 | Japan | **DRB1*0803** |
| *Begovich et al.* | 1994 | 582 | US | **DRB1*0801**<br>**DRB1*0901** |
| *Donaldson et al.* | 2001 | 266 | UK | **DRB1*0801**<br>**DQA1*0401/DQB1*0402**<br>**DRB1*0101/DQA1*0401/DQB1*0402** |
| *Stone et al.* | 2002 | 370 | Canada | **DRB1*08** |
| *Wassmuth et al.* | 2002 | 256 | Sweden | **DQB1*0402**<br>**DRB*08** |
| *Invernizzi et al.* | 2003 | 707 | Italy | DRB*11 |
| *Invernizzi et al.* | 2003 | 670 | Italy | **B*15**<br>**B*41**<br>**B*55**<br>**B*58** |
| *Mullarkey et al.* | 2005 | 453 | US | **DQB1*04**<br>**DRB1*0801**<br>DQA1*0102<br>DQB1*0301<br>DQB1*0602<br>DRB1*1501 |
| *Donaldson et al.* | 2006 | 411 | UK | **DQA1*0401** |
| *Donaldson et al.* | 2006 | 175 | Italy | **DQA1*0401**<br>**DQB1*0402**<br>**DRB1*08**<br>DQB1*0301<br>DRB1*11<br>DRB1*13 |
| *Donaldson et al.* | 2006 | 350 | Italy | **DRB1*0801/DQA1*0401/DQB1*0402**<br>DRB1*11/DQA1*0501/DQB1*0301<br>DRB1*13/DQA1*0103/DQB1*0603 |
| *Donaldson et al.* | 2006 | 648 | UK | **DQB1*0402**<br>**DRB1*08**<br>DQB1*0301<br>DRB1*13 |
| *Donaldson et al.* | 2006 | 1296 | UK | **DQA1*0401/DQB1*0402**<br>**DRB1*0801/DQA1*0401/DQB1*0402** |
| *Invernizzi et al.* | 2008 | 2656 | Italy | **DRB1*08**<br>DRB1*13 |
| *Nakamura et al.* | 2010 | 1184 | Japan | **DRB1*0405**<br>**DRB1*0803**<br>DRB1*11<br>DRB1*1101<br>DRB1*1302<br>DRB1*1501 |
| *Umemura et al.* | 2012 | 752 | Japan | **DRB1*0405/DQB1*0401**<br>**DRB1*0803/DQB1*0601**<br>DRB1*1101/DQB1*0301<br>DRB1*1302/DQB1*0604 |
| *Liu et al.* | 2012 | 11275 | UK | **DQA1*0401**<br>**DQB1*0402**<br>**DQB1*0302**<br>**DRB1*0404**<br>DQB1*0602<br>DQB1*0301 |

*(Continued)*

TABLE 2 Continued

| *Reference* | *Year* | *Total subjects (cases+controls)* | *Country* | *HLA associations* |
|---|---|---|---|---|
| | | | | DRB1*1501<br>DRB1*1101<br>DRB1*1104<br>DQA1*0102<br>DQA1*0501 |
| *Invernizzi et al.* | 2012 | 2116 | US | **DRB1*08**<br>**DRB1*14**<br>DRB1*11 |
| *Invernizzi et al.* | 2012 | – | Italy | **DRB1*08**<br>**DRB1*14**<br>DRB1*11 |
| *Zhao et al.* | 2014 | 645 | China | **DRB1*0701/DQB1*0202**<br>**DRB1*0803/DQB1*0601**<br>DRB1*1202/DQB1*0301 |
| *Almasio et al.* | 2016 | 265 | Italy | **DRB1*07**<br>**DRB1*08** |
| *Clemente et al.* | 2017 | 218 | Sardinia | **DRB1*0301/DQB1*0201** |
| *Yasunami et al.* | 2017 | 2392 | Japan | **DRB1*0405/DQB1*0401**<br>**DRB1*0803/DQB1*0601**<br>DRB1*1302/DQB1*0604<br>DRB1*1403/DQB1*0301<br>Not(DRB1*1403)/DQB1*0301 |
| *Darlay et al.* | 2018 | 11275 | UK | **DPB1*0301**<br>**DPB1*0601**<br>**DPB1*1001**<br>**DPB1*1701**<br>**C*0401**<br>**DPA*0201**<br>DPB1*0401 |
| *Li et al.* | 2022 | – | – | **DRB1*0701**<br>**DRB1*1401**<br>**DRB1*1405** |

Studies are ordered by year. HLA variants in bold represent predisposing associations, those underlined represent protective ones. Only significant associations (P<0.05) are reported. Approximately, the pre-GWAS era corresponds to years 1992-2009, while GWAS era starts from 2009; post-GWAs technologies have been employed in PBC from years 2021.

the allele frequencies in cases and controls. Most of human multifactorial diseases have a complex genetic architecture, with several risk variants contributing little to the disease per se and tagging mostly regulatory regions of the genome.

From 2009 up to the present date, GWAS in PBC have included individuals of European origin (Italy, United Kingdom, USA, Canada) and East Asians (China, Japan), and further analyses, including fine-mapping studies and genome-wide meta-analysis have expanded the list identifying up to 50 genome-wide significant associated variants (12–19, 31–33). Genome wide association studies confirmed that the MHC region remains by far the strongest genetic contributor to PBC susceptibility in terms of OR (Figure 1). The HLA-*DRB1* genes (alleles *08, *11, and *14) have been reported for most of the *DRB1* association signal; *DRB1**08 is the strongest predisposing allele, whereas *DRB1**11 is the protective one (34). A more recent meta-analysis from Li et al. (35) identified HLA *DR*07* as risk factor in Asian and European populations, and HLA *DR*08* associated with PBC in Asian, American and European subgroups; on the contrary, a protective role of HLA *DR*11* and **13* was described in Asian and European populations, while HLA *DR**12 decreased risk of PBC in Asian and **15* in European and Americans populations. It is of note that the HLA signals *DR*08*, *DRB1*11*, and **13* have been confirmed across different populations, except in Sardinians (36). Furthermore, studies based on UK PBC consortium confirmed the results of the pre-GWAS era. Strong associations were found for HLA-*DQA1* (alleles *04:01), conferring an approximately threefold increased disease risk, HLA-*DQB1* (alleles *04:02, *03:02) and HLA-*DRB1* (alleles *04:04). Conversely, HLA-*DQB1* (alleles *06:02, *03:01), HLA-*DRB1* (alleles *15:01, *11:01, *11:04), HLA-*DQA1* (alleles *01:02, *05:01) have been reported as protective ones (18).

Subsequently, HLA-*DPB1* (alleles *03:01, *06:01, *10:01, *17:01 as causative and alleles *04:01 as protective), HLA-*C* (allele *04:01) and HLA-*DPA* (allele *02:01) were found to be significantly associated within the same cohort (37). In a Chinese

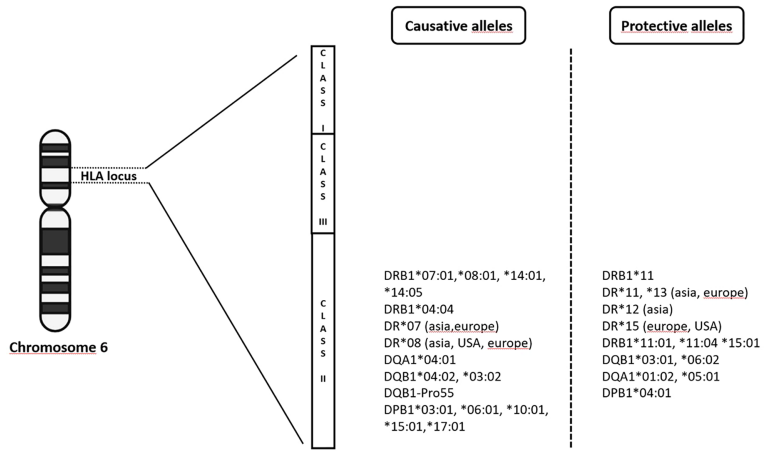


FIGURE 1
The complex milieux of HLA alleles and its association with PBC (increased vs protective risk).

cohort, Qiu et al. found 179 SNPs in the MHC region and described the most significant association with the HLA-DRA locus, while a SNP in the HLA-*DRPB1* was the second most significant locus in the MHC region (16).

GWAS allow to associate genetic variants with any phenotype of interest. PBC is typically characterized by the positivity for AMA and/or PBC-specific anti-nuclear antibodies (against the gp210 and sp100 proteins) (38). Positivity for these autoantibodies may occur even before the presence of overt disease and be an isolated laboratory finding. In a Chinese cohort, Wang et al. studied the genetic predisposition to sp100 positivity and identified HLA-*DRB1**03:01, *DRB1**15:01, *DRB1**01 and *DPB1**03:01 alleles as the most associated ones (38).

Generally speaking, since association does not mean causation, the region where the leading SNP has been found is usually dissected by the so-called "fine mapping strategy", that aims to identify the potential biological role of the genetic variant associated with the disease (39). Fine-mapping of the MHC region in Han Chinese court confirmed that HLA-*DRB1* and/or HLA-*DQB1* contributed the strongest signals, and that HLA-*DPB1* was a separate independent locus (40). In addition, the authors performed logistic regression to identify independent associations with amino acid polymorphisms in MHC genes. Three residues (HLA-DRβ1-Ala74, HLA-DQβ1-Pro55 and HLA-DPβ1-Asp84) were found to be independently associated with PBC and all three were located in the peptide-binding groove of MHC II molecules. The 3D protein structure model and electrostatic potentials calculation revealed alternations in antigen-binding affinity leading to an instability of the MHC proteins, that has been proposed as a pathogenetic mechanism in HLA-mediate autoimmune disease (41).

## Post-GWAS era

GWAS have some intrinsic limitations. First of all, GWAS results only account for few percent of the whole genetic risk (the remaining being called "missing heritability") (42). One reason is that diseases are likely to have some of their genetic risk determined by rare genetic variants that are not included in GWAS (43). Second, GWAS generate hypotheses but require fine-mapping downstream approaches to put their results in the right biological context. Third, many ethnic groups have not been included in GWAS for decades, leaving out a huge number of underserved populations (44). On top of the ethical concerns, when GWAS have been performed on populations with different genetic backgrounds only partial overlap with historical ones has been found, revealing novel variants and generating novel candidates for further dissection.

For all of these reasons, several "post-GWAS" strategies have been developed (45). As far as the study of HLA is concerned, there is mounting interest in studying how genetic alterations result in altered peptides. This is becoming more and more relevant also for clinical purposes, since typing HLA at the amino acid level can tease apart different proteins that can lead to allogeneic responses. For example, as previously described, amino acid alterations located in the antigen-binding site of MHC molecules can lead to instability of the molecule that more

likely might form MHC-self-epitope complexes, conferring a risk for autoimmunity (41).

Nonetheless, precise HLA typing remains very challenging due to the high density of polymorphisms in HLA genes, sequence similarity among these genes, and to the extreme level of linkage disequilibrium of the locus (46). High resolution HLA typing is possible with various technologies that are becoming more and more efficient with an increasing reduction in cost. Next-generation sequencing (NGS) methods have been increasingly replacing previous technologies (such as DNA-based typing and sequence-based typing methods) allowing an increasingly precise HLA typing, bringing many advancements to the field of HLA genotyping within relatively few years (47).

Other promising approaches have been under development. For example, the PCR-NGS approach implies the use of standard polymerase chain reaction (PCR) to capture regions of interest, and the resultant amplicons are then subjected to NGS (48). More recently, a new technology called HLAscan has been proposed. HLAscan is a typing multi-step method based on alignment approach, in which short selected sequence reads are aligned with reference HLA alleles obtained from a database and then allele types are determined based on the numbers and distribution patterns of the reads on each reference target. HLAscan demonstrated not only to outperform the established NGS-based methods but also to complete other sequencing-based typing methods (49).

World-wide efforts in developing NGS technologies have dramatically increased the availability of whole-genome sequencing (WGS) and whole-exome sequencing (WES) data. WES and WGS have proved to be effective strategies in discovering rare variants that produce large effects, even in non-monogenic rare disease (50) and in isolated population (51). Li et al. applied WES in three PBC families and associated three new HLA variants (HLA-*DRB1**07:01, *14:01 and *14:05) to PBC (52). The authors of this study also describe the amino acid variants located in the peptide-binding site of the MHC molecule and found that *DRB1**07:01 was strongly associated with clinical manifestations. Of note, Wang et al. used WES on 90 individuals including 30 PBC probands and identified 19 unreported genes harboring validated *de novo* mutations (DNMGs) (53). Subsequently, the authors interrogated the co-expression dynamics of the DNMGs utilizing a published transcriptome dataset of the $CD4^+$ T cells, since they play a critical role in PBC (54). In addition, they found that transcription factor MEF2D and the DNA repair gene PARP2 were highlighted as hub genes and identified to be up- and down-regulated respectively in peripheral blood data. This transcription alteration seems to be the trigger of a series of molecular and cellular processes that have pivotal roles in PBC pathophysiology (53). This represents a small example of the potential of WES/WGS to further improve the understanding of the genetic predisposition to the disease.

## Geoepidemiology

PBC presents different prevalence and incidence rates across populations and geographical areas. Geo-epidemiology has focused on potential environmental and socioeconomic risk factors (55, 56). Studies from the North-East of England have identified a higher prevalence of PBC in urban areas that have a strong coal-mining heritage (55), whereas a study in New York City found clusters of PBC patients in zip codes that contained, or were adjacent to, superfund toxic waste sites (56).

Other environmental factors associated with increased risk of PBC were cigarette smoking, hair dyes, the use of hormone replacement therapies, and recurrent urinary tract infections (UTI) (3). As it concerns the latter molecular mimicry analysis has shown that PBC autoantigene pyruvate dehydrogenase complex E2 subunit (PDC-E2) has common structure with the Escherichia coli E2 subunit of the 2-oxo-acid dehydrogenase complexes (57). This suggested that the infection with E. coli, a predominant pathogen in most cases with UTI, is a key factor in breaking immunological tolerance in PBC (58).

In addition to environmental factors, there is known variation in allele frequencies among different ethnic groups. Unfortunately, PBC GWAS have mostly been performed on subjects of European ancestry and more recently on east-Asians (Han Chinese and Japanese). At the time of writing, genetic studies on "neglected population" such as African, Latin American or Australian population are still lacking.

## The role of HLA in PBC pathogenesis: Imperfect HLA interactions

The most recent model of PBC pathogenesis comprises two phases: an initiation phase, characterized by autoimmune phenomena driven by defects in biliary homeostasis and aberrant antigen presentation; and a progression phase, driven by the retention of bile acids (2). Based on this model, one would expect that HLA would play its major pathogenetic role at the initiation phase. Despite the association between HLA and autoimmune diseases have been known since 1970s (59), the exact pathogenic mechanism that links HLA and disease onset remains largely elusive (60). The scientific progress in genetic and molecular profiling technologies represents a big hope for the future, since it is gradually unveiling the complex interaction between HLA, peptides and TCR (61). Several structural and associated functional studies were conducted, with more than 50 unique TCR-peptide-HLA structures recognized (62).

A variety of physiologic and steric factors can affect the TCR-peptide-HLA interface (61). Further, several mechanisms of imperfect HLA interactions were identified as potential

disease triggers in several autoimmune disorders. They include: i) molecular mimicry and mechanical binding alterations (HLA–peptide–TCR binding orientation, low-affinity peptide binding), ii) post-translational epitope modification or iii) generation of hybrid peptides (63) (Figure 2). As for the latter, no data are available in PBC. We will thus briefly focus on the first two mechanisms.

## Molecular mimicry and mechanical binding alterations

Molecular mimicry is a well-accepted mechanism linking infections and autoimmunity onset. It consists in the antibody- and cellular-mediated immune response against self-protein(s) sharing a sufficient degree of similarity with epitopes of infectious agents (64). Almost all patients with PBC have autoantibodies recognizing the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) or other related mitochondrial enzymes complexes. Thus, cross reactivity should not be a surprise since such mitochondrial enzymes have conserved sequences shared across different species (65). Shimoda et al. nicely demonstrated the mechanism of molecular mimicry of AMA and antinuclear autoantibodies in PBC, and also showed that the mimicry peptide can become the immunodominant T-cell epitope (57, 66). Different bacterial strains and xenobiotics were considered as possible causative of cross reaction in PBC. Among them, E. coli and *Novosphingobium aromaticivorans* are considered among the best candidates for the induction of PBC (67–71).

Along with molecular mimicry, mechanical binding alterations were considered among the triggers of autoimmune disorders (63). Somatic hypermutation (SHM) is a process occurring within germinal centres, aimed at producing high-affinity antibodies *via* the introduction of somatic mutations in the rearranged region of the Ig genes. Activation-induced cytidine deaminase (AID) is required for SHM to occur, and it leads to DNA mutations by conversion of cytosine into uracil (72). While some studies showed that AID can contribute to the loss of self-reactivity in mice and humans (73–76), other have attributed a role of AID and SHM deficiencies in autoimmune disease onset and severity (77–81). As for PBC, a single work assessed a limited SHMs in class-switched isotypes in PBC patients, which may be indicative of low-affinity Ag binding (82). To the best of our knowledge, data on HLA–peptide–TCR binding orientation in PBC are still lacking.

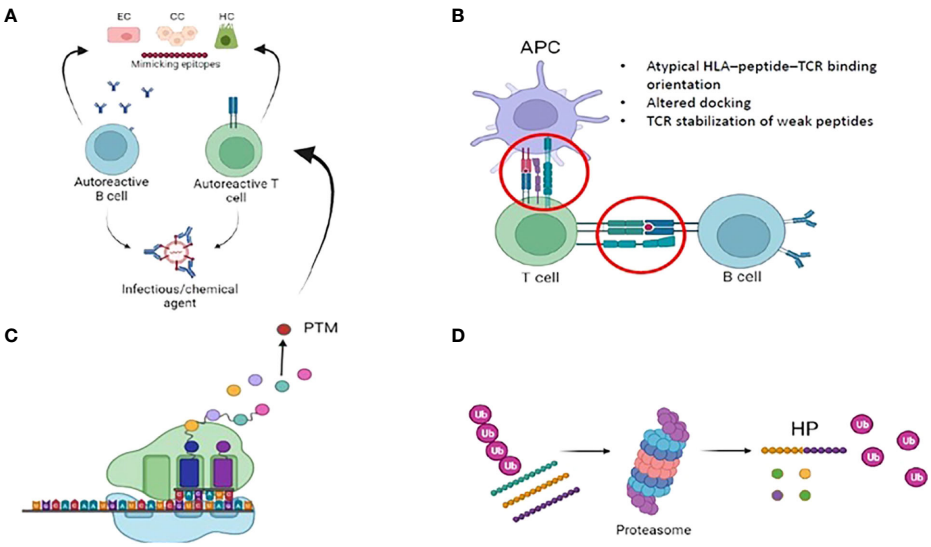


FIGURE 2
Imperfect HLA interactions and autoimmunity: **(A)** Molecular mimicry: Foreign antigens (i.e. either from infecious or chemical agents) with similarities to self-antigens are primed by T and B cells, that autoreactive and trigger autoimmunity. **(B)** Immunological synapse and autoimmunity: several mechanisms of the complex interplay between APC, B- and T- cells might lead to the activation of autoimmune B and T cells. Among them, mechanical binding alterations (atypical binding orientation, altered docking) and TCR stabilization of weak peptide. Weak HLA binding of self-peptides might contribute to autoimmunity by allowing the escape of autoreactive T-cellsfrom the thymus. **(C)** Post translational modifications (PTM): they are spontaneous or enzymatically induced modifications of one or more amino acids occuring after protein biosynthesis. After PTM, these proteins become modified self-antigens, and they do not "tolerize" developing thymocytes. Consequently, modified self-antigens can be taken and processed by APC, that will present them to autoreacitve T and B cells. **(D)**: Generation of hybrid peptides: proteasomal-mediated degradation and splicing of intracellular self-peptides can promote the generation of non-self-hybrid peptides. This mechanism occurs with high protein concentrations in a confined environment, that favours protease-mediated peptide fusion.

## Post-translational epitope modification

Post-translational modification refers to the addition of a functional group to a protein or to the proteolytic processing and folding necessary its functional maturation. Acetylation, methylation, phosphorylation, ubiquitination and sumoylation are among the most common functional group additions. These modifications have fundamental roles in chromatin structure and function.

Reduced methylation levels of the cluster of differentiation 40 ligand (CD40L) promoter were described in peripheral CD4 T cells in PBC patients, and this correlated to increased immunoglobulin M (IgM) serum concentration, a typical serological finding of PBC (83). An increased variety of methylation patterns were on chromosome X of a small cohort of monozygotic twins with PBC (84). A scan of the X chromosome revealed a hypomethylation of the promoter of a C-X-C motif chemokine receptor 3 (CXCR3) (85). CXCR3 plays a key role in dysimmunity, and increased levels of this receptor were described among PBC patients (86).

T cells from PBC patients showed an increased concentration of β-arrestin 1, a protein involved in CD4+ T cells histone acetylation, and its overexpression correlated to hepatic disease severity (87). Upregulated βarr1 in PBC associated to decreased levels of TNF-related apoptosis-inducing ligand (TRAIL), involved in autoimmunity prevention through cell cycle arrest (87). The role of non-coding RNAs (ncRNAs) was largely studied in PBC. They are RNAs not translated into proteins. Scientific focus was directed towards microRNAs (miRNAs), a specific class of ncRNA with the ability to regulate gene expression (88). Among miRNAs, MiR-506 overexpression was described in PBC. This specific miRNA targets the anion exchanger 2 (AE2), an important protein involved in biliary epithelium homeostasis (89). Of note, miR-506 targeted cholangiocytes showed an higher expression of pro-inflammatory and pro-fibrotic markers (90). A novel and interesting topic in PBC is represented by the connection between epigenetic alterations and X chromosome monosomy/inactivation abnormalities (91). Deep investigation on the impact of epigenetic modifications in X chromosome alterations in PBC might reveal important insights on disease specific sex imbalance and pathogenesis.

# The role of biliary epithelial cells in PBC pathogenesis

The recognition of self-peptides selectively presented by susceptible HLA has gained scientific interest for a long time. Specific HLA loci, when combined with certain molecules, may confer either protection or susceptibility to develop autoimmunity. In rheumatoid arthritis, variants at 71β position of certain HLA haplotypes are responsible for the presentation of citrullinated peptides, recognized as non-self by T cells (92). Similarly, the presentation of the reduced form of insulin to specific DRB1 protein haplotypes correlated to insulin autoimmune syndrome (93). As for PBC interesting data are emerging on the role of biliary epithelial cells [BECs, aka cholangiocytes (CC)] as possible triggers of autoimmunity. Cholangiocytes line the biliary tree from the small intrahepatic Hering canals to the large extrahepatic bile ducts (94). They are endowed with the ability to regulate bile production, fluidity, and homeostasis (95). Evidence showed that CC actively participate in the innate and adaptive immune responses, acting as innate immune cells, antigen presenting cells, and expressing cytokines, chemokines, and adhesion molecules (96). In this way, they protect hepatocytes from potentially toxic bile acids, microbial products, and the direct translocation of gut-derived microbes (97). BECs constitutively express HLA class I associated to CD8 cytotoxic activity, as it occurs for all mammalian epithelial cells. However, differently from other epithelial cells, they are capable of antigen-uptake and can upregulate HLA class II peptides in response to the local release of inflammatory cytokines, in particular IFN-γ and IL-2 (98).

This process was described in several autoimmune cholangiopathies, including PBC (99–102). Despite this, several studies failed to demonstrate that HLA class II expressed by BECs can directly present peptide antigens to T cells (54, 101, 103). This might occur secondary to the lack of B7-1 (CD80) and B7-2 (CD86) co-stimulatory ligands, required for T cell activation. The inefficient peptide antigen presentation, together with the expression of inhibitory molecule such as PDL1 and PDL2, result in T-cell anergy. This suggested a possible role of BECs as accessory cells rather than proper antigen presenting cells, at least for peptide antigen presentation (104–106).

A breakthrough in the recent history of immunology was the discovery of CD1-restricted T cells, capable of a subset of HLA I-like molecules with the ability to present self and microbial lipid antigens to T cells (107). The scientific excitement associated with this discovery grew dramatically with the recognition of CD1d-restricted NKT cells, a subset of T-lymphocytes bridging innate and adaptive immunity (108). Most NKT can recognize self-lipid antigens, but the antigen capability to activate NKT and trigger cytokine release depends largely on TCR signalling strength and the presence of co-stimulatory signals (109). NKT can rapidly express pro- and anti-inflammatory cytokines and are endowed with split roles in several pathologic conditions, including autoimmune disorders and malignancies. They can inhibit or exacerbate autoimmunity, and this feature makes them an interesting target for treatment (110, 111).

Cholangiocytes can present lipid antigens through CD1d and the HLA-related receptor MR1, respectively (112–114). In particular, MR1 activates a specific subset of T cells, the mucosal

associated invariant T cells (MAIT), that are located in the peribiliary sinusoids close to the portal tracts, thus being in straight contact with gut bacteria antigens. Peribiliary MAIT localization was described in several hepatic disorders including PBC, autoimmune hepatitis and primary sclerosing cholangitis (115).

As for PBC, MAIT cells seem to have a protective, since their levels is reduced as compared to healthy livers (116).

CD1d is upregulated on biliary epithelial cells during the early stages of PBC (113). *In vitro* models showed the ability of CD1d to activate natural killer T cells through the presentation of endogenous and microbiome-derived lipid antigens (112). Whether the expression of HLA class II molecules might represent an epiphenomenon of BECs activation, or it might link cholangiocytes and autoimmunity is unknown, and further studies are awaited.

## The strength of unity in autoimmunity: Shared HLA recognition

Specific HLA subtypes are shared among different autoimmune disorders (117). This feature has attracted the scientific interest in the search for molecular mechanisms behind this correlation (63). Conversely, several MHC–I-pathies share immunopathogenic basis despite different HLA genotypes (118). The ability to compare HLA commonalities and differences among autoimmune diseases can translate into the recognition of shared or distinct pathophysiological pathways, thus increasing the understanding of disease aetiology and therapy. As for HLA class II, different allele interactions were identified for multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and coeliac disease (119). However, large data collection and sharing enabled by technological advances will ease the process of common disease pattern recognition.

## The role of HLA-mediated antigen recognition

The pool of endogenous and exogenous antigens presented by each HLA molecule varies enormously not only among different subjects but also within different tissues of the same subject. The rapid pace of epitope recognition fostered by novel quantitative mass spectrometry technologies enabled the recognition of millions of peptide repertoires, collected from blood and target tissue samples (120). In chronic autoimmune disorders, epitope heterogeneity coupled with HLA polymorphisms leads to a continuous competition of T cells for the most immunodominant epitopes. This resulted in the development of a large T cell clonotypes repertoire, consequence of lifetime modifications occurring from the early thymic T-cell development to specific events occurring in peripheral tissues (121). In liver tissues derived from PBC patients, disease-associated clonotypes were detected irrespectively of the presence of DRB1*08:01/DRB1*04:04 susceptibility alleles, with three clonotypes detected in 40% of the cases. Further, antigen-driven clonal selection and expansion was evidenced, with HLA restricted autoreactive T cells specific for the PDC-E2 (122). In addition, T-cell repertoires differ among autoimmune liver diseases (123), and this observation led to the concept of disease-specific imprinting from antigenic repertoires.

To conclude, the recognition of PBC-specific HLA susceptibility can translate into specific T-cell repertoires. This might consequently lead to the recognition and selective targeting of disease-specific T-cells, with possible treatment implications for the future.

## The imperfect HLA-antigen-TCR binding behind autoimmunity

It has been long recognized that the strength of HLA-peptide-TCR interaction can influence the thymic fate of a TCR (124). In particular, weaker interactions promote positive selection and T cell survival, while stronger ones drive clonal deletion and differentiation into Treg phenotype.

The study of highly polymorphic TCR sequences and HLA alleles was enabled only in recent years, thanks to technological advances in deep-sequencing technologies (125). TCR receptors consist of an α- and a β-chain. They include three highly variable peptide loops, each encoded by a specific gene, that protrude toward the pMHC complex. Of them, the complementarity-determining region (CDR3β) in the β chain is the most variable, and it mediates antigen recognition (126). Hydrophobicity at specific positions of the β–chain CDR3β was demonstrated to promote reactivity to self-peptides (127, 128). Recent evidence highlighted the role of TCR β variable gene (TRBV), encoding for a region of CDR3β, in the mechanism of autoimmunity. In particular, TRBV shapes the TCR affinity to conserved HLA sites, thus altering their propensity towards self vs non-self-activation (129). A study of peptide-HLA libraries coupled to deep-sequencing analysis attested a higher tolerance for substitutions to peptide residues that are located distally to the TCR-binding site, and some of them were identified through computational analysis (130). The TCR-peptide-HLA docking mechanism is not always perfect, and engagement of homologous antigens *via* distinct docking geometries was described. TCR-antigen cross reaction might lead to the breakdown of immune tolerance, potentially triggering autoimmunity (131, 132). A recent study used NGS to

characterize and compare the TCRβ chain of peripheral T cells in PBC patients vs controls (68). Results showed that PBC patients carried TCRβ with reduced CDR3 length and that TCRβs bearing shorter CDR3 loops, which are associated with abnormal insertion length at the variable, diversity and joining gene segments in the β-chain locus. The functional outcome of this structural alteration is yet to be demonstrated.

## Exploiting the HLA-peptide-TCR binding to modulate autoimmunity: Antigen specific immunotolerance

Antigen-specific immunotolerance (ASI) represents one of the most promising therapeutic approaches for autoimmune disorders. It has been long practiced in the field of allergy (133, 134), and it consists in the promotion of antigenic specific tolerance by inhibiting autoantigen-specific immune response, while sparing the rest of the immune system. In fact, despite the revolutionary potential of immune suppressive treatments in autoimmune and inflammatory disorders, those are predominantly symptomatic approaches not acting on disease cause and associating to several side effects. The inhibition of autoantigen-specific immune response though ASI represents a clever, innovative, and safer approach. It mainly relies on the administration of antigenic epitopes derived from the same proteins targeted by the autoreactive lymphocytes. Prior to antigen administration, specific antigenic conditioning is required to induce a regulatory response capable to restore homeostasis and to aid the deletion of autoreactive cones. The HLA background has a strong influence on the efficacy of ASI, since HLA heterogeneity largely shapes the antigen-specific immune responses. Individual HLA background should be considered in clinical trials exploiting ASI, and early tolerization should be encouraged to reduce the likelihood of epitope spreading.

The use of ASI recently increased also in autoimmune disorders, after a preliminary success in experimental models (135). So far, ASI focused on antigen presenting cells (APC), in particular dendritic cells (DC), and CD4 T cells, responsible for the onset and perpetuation of most autoimmune-related processes (136). Tolerogenic vaccines represent the mainstay of ASI, and several vaccines platforms were built throughout the years (Figure 3). Since APC represent the main actors of antigen specific tolerance, strong efforts were directed towards the recognition and delivering of autoantigens to specific APC subtypes (136–138). There are several mechanisms of action behind the induction of antigen-specific tolerance, each with a specific antigen delivery approach (139). A major limit of ASI in most autoimmune disorders is the poor knowledge of the whole autoantigen panel involved and the uncertainty of whether tolerance towards the recognized autoantigen/s is enough to reverse autoimmunity, since several autoimmune disorders have autoantibodies associated with disease but without a role in disease pathogenesis (140). ASI should rely on antigens capable of modulating CD4 T cells without engaging pathogenic B or T cells. In this way, a correct balance between efficacy and safety is guaranteed.

Among the different antigen delivery approaches, the use of nanoparticles seems promising, with great interest for tolerogenic nanoparticles (tNPs). A particular subtype of tNPs, called tolerogenic immune modifying nanoparticles (TIMP) were designed to achieve antigen-specific and site-specific immune tolerance (141).

TIMP use has steeply increased, emerging from cell-based to synthetic antigen carriers and from animal models to clinical trials. In particular, the role of TIMP was studied in some autoimmune disorders, such as experimental autoimmune encephalomyelitis (the mouse model for multiple sclerosis), type 1 diabetes, systemic lupus erythematosus and multiple sclerosis (142–149). Of note, up to 99% of nanoparticles pass through the liver, and most of them are taken up and internalized by scavenger receptors of hepatic Kupffer cells (150, 151).

The tolerogenic nature of liver fosters the induction of antigen-specific regulatory T cells (Tregs), a subset of CD4+ T cells with a critical role in maintaining immune system homeostasis and immune tolerance. The contact between nanoparticles carrying autoantigens and Tregs favours the induction and activation of latter (152). PBC represents an ideal target disease for nanotechnology, since autoantibodies are disease- and organ-specific, thus increasing the specificity of nanoparticles-delivery. The major actors of PBC immunopathogenesis are AMA, T cells and biliary epithelial cells, while the inner lipoyl domain of PDC-E2 is the dominant autoepitope (153). The recognition of the correct timing and dosing of antigen delivered by nanoparticles are limiting steps in the process of immunogenicity, and research is ongoing at an exciting pace. TIMP is a potentially safe approach, and it represents a potential shift from immunosuppressive to tolerance inducing therapies in autoimmune disorders (154–156). As mentioned above, Tregs orchestrate the immune system homeostasis. They express high levels of transcription factor Forkhead box protein P3 (FOXP3), critical for their thymic development and activity (157). FOXP3+ Tregs exert a suppressive activity for autoreactive T and B cells (158), and its absence results in severe autoimmune and immune-related disorders (159, 160). Treg dysfunction might be responsible for the uncontrolled T cell reaction against cholangiocytes, as showed by Jeffrey et al. (161). PBC patients showed a reduced level of peripheral Tregs (162, 163), and the silencing or reduced activity of FOXP3 in murine models led to peri-

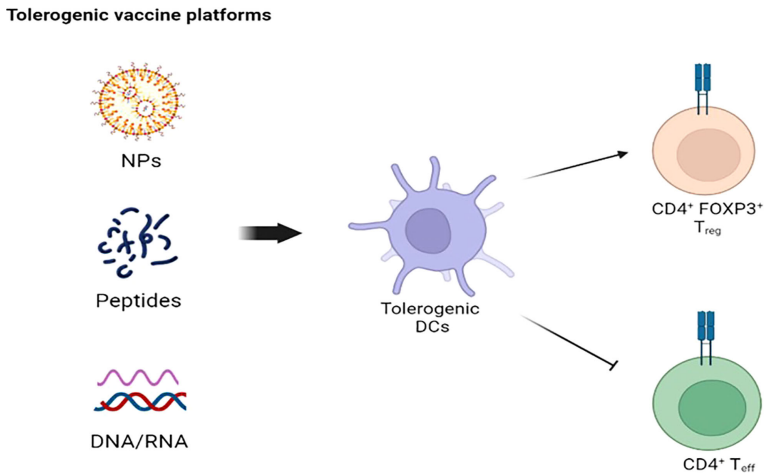


FIGURE 3
Tolerogenic vaccine platforms: The goal of NPs-, peptides-, and DNA/RNA-based platformes is to stimulate tolerogenic DCs to suppress $CD4^+$ T cell-mediated autoimmunity. NPS, nanoparticles; DC, dendritic cells.

biliary immune damage and AMA production (164, 165). Of interest, reduced levels of Tregs were recorders also in the peripheral blood of daughters and sisters of PBC patients (162, 163). The expansion of Tregs represents a promising treatment approach in autoimmune disorders, even if several barriers need to be overcome to achieve a high quality Treg cell therapy. Among them, mentions should be done to the difficulty of Treg expansion and the lack of Treg-exclusive markers (136).

## Conclusions

PBC is a complex autoimmune disorder with high inter- and intra- individual variability. HLA has long been studied in PBC, and associations were first described in the pre-GWAS era and largely expanded thanks to the rapid spread of GWAS platforms. However, such platforms are not informative on the causal genes or the disease mechanisms, explain only a small fraction of the missing heritability, do not allow to assess rare disease variants, and were not implemented for several populations worldwide. The rapid pace of technological innovation, with the development of novel sophisticated sequencing technologies will gradually unveiling the "hidden" genomic signatures, possibly enabling a complete disease HLA (and non-) mapping. The role of HLA in PBC, as it occurs for most autoimmune disorders, is still to be determined and detailed. The HLA-peptide-TCR interaction has been the focus for long time, but we are far from an exhaustive explanation on how it might be involved in autoimmunity. While PBC pathogenesis remains far from being determined, there is a rapid and constant surge of novel treatment approaches aimed at limiting the autoimmune clones to guarantee antigen specific immunotolerance. Among them, scientific excitement surrounds the discovery of tolerogenic vaccine platforms aimed at the expansion of Tregs.

## Author contributions

GM and AP equally searched the literature and contributed to the first draft of the manuscript. All authors provided critical feedbacks and helped to shape the research, analysis and manuscript. PI, MEG, RA and AG reviewed for important intellectual contents. All the authors approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Disease predisposition of human leukocyte antigen class II genes influences the gut microbiota composition in patients with primary biliary cholangitis

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**Background:** The human leukocyte antigen (HLA) susceptibility gene is the main genetic risk factor for primary biliary cholangitis (PBC). The prognosis of patients with PBC is linked to gut microbiota dysbiosis. However, whether the HLA alleles are associated with the gut microbiota distribution and disease severity remains unknown.

**Methods:** A cohort of 964 Chinese patients with PBC was enrolled at Beijing YouAn Hospital, Beijing, China. High-resolution genotyping of the HLA class I and class II loci from 151 of these patients was performed using sequence-based PCR. Stool samples were collected from 43 of the 151 fully HLA-typed patients to analyze their microbiota compositions *via* 16S RNA gene sequencing.

**Results:** Of the 964 patients, the male:female ratio was 114:850, and 342 of these patients (35.5%) had already developed liver cirrhosis (LC) before enrollment. Patients with PBC showed a significantly higher frequency of HLA *DRB1*08:03* than did the controls (21.2% vs. 9.0%, $P$=0.0001). HLA-*DRB1*03:01*, *DRB1*07:01*, *DRB1*14:05*, and *DRB1*14:54* frequencies were also increased but did not reach significance after Bonferroni's correction. Conversely, the *DQB1*03:01* frequency was significantly lower in patients with PBC than in the controls (24.5% vs. 39.2%, $P$=0.0010). The patients' gut microbiota were analyzed from four perspectives. The microbial community abundances were significantly lower in FHRAC-positive patients (patients with a combination of five HLA *DRB1* high-risk alleles) than in FHRAC-negative patients ($P$<0.05). Of the top 10 microbial genera, *Lachnospiraceae_incertae_sedis* was higher in the FHRAC-positive patients than in the FHRAC-negative patients ($P$<0.05). linear

discriminant analysis (LDA) effect-size (LEfSe) analysis showed different microbes at different levels in the FHRAC-negative patients but not in the FHRAC-positive patients. *DQB1*03:01*-positive patients contained mostly Lactobacillaceae at the family level. A comparison of the FHRAC-positive patients with and without liver cirrhosis showed that the abundances of *Veillonella* were significantly higher in patients with cirrhosis and FHRAC than in those without cirrhosis and are FHRAC-negative.

**Conclusion:** The HLA class II genes may influence the gut microbiota compositions in patients with PBC. Differential gut microbiota were expressed at different taxonomic levels. Some bacterial abundances may be increased in FHRAC-positive patients with PBC and cirrhosis.

KEYWORDS

**primary biliary cholangitis, human leukocyte antigen, gut microbiota, susceptibility gene, bioinformatic analysis**

# Introduction

Primary biliary cholangitis (PBC) is an autoimmune liver disease, with its hallmark being the serological signature of the antimitochondrial antibody (AMA) and specific bile duct pathology. The disease is chronic and often progressive, resulting in end-stage liver disease and associated complications. In China, a meta-analysis estimated that the prevalence of PBC was 20.5/100,000 persons (1). In European populations, the estimated incidence is 1–2/100,000 persons annually, and commonly cited ranges for PBC incidence and prevalence are 0.3–5.8 and 1.9–40.2/100,000 persons, respectively (2). Interactions among environmental, genetic/epigenetic, and immunological factors play crucial roles in the PBC pathogenesis, although the factors leading up to disease initiation and progression mechanisms are poorly understood.

Epidemiological studies from different countries have provided genetic evidence based on familial clustering and suggest that PBC results from the combination of "bad genes and bad luck". In researching the genetic basis of PBC, case-control studies have revealed an association between human leukocyte antigen (HLA) alleles and PBC development. Specifically, members of the *DRB1**08 allele family, including *DRB1**08:01, *DRB1**08:03, *DRB1**14, and *DPB1**03:01, were identified as susceptible alleles, and *DRB1**11 and *DRB1**13 were identified as protective alleles (3). HLA-*DRB1**08:03, HLA-*DRB1**07:01, and *DPB1**17:01 were identified as the major susceptible alleles, and *DQB1*03:01* and the *DRB1*12:02-DQB1*03:01* haplotype were significantly decreased in a different cohort of Chinese patients (4, 5).

The gut microbiota has been proposed as a potential environmental component of PBC because it influences patients' treatment and prognosis. Li et al. suggested that microbiota alterations were closely related to beneficial responses to PBC, highlighting the possibility of exploring bile acid–microbiota interactions for treating patients (6). Lammert et al. reported that microbiota alterations might correspond to liver fibrosis progression (7). Previous studies have also suggested changes in microbiota compositions in autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, and celiac disease, which are at least partially due to the effects of susceptible HLAs (8, 9). We previously reported that a distinct gut microbiome profile was associated with PBC prognosis (10). However, whether the HLA alleles influence the gut microbiota distribution, and whether a link exists between disease development, severity, and gut microbiota alterations in patients with PBC remain unknown.

In the present study, using the PBC cohort we established during the past decade, we analyzed the HLA genotyping and gut microbiota in a proportion of these well-defined patients. The results showed associations between HLA alleles, microbiota compositions, and functional characteristics.

# Methods

## Patients and samples

A cohort of 964 Chinese patients with PBC was enrolled from 2010 to 2020 in Beijing YouAn Hospital, Beijing, China. The

average follow-up time was 40.1 ± 27.5 months; the longest was 107.6 months. The male:female ratio was 114:850, and the average age was 56.5 ± 10.9 years. Of the 964 patients, 151 underwent HLA genotyping. Forty-three of the 151 fully HLA-typed patients were enrolled, and their stool samples were analyzed. The stool samples were collected from April 2019 to December 2021 and stored at −80°C. The patients' gut microbiota were analyzed from the samples shortly after collection.

Inclusion criteria were that (1) all patients had a definite diagnosis of PBC based on the European Association for the Study of the Liver Clinical Practice Guidelines of PBC (2); (2) all were aged >18 years; and (3) all were of Chinese nationality, with most being from northern China. Exclusion criteria were (1) liver diseases caused by hepatitis viruses or drugs, alcoholic and non-alcoholic fatty liver disease; (2) hepatocellular carcinoma or liver metastases; (3) severe cardiac or renal insufficiency; (4) intestinal diseases or intestinal surgery; and (5) antibiotics or intestinal microecologics used within 2 weeks prior. Beijing YouAn Hospital (Ethics: No. [2012] 44 and No. LL-2018-044-K) approved this study. All enrolled patients signed their informed consent.

## Serum biochemical and immunologic parameters

Routine laboratory tests were performed for alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), albumin (ALB), total bile acid (TBA), creatinine (Cr), white blood cells (WBCs), hemoglobin (HB), and platelets (PLTs). AMA, antinuclear antibody (ANA), anti-gp210, and anti-sp100 were detected by indirect immunofluorescence and immunoblotting assays (EUROIMMUN, Lübeck, Germany), and immunoglobulin (Ig) M and IgG were included as serum parameters.

## DNA extraction and HLA genotyping

Genomic DNA was purified from peripheral whole blood using the hydrochloride method as described previously (4, 11). High-resolution genotyping of the HLA class I (A, B, C) and class II (DRB1 and DQB1) loci was performed using sequence-based PCR (Beijing Genomics Institute-Shenzhen [BGI-Shenzhen]; Shenzhen, China). Data from 500 healthy people were used as controls (4).

## Stool analysis of the 16s RNA gene

Microbial community DNA was extracted using a MagPure Stool DNA KF kit B (Magen, Guangzhou, China) following the manufacturer's instructions. DNA was quantified with a Qubit Fluorometer using a Qubit® dsDNA BR Assay kit (Invitrogen, California, USA), and the quality was checked by running aliquots on 1% agarose gel.

Variable regions of the V4 region of the bacterial 16S rRNA gene were amplified with degenerate PCR primers: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Both forward and reverse primers were tagged with Illumina adapter, a pad, and linker sequences. PCR enrichment was performed in a 50-μl reaction volume containing a 30-ng template, fusion PCR primer, and PCR master mix. PCR cycling conditions were 95°C for 3 min, 30 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were purified using Agencourt AMPure XP beads and eluted in an elution buffer. Libraries were qualified using the Agilent Technologies 2100 bioanalyzer. The validated libraries were used for sequencing on the Illumina platform (HiSeq 2500) following the manufacturer's standard protocols and generating 2 × 250-bp paired-end reads (BGI-Shenzhen; Shenzhen, China).

## Data bioinformatics analysis

High-quality clean data were filtered and retained for analysis. Reads were spliced into tags. Operational taxonomic units (OTUs) with a 97% similarity cutoff were used. Tags were clustered into OTUs *via* USEARCH (v7.0.1090). Species were annotated by comparing OTUs with the GreenGene database (v.201305). From the OTUs and annotation results, α- and β-diversities were calculated using the Wilcoxon and Wilcoxon rank-sum tests, respectively. The differential genera and predicted pathways were screened using the Wilcoxon test ($P < 0.05$) and |log (fold change [FC]) |>1 through PICRUSt2 v2.2.0-b, R (v3.4.10). Differential microbiotas were analyzed through LDA effect size (LEfSe) (https://huttenhower.sph.harvard.edu/galaxy/). At the class level, microbes whose relative abundances were <0.5% in all samples were merged into "Others". Spearman's correlation coefficient analysis of the species revealed important patterns and relationships among dominant species (BGI-Shenzhen, Shenzhen, China).

## Statistical analysis

Continuous variables are presented as the mean ± standard deviation. All statistical analyses were performed using the SPSS17.0 software, and $P<0.05$ was considered statistically significant.

SPASS 17.0 software was used for the HLA genotyping statistical analysis. Allele distributions were compared between patients and controls, using the chi-square or Fisher's exact tests.

A two-sided $P<0.05$ was considered statistically significant. For multiple testing, *P*-values were corrected (*Pc*) by the number of comparisons according to Bonferroni's inequality method. Association strengths were estimated by calculating the odds ratio (OR) and 95% confidence interval (CI).

In the gut microbiota analyses, continuous variables are expressed as means and standard deviations. Correlation coefficients ≥0.29 or ≤−0.29 were considered relevant. $P<0.05$ was considered statistically significant. All statistical analyses were performed using an SPSS software (v. 19). α-diversity and β-diversity analyses and partial least-squares-discriminant analysis (PLS-DA) were performed using R (v.3.2.1).

# Results

## Baseline characteristics of the 964 patients with PBC

Of the patients in our cohort, 60.6% (584/964) were enrolled at initial diagnosis, 39.4% (380/964) were enrolled while undergoing treatment, 73.5% (709/964) had elevated serum ALP, and 55.9% (514/920) had elevated serum IgM (baseline results of IgM were absent from 44 patients). The specific autoantibodies AMA (or AMA-M2), anti-gp210, and anti-sp100 were detected in 92.7%, 28.2%, and 12.7% of patients, respectively. Of the 964 patients, 342 (35.5%) had developed liver cirrhosis (LC) prior to enrollment. From this cohort, we previously reported the HLA alleles and their associations with ANA (4), gut microbiota alterations and elevated serum bilirubin (10), and the clinical characteristics of the patients with anti-gp210 (12). Here, we further explored the association between the HLA alleles and the microbiota in these patients (Figure 1).

## HLA allele distribution

Patients with PBC showed a significantly higher frequency of HLA *DRB1*08:03* than did the controls (21.2% vs. 9.0%, $P=0.0001/Pc=0.0063$, OR= 2.71, 95% CI:1.59–4.58). HLA-*DRB1*03:01*(13.1% vs. 7.8%, $P=0.049$), *DRB1*07:01* (29.1% vs. 20.2%, $P=0.0254$), *DRB1*14:05* (8.3% vs. 4.2%, $P=0.05$), *DRB1*14:54* (9.9% vs. 5.2%, $P=0.05$), and *DQB1*06:01* (25.8% vs. 17.8%, $P=0.0353$) frequencies were increased in patients with PBC, but did not reach significance after Bonferroni's correction. The HLA *DQB1*03:01* frequency was significantly lower in patients with PBC than in the controls (24.5% vs. 39.2%, $P=0.0010/Pc=0.0153$, OR=0.50, 95% CI:0.32–0.77). *DRB1*11:01* (5.3% vs. 10.6%, $P=0.05$) and *DRB1*12:02* (7.3% vs. 14.8%, $P=0.0185$) were less common in patients with PBC than in the controls.

## Gut microbiota analysis in patients with PBC with different HLA alleles

The intestinal flora of 43 patients were analyzed from four perspectives. The first was patients who were positive vs. negative for a combination of five HLA DRB1 alleles occurring at high-risk. Of these 43 patients, only seven carried susceptible *DRB1*08:03*, a combination group of these five high-risk HLA *DRB1* alleles: *DRB1*03:01* (5/43), *DRB1*07:01* (9/43), *DRB1*08:03* (7/43), *DRB1*14:05* (4/43), and *DRB1*14:54* (4/43) was set up (abbreviation: Group FHRAC). Of the 43 patients, 25 were positive for any of the five alleles, and 18 were negative for all five alleles. We further analyzed patients who were positive vs. negative for the "protective" allele *DQB1*03:01*, patients who were FHRAC-positive with and without LC, and patients who were FHRAC-positive with LC vs. patients who were FHRAC-negative without LC.

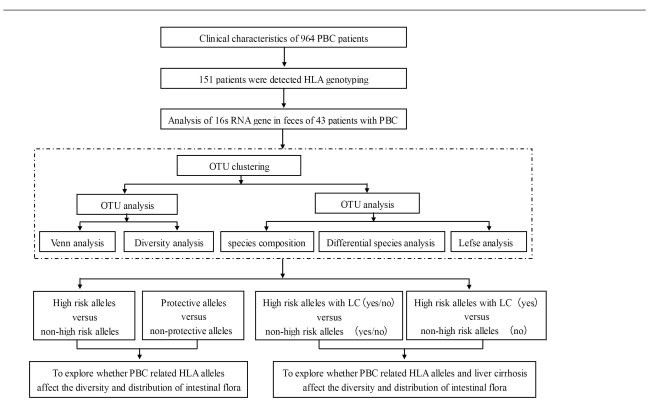


FIGURE 1
The study flowchart.

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## Gut microbiota in patients with vs. without high-risk HLA alleles

### (1) Baseline characteristics

FHRAC-positive (n=25) and FHRAC-negative (n=18) patients were classified according to their conditions. The mean sex, age, body mass index (BMI), ALT, AST, TBIL, ALP, GGT, ALB, TBA, Cr, WBCs, HB, PLT, anti-sp100, IgM, and IgG did not significantly differ between the two groups. However, anti-gp210 occurred more frequently in FHRAC-negative than in FHRAC-positive patients (Table 1).

### (2) Bacterial richness and diversity analyses

Five hundred and ten shared "universal" OTUs were found in both FHRAC-positive and FHRAC-negative patients. FHRAC-positive patients contained more unique OTUs than did FHRAC-negative patients (554 vs. 538; Figure 2A).

The α-diversity analysis showed no significant differences on the Simpson's, Shannon's, Sobs, abundance-based coverage estimator (ACE), Chao, or other observed species indexes between the two groups ($P$>0.05; Figure 2B). However, the microbial community abundance was significantly lower in FHRAC-positive patients than in FHRAC-negative patients ($P$<0.05; Figure 2C).

### (3) Comparison of bacterial compositions

Twenty-seven microorganismal genera were detected. In the FHRAC-positive patients, *Bacteroides*, *Escherichia*, Others, *Faecalibacterium*, *Phascolarctobacterium*, *Prevotella*, *Lachnospiraceae incertae*, *Megamonas*, *Veillonella*, and *Megasphaera* were the top genera, constituting 79% of all genera. *Escherichia*, Others, *Bacteroides*, *Megamonas*, *Prevotella*, *Faecalibacterium*, *Gemmiger*, *Ruminococcus*, *Megasphaera*, and *Citrobacter* were the top genera in the FHRAC-negative patients (78%; Figure 2D and Supplementary Table S1A). The Wilcoxon test was used to compare the significantly different top 10 genera. *Lachnospiraceae_incertae_sedis* was higher in FHRAC-positive patients than in FHRAC-negative patients (Figure 2E and Supplementary Table S1B).

LEfSe analysis showed that samples from the FHRAC-negative patients mainly contained Epsilonproteobacteria at the class level, Campylobacterales and Actinomycetales at the order level, and Campylobacteraceae at the family level (Figure 2F). *Lachnospiraceae_incertae_sedis*, *Anaerostipes*, *Actinomycetales*, *Desulfovibrio*, *Barnesiella*, *Lachnospira*, *Campylobacteraceae*, *Epsilonproteobacteria*, *Campylobacterales*, *Campylobacter*, and *Klebsiella* were differentially expressed among the taxonomic levels (Figure 2G). LEfSe analysis showed no distinguishing characteristics among the FHRAC-positive patients.

TABLE 1 The baseline characteristics of PBC in FHRAC-positive and FHRAC-negative groups.

| Variables | FHRAC-positive ( n = 25 ) | FHRAC-negative ( n = 18 ) | *P* -value |
|---|---|---|---|
| Gender (female/male) | 22 (91.67%)/3 (8.33%) | 17 (94.4%)/1 (5.6%) | 0.473 |
| Age (years) | 54.09 ± 10.35 | 54.63 ± 9.53 | 0.862 |
| γ-GT (U/L) | 263.68 ± 322.73 | 297.34 ± 243.70 | 0.712 |
| ALB (g/L) | 40.36 ± 7.64 | 39.19 ± 6.76 | 0.605 |
| ALT (U/L) | 70.14 ± 73.68 | 63.07 ± 49.94 | 0.726 |
| ALP (U/L) | 316.90 ± 258.68 | 277.81 ± 123.39 | 0.514 |
| AST (U/L) | 90.59 ± 86.14 | 79.11 ± 46.26 | 0.610 |
| GLO (g/L) | 37.74 ± 8.70 | 36.72 ± 7.36 | 0.689 |
| TBIL (μmol/L) | 30.62 ± 23.43 | 43.27 ± 41.00 | 0.207 |
| TBA (μmol/L) | 37.15 ± 40.51 | 58.79 ± 49.85 | 0.124 |
| Cr (μmol/L) | 47.46 ± 10.53 | 48.94 ± 12.08 | 0.678 |
| WBC (*10^9/L) | 4.66 ± 1.45 | 4.60 ± 2.38 | 0.915 |
| HB (g/L) | 121.76 ± 20.01 | 112.67 ± 14.66 | 0.110 |
| PLT (*$10^9$/L) | 185.56 ± 79.24 | 171.83 ± 97.85 | 0.614 |
| IgA (g/L) | 3.79 ± 1.87 | 3.69 ± 1.65 | 0.865 |
| IgG (g/L) | 20.43 ± 10.14 | 17.49 ± 4.54 | 0.287 |
| IgM (g/L) | 4.01 ± 3.79 | 4.39 ± 2.98 | 0.738 |
| PT (s) | 12.03 ± 1.71 | 11.34 ± 1.62 | 0.636 |
| INR | 1.09 ± 0.167 | 1.02 ± 0.146 | 0.703 |
| Anti-gp210 (neg/pos) | 21 (84%)/4 (16%) | 8 (44.44%)/10 (55.56%) | 0.006* |
| Anti-sp100 (neg/pos) | 20 (80%)/5 (20%) | 17 (94.44%)/1 (5.56%) | 0.177 |
| Cirrhosis (no/yes) | 20 (80%)/5 (20%) | 13 (72.22%)/5 (27.78%) | 0.406 |

PBC, primary biliary cholangitis; γ-GT, γ-glutamyl transpeptidase; ALB, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GLO, globulin; TBIL, total bilirubin; TBA, total bile acid; Cr, creatinine; WBC, white blood cell; HB, hemoglobin; PLT, platelet; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; PT, prothrombin time; INR, international standardized ratio; neg, negative; pos, positive; *P < 0.05.

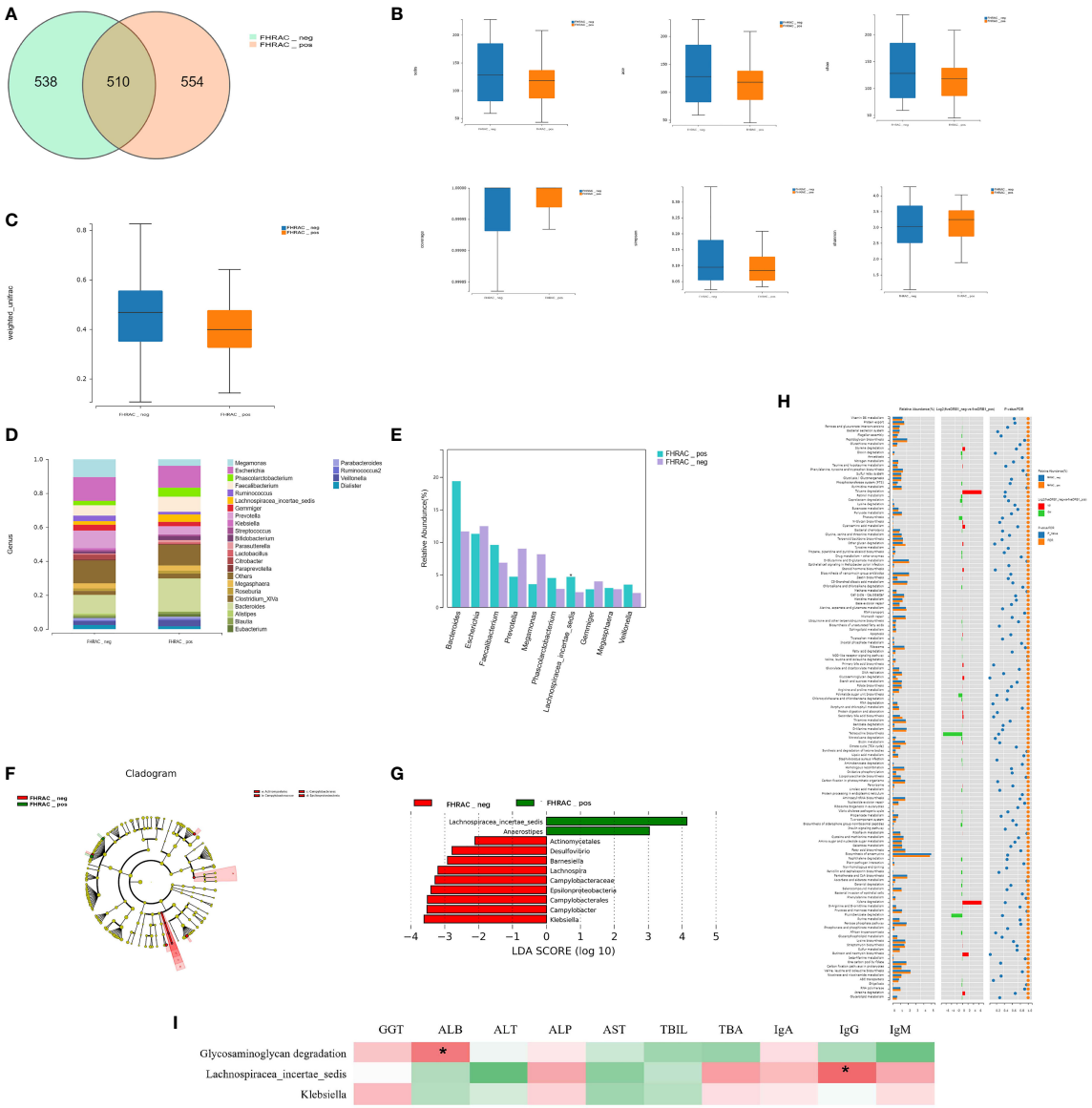


FIGURE 2
Bacterial richness and diversity analysis. **(A)** We found 510 shared universal OTUs in samples from both FHRAC-positive and FHRAC-negative patients. FHRAC-positive patients contained more unique OTUs than did FHRAC-negative patients (554 vs. 538 OTUs). **(B)** α-diversity analysis showed no significant differences in the Simpson's, Shannon's, Sobs, abundance-based coverage estimator (ACE), Chao, or other observed species indexes between the two groups ($P > 0.05$). **(C)** Microbial community diversity in the FHRAC-positive patients was significantly lower than that of the FHRAC-negative patients ($P < 0.05$). **(D)** Twenty-seven microbial genera were detected. In FHRAC-positive patients, *Bacteroides*, *Escherichia*, Others, *Faecalibacterium*, *Phascolarctobacterium*, *Prevotella*, *Lachnospiraceae incertae*, *Megamonas*, *Veillonella*, and *Megasphaera* were the top genera, constituting 79% of the taxa. *Escherichia*, Others, *Bacteroides*, *Megamonas*, *Prevotella*, *Faecalibacterium*, *Gemmiger*, *Ruminococcus*, *Megasphaera* and *Citrobacter* were the top genera in the FHRAC-negative patients (78%). **(E)** The Wilcoxon test was used to compare the significantly different top 10 genera. *Lachnospiraceae_incertae_sedis* was higher in FHRAC-positive patients than in FHRAC-negative patients. **(F)** linear discriminant analysis (LDA) effect-size (LEfSe) analysis showed that FHRAC-negative patient samples contained mainly Epsilonproteobacteria at the class level, Campylobacterales and Actinomycetales at the order level and Campylobacteraceae at the family level. **(G)** *Lachnospiraceae_incertae_sedis*, *Anaerostipes*, *Actinomycetales*, *Desulfovibrio*, *Barnesiella*, *Lachnospira*, *Campylobacteraceae*, *Epsilonproteobacteria*, *Campylobacterales*, *Campylobacter*, and *Klebsiella* were differentially expressed among the taxonomic levels. **(H)** We detected 157 metabolites or pathways at the KEGG level 3. The Wilcoxon test was used to compare the significantly different features. The differentially expressed microbiota were closely related to four differential metabolic pathways, including glycosaminoglycan degradation. **(I)** The Spearman's correlation coefficient showed an association between clinical manifestations and microbial taxa. Glycosaminoglycan degradation was positively correlated with albumin (ALB). *Lachnospiraceae_incertae_sedis* was positively correlated with IgG. *P<0.05.

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### (4) Association between featured bacterial taxa and pathways

PICRUSt was used to predict the functional pathways based on the 16S rRNA gene to obtain information on metabolites or pathways that may be involved in PBC. We detected 157 metabolites or pathways at the Kyoto Encyclopedia of Genes and Genomes (KEGG) level 3. The Wilcoxon test was used to compare the significantly different features. The differentially expressed microbes were closely related to four differential metabolic pathways, including glycosaminoglycan degradation (Figure 2H). The Spearman's correlation coefficient revealed an association between clinical manifestations and microbial taxa. Glycosaminoglycan degradation was positively correlated with ALB, and *Lachnospiraceae_incertae_sedis* was positively correlated with IgG (Figure 2I). In FHRAC-positive patients, *Lachnospiraceae_incertae_sedis* and *Anaerostipes* were significantly decreased, and *Campylobacter*, *Lachnospira*, *Desulfovibrio*, *Klebsiella*, and *Barnesiella* were significantly increased. *Lachnospiraceae_incertae_sedis* may affect liver function in patients with PBC by affecting glycosaminoglycan metabolism.

## Gut microbiota compositions in patients with vs. without the protective allele *DQB1*03:01*

### (1) Baseline characteristics

Of 42 patients, 13 were *DQB1*03:01*-positive, and 29 were *DQB1*03:01*-negative. The mean sex, age, BMI, ALT, AST, GGT, ALB, Cr, WBCs, HB, PLT, anti-sp100, anti-gp210, TBA, IgM, and IgG did not significantly differ between the two groups. However, TBIL and ALP levels were higher in the *DQB1*03:01*-negative than in the *DQB1*03:01*-positive patients (Table 2).

### (2) Richness and diversity analyses

We detected 446 shared universal OTUs in both *DQB1*03:01*-positive and *DQB1*03:01*-negative patients. *DQB1*03:01*-negative patients contained more exclusive OTUs than did *DQB1*03:01*-positive patients (843 vs. 303; Figure 3A).

The α-diversity analysis revealed some differences that were not significant by the Simpson's, Shannon's, Sobs, ACE, Chao, or other observed species indexes between the two groups ($P$>0.05; Figure 3B). Likewise, the microbial communities did not statistically differ between these two groups ($P$>0.05; Figure 3C).

TABLE 2 The baseline characteristics of PBC in the *DQB1* 03:01*-positive and *DQB1* 03:01*-negative groups.

| Variables | *DQB1* 03:01*-positive ( n = 13 ) | *DQB1* 03:01*-negative ( n = 29 ) | *P* -value |
|---|---|---|---|
| Gender (female/male) | 12 (92.31%)/1 (7.69%) | 26 (89.66%)/3 (10.34%) | 0.787 |
| Age (years) | 55.73 ± 7.19 | 54.11 ± 10.88 | 0.626 |
| γ-GT (U/L) | 203.49 ± 186.75 | 295.96 ± 317.88 | 0.337 |
| ALB (g/L) | 39.26 ± 6.26 | 40.03 ± 7.81 | 0.755 |
| ALT (U/L) | 60.05 ± 52.33 | 70.69 ± 70.52 | 0.630 |
| ALP (U/L) | 228.73 ± 108.53 | 320.47 ± 234.10 | 0.090 |
| AST (U/L) | 68.95 ± 40.86 | 92.88 ± 82.48 | 0.329 |
| GLO (g/L) | 41.26 ± 7.79 | 35.84 ± 7.75 | 0.043 |
| TBIL (μmol/L) | 21.47 ± 9.40 | 42.88 ± 36.86 | 0.006* |
| TBA (μmol/L) | 33.12 ± 25.64 | 53.09 ± 51.47 | 0.102 |
| Cr (μmol/L) | 51.85 ± 14.77 | 46.71 ± 9.10 | 0.185 |
| WBC (*$10^9$/L) | 4.85 ± 2.24 | 4.52 ± 1.75 | 0.612 |
| HB (g/L) | 116.15 ± 11.54 | 119.10 ± 21.00 | 0.562 |
| PLT (*$10^9$/L) | 178.46 ± 95.40 | 179.93 ± 85.774 | 0.961 |
| IgA (g/L) | 3.82 ± 1.52 | 3.81 ± 1.859 | 0.982 |
| IgG (g/L) | 23.22 ± 13.00 | 17.81 ± 5.18 | 0.208 |
| IgM (g/L) | 4.86 ± 3.35 | 3.99 ± 3.53 | 0.488 |
| INR | 1.03 ± 0.14 | 1.07 ± 0.17 | 0.522 |
| PT (s) | 11.56 ± 1.55 | 11.85 ± 1.81 | 0.638 |
| Anti-gp210 (neg/pos) | 5 (71.4%)/2 (28.6%) | 24 (66.7%)/12 (33.4%) | 0.345 |
| Anti-sp100 (neg/pos) | 6 (85.7%)/1 (14.2%) | 31 (86.1%)/5 (13.9%) | 0.414 |

PBC, primary biliary cholangitis; γ-GT, γ-glutamyl transpeptidase; ALB, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GLO, globulin; TBIL, total bilirubin; TBA, total bile acid; Cr, creatinine; WBC, white blood cell; HB, hemoglobin; PLT, platelet; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; INR, international standardized ratio; PT, prothrombin time; neg, negative; pos, positive; *, $P < 0.05$.

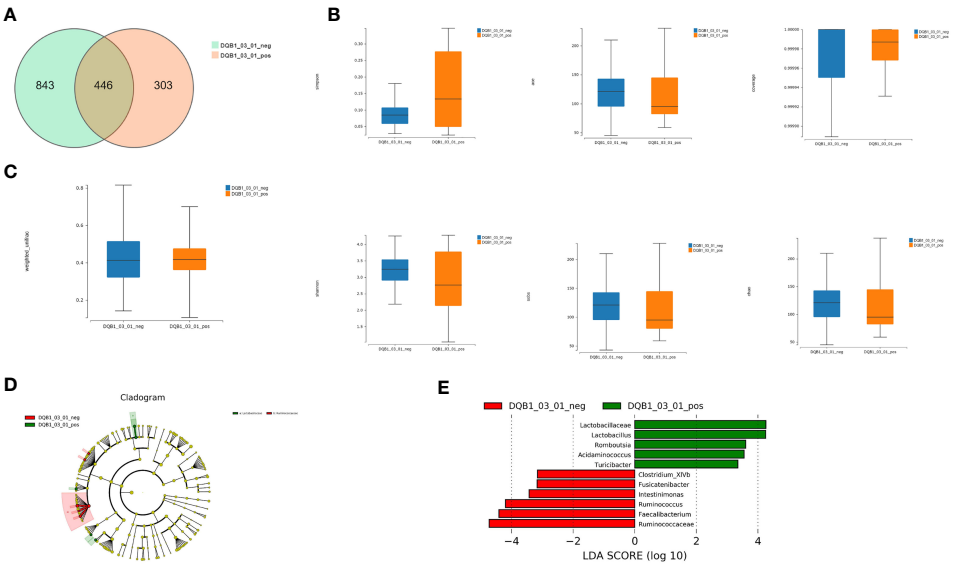


FIGURE 3
Richness and diversity analysis. **(A)** We detected 446 shared universal OTUs in samples from both *DQB1*03:01*-positive and *DQB1*03:01*-negative patients. *DQB1*03:01*-negative patients contained more unique OTUs than did *DQB1*03:01*-positive patients (843 vs. 303 OTUs). **(B)** α-diversity analysis revealed non-significant differences in the Simpson's, Shannon's, Sobs, ACE, Chao, and other observed species indexes between the two patient groups ($P>0.05$). **(C)** Microbial communities did not statistically differ between these two groups ($P>0.05$). **(D)** LEfSe analysis showed that the *DQB1*03:01*-negative patient samples contained mainly Ruminococcaceae at the family level, and the *DQB1*03:01*-positive samples contained mainly Lactobacillaceae. **(E)** Lactobacillaceae, *Lactobacillus*, *Romboutsia*, *Acidaminococcus*, *Turicibacter*, *Clostridium_XlVb*, *Fusicatenibacter*, *Intestinimonas*, *Ruminococcus*, *Faecalibacterium*, and Ruminococcaceae were differentially expressed among the taxonomic levels.

### (3) Comparison of bacterial compositions

LEfSe analysis showed that samples from the *DQB1*03:01*-negative patients mainly contained Ruminococcaceae at the family level, and the *DQB1*03:01*-positive samples mainly contained Lactobacillaceae (Figure 3D). Lactobacillaceae, *Lactobacillus*, *Romboutsia*, *Acidaminococcus*, *Turicibacter*, *Clostridium_XlVb*, *Fusicatenibacter*, *Intestinimonas*, *Ruminococcus*, *Faecalibacterium*, and Ruminococcaceae were differentially expressed among the taxonomic levels (Figure 3E).

Although bacterial richness did not significantly differ between the *DQB1*03:01*-positive and *DQB1*03:01*-negative patients, *Lactobacillus*, *Romboutsia*, *Turicibacter*, and *Acidaminococcus* were increased, and *Clostridium_XlVb*, *Ruminococcus*, *Faecalibacterium*, and *Fusicatenibacter* were significantly decreased in patients with the protective *DQB1*03:01* allele.

## Gut microbiota compositions in patients carrying high-risk alleles with or without LC

The 43 patients were divided into four subgroups: FHRAC-positive with LC (n=5), FHRAC-negative with LC (n=5), FHRAC-positive without LC (n=20), and FHRAC-negative without LC (n=13).

### (1) Bacterial richness and diversity analyses

We detected 135 shared universal OTUs in the four subgroups (Figure 4A). The α-diversity analysis showed no significant differences in the Simpson's, Shannon's, Sobs, ACE, Chao, and other observed species indexes among the four groups ($P>0.05$; Figure 4B). However, the microbial communities differed significantly among the four groups ($P<0.05$; Figure 4C and Supplementary Table S3A).

### (2) Comparison of bacterial compositions

(2.1) Twenty-seven bacterial genera were detected in total (Figure 4D). The relative abundances of *Veillonella* differed significantly among the groups at 13.05% for FHRAC-positive patients with LC, 7.18% for FHRAC-negative patients with LC, 0.28% for FHRAC-positive patients without LC, and 1.08% for FHRAC-negative patients without LC ($P<0.05$; Figure 4E and Supplementary Table S3B).

(2.2) Thirty-two bacterial species were detected in total (Figure 4F). The relative abundance of *Veillonella_atypica* differed significantly among the groups at 9.24% for FHRAC-positive patients with LC, 1.40% for FHRAC-negative patients

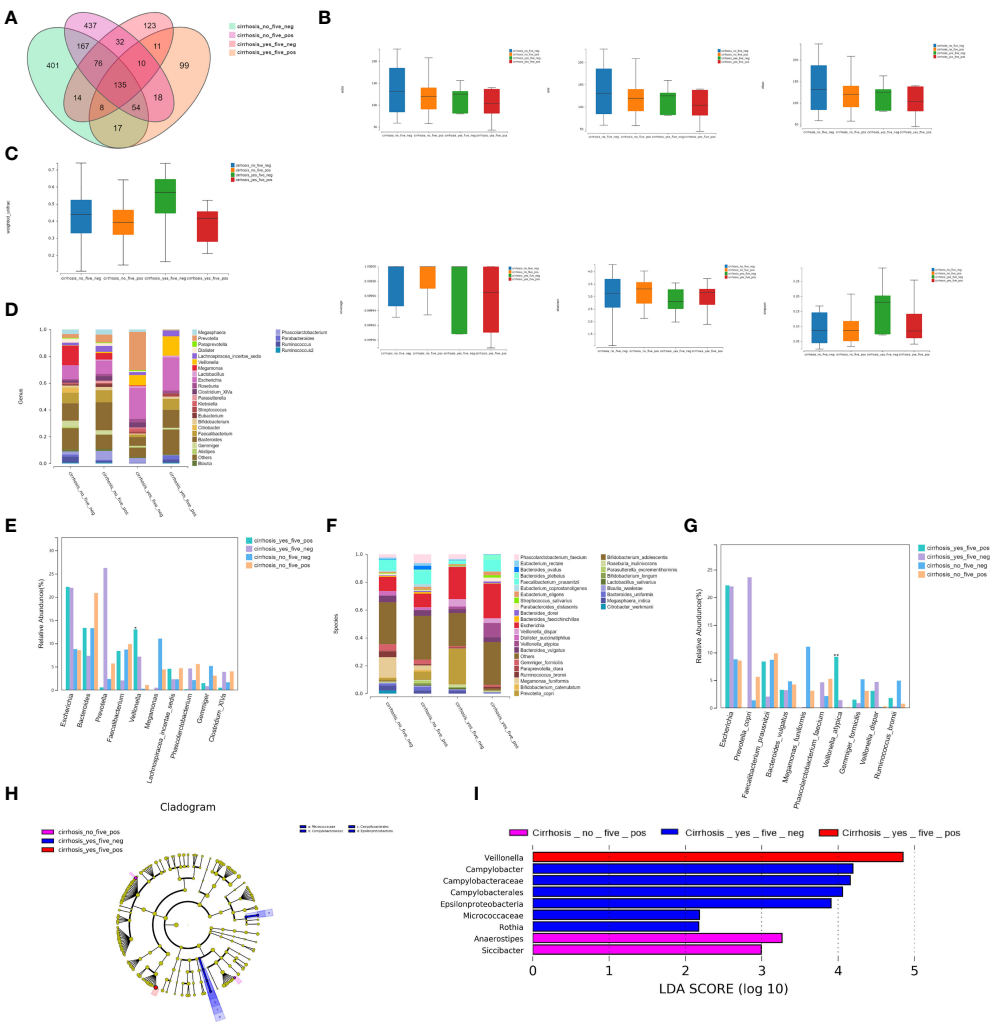


FIGURE 4
Bacterial richness and diversity analysis. **(A)** We found 135 shared universal OTUs among the four patient subgroups. **(B)** α-diversity analysis showed no significant differences in the Simpson's, Shannon's, Sobs, ACE, Chao, or other observed species indexes between the four groups ($P>0.05$). **(C)** The microbial communities differed statistically among the four groups ($P<0.05$). **(D)** Twenty-seven bacterial genera were detected. **(E)** The relative abundances of *Veillonella* were 13.05% for FHRAC-positive patients with LC, 7.18% for FHRAC-negative patients with LC, 0.28% for FHRAC-positive patients without LC, and 1.08% for FHRAC-negative patients without LC (all $P<0.05$). **(F)** Thirty-two bacterial species were detected. **(G)** The relative abundances of *Veillonella atypica* were 9.24%for FHRAC-positive patients with LC, 1.40% for FHRAC-negative patients with LC, 0.05% for FHRAC-positive patients without LC, and 0.10% for FHRAC-negative patients without LC (all $P<0.05$). **(H)** LEfSe analysis showed that samples from the FHRAC-negative patients with LC contained mainly Campylobacteraceae at the class level, Campylobacterales at the order level, and Micrococcaceae and Epsilonproteobacteria at the family level. **(I)** *Anaerostipes*, *Campylobacter*, *Campylobacteraceae*, *Epsilonproteobacteria*, *Veillonella*, *Rothia*, *Siccibacter*, *Micrococcaceae*, and *Campylobacterales* were differentially expressed among the taxonomic levels.

with LC, 0.05% for FHRAC-positive patients without LC, and 0.10% for FHRAC-negative patients without LC ($P<0.05$; Figure 4G and Supplementary Table S3C).

LEfSe analysis showed that samples from FHRAC-negative patients with LC mainly contained Campylobacteraceae at the class level, Campylobacterales at the order level, and Micrococcaceae and Epsilonproteobacteria at the family level (Figure 4H). *Anaerostipes*, *Campylobacter*, Campylobacteraceae, *Epsilonproteobacteria*, *Veillonella*, *Rothia*, *Siccibacter*, Micrococcaceae, and Campylobacterales were differentially expressed among the taxonomic levels (Figure 4I).

## Gut microbiota compositions in patients carrying high-risk alleles with LC vs. without high-risk alleles and LC

### (1) Bacterial richness and diversity analyses

We detected 214 shared universal OTUs among the FHRAC-positive patients with LC (n=5) and FHRAC-negative patients without LC (n=13). The samples from FHRAC-positive patients with LC contained fewer unique OTUs than did those from FHRAC-negative patients without LC (138 vs. 658; Figure 5A).

The α-diversity analysis showed no significant differences on the Simpson's, Shannon's, Sobs, ACE, Chao, or other coverage indexes between the two groups ($P$>0.05; Figure 5B). The microbial communities did not differ significantly between the two groups ($P$>0.05; Figure 5C). However, FHRAC-positive patients with LC exhibited unique diversity and bacterial

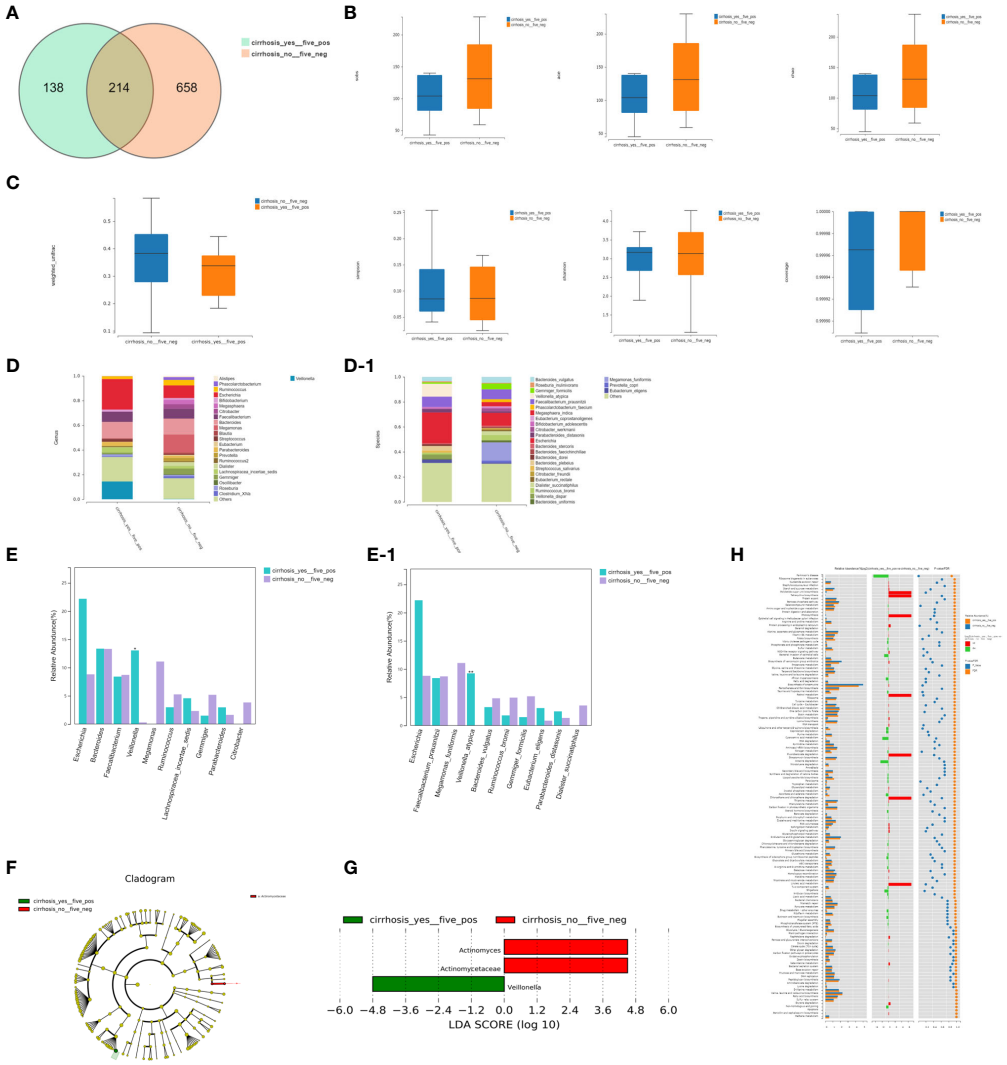


FIGURE 5
Bacterial richness, diversity, and function. **(A)** We detected 214 shared universal OTUs in FHRAC-positive patients with LC (n=5) and FHRAC-negative patients without LC (n = 13). Samples from FHRAC-positive patients with LC contained fewer unique OTUs than did FHRAC-negative patients without LC (138 vs. 658). **(B)** α-diversity analysis showed no significant differences in the Simpson's, Shannon's, Sobs, ACE, Chao, or other coverage indexes between the two groups ($P$ > 0.05). **(C)** The microbial communities did not statistically differ between the two groups ($P$>0.05). **(D, D-1)** Twenty-four bacterial genera and 27 bacterial species were detected **(E, E-1)**. The abundances of *Veillonella* at the genus level and *Veillonella atypica* at the species level were greater in the FHRAC-positive patients with LC than in the FHRAC-negative patients without LC. **(F)** LEfSe analysis showed that the samples from the FHRAC-negative patients without LC mainly contained Antinomycetaceae at the family level. **(G)** Three flora were differentially expressed among the taxonomic levels. **(H)** We detected 151 metabolites or pathways at the KEGG level 3. The Wilcoxon test was used to compare the significantly different features. The differentially expressed microbiota were closely related to four differential metabolic pathways: plant hormone signal transduction, Parkinson's disease, spliceosomes, and protein processing in the endoplasmic reticulum.

compositions compared with those of FHRAC-negative patients without LC.

### (2) Comparison of bacterial compositions

Twenty-four bacterial genera and 27 bacterial species were detected in total (Figures 5D, 5D-1), with some significantly different flora at both levels. The abundances of *Veillonella* at the genus level and *Veillonella atypica* at the species level were higher in FHRAC-positive patients with LC than in FHRAC-negative patients without LC (Figures 5E, 5E-1).

LEfSe analysis showed that samples from the FHRAC-negative patients without LC mainly contained Antinomycetaceae at the family level (Figure 5F). Three flora were differentially expressed among the taxonomic levels (Figure 5G).

### (3) Association between featured bacterial taxa and pathways

We used PICRUSt to predict the functional pathways based on the 16S rRNA gene to obtain information on the metabolites or pathways potentially involved with PBC, and 151 metabolites or pathways were detected at the KEGG level 3. We used the Wilcoxon test to compare the significantly different features. The differentially expressed microbiota were closely related to four differential metabolic pathways: plant hormone signal transduction, Parkinson's disease, spliceosomes, and protein processing in the endoplasmic reticulum (Figure 5H).

## Discussion

We analyzed the associations between the HLA class II genes (including susceptible, high-risk, and protective alleles) and the gut microbiome compositions and distributions in patients with PBC. Some characteristics of the HLA class II genes and gut microbiome distributions in patients with PBC and LC were observed. First, we confirmed HLA-*DRB1* high-risk alleles in these patients. The results suggested that the gut species richness and microbiome compositions were lower in patients with high-risk alleles than in those without high-risk alleles. Next, using LEfSe analysis of taxonomic classifications, significant associations were noted between patients with high-risk alleles and those with protective alleles. Furthermore, in patients with PBC and LC with high-risk alleles, some flora were increased significantly compared with those of patients without high-risk alleles and LC. Finally, we observed different flora distributions with biochemical markers and pathways.

The pathogenesis and progression mechanisms of PBC are complex and poorly understood. However, environmental influences likely play significant roles in driving PBC development and progression, interacting with immunogenetic and epigenetic risks (12–14). Previous reports described the correlation between susceptible HLA genes and PBC and between intestinal flora and PBC, and similar studies have been conducted in other diseases with LC (15–17). We focused on the microbiota composition and functional features in patients with PBC with susceptible or protective HLA alleles. To avoid having too few samples of a single allele affecting the statistical results, we set up an FHRAC group, which combined five high-risk HLA-DRB1 alleles (*DRB1*03:01*, *DRB1*07:01*, *DRB1*08:03*, *DRB1*14:05*, and *DRB1*14:54*), then analyzed the associations between these high-risk HLA alleles and patients' gut microbiota. In the FHRAC group, species richness and microbiome compositions were decreased in patients with high-risk HLA alleles compared with those in patients without high-risk HLA alleles. Thus, the HLA class II genes may influence the gut microbiome compositions of patients with PBC.

Microbiota compositions are reported to be significantly correlated with poor prognoses for patients with PBC (10). Some intestinal microflora have been associated with bile acid metabolism in PBC, and some microbes produced by short-chain fatty acids were significantly decreased in patients with PBC with inferior remission (6). Chen et al. reported decreased taurine-conjugated TBA in patients with PBC treated with ursodeoxycholic acid (UDCA) (18), raising questions regarding the distribution characteristics of the microbiome compositions of patients with PBC influenced by the HLA II gene. We further analyzed the different microbiota between the HLA groups and found that the gut species richness and microbiome compositions were lower in patients with high-risk alleles than in those without high-risk alleles.

LEfSe analysis of the taxonomic classification levels showed significant associations between patients with high-risk HLA genes. *Lachnospiraceae_incertae_sedis* and *Anaerostipes* were significantly decreased, whereas *Campylobacter*, *Lachnospira*, *Desulfovibrio*, *Klebsiella* and *Barnesiella* were significantly increased in FHRAC-positive patients. Additionally, *Lactobacillus*, *Romboutsia*, *Turicibacter*, and *Acidaminococcus* were increased, whereas *Clostridium_XlVb*, *Ruminococcus*, *Faecalibacterium*, and *Fusicatenibacter* were decreased in patients carrying *DQB1*03:01*.

Does genetic susceptibility affect disease progression? Genetic predisposition to autoimmune hepatitis (AIH) has been associated with HLA alleles. Ma et al. (19) studied 236 children of European ancestry. Possession of homozygous *DRB1*03* or of *DRB1*13* was associated with fibrosis at disease onset, and possession of both genes along with *DRB1*07* was associated with a more severe disease in three subgroups of patients with AIH, including type 1 and type 2 AIH and autoimmune cholangitis. Therefore, we analyzed the interactions between LC, HLA, and the intestinal flora in patients with PBC. First, patients were divided into four groups, then two subgroups according to the presence of LC and HLA alleles. Interestingly, their microbiome compositions differed significantly. *Veillonella* and *Veillonella atypica* abundances differed among the four groups, with the highest in patients with LC and

FHRAC, and the lowest in patients without LC and FHRAC. Antinomycetaceae abundances also differed. Because of limitations in our four-group analysis, we further analyzed these two subgroups. *Veillonella* were significantly higher in patients with LC and FHRAC than in those without LC or FHRAC. A literature reviewed revealed that the levels of secondary bile acids such as deoxycholic acid (DCA) and conjugated DCA were inversely correlated with PBC-enriched gut microbes (*Veillonella*) (18). *Veillonella*, which is associated with chronic inflammatory and fibrotic conditions, was enriched in primary sclerosing cholangitis (20). *Veillonella* is also reported to be associated with urinary tract infections (UTIs) (21). UTI appears to be the only bacterial infection identified as a risk factor for PBC. *Escherichia coli* is a predominant pathogen in most UTI cases, and its infection is a key factor in breaking immunological tolerance through molecular mimicry mechanisms (22). Our results showed for the first time that *Veillonella* was increased in patients with PBC and LC carrying susceptible HLA genes. However, this finding requires further investigation in larger samples of patients with PBC to identify its pathway.

A significant proportion of the microbiota can be linked to maintaining intestinal and cellular homeostasis. The microbiota helps modulate histone deacetylases, thus affecting immune cell immigration, chemotaxis, programmed cell death, cytokine production, and cell attachment. Therefore, the relative abundances of differentially expressed microbes in diseases should be considered in disease treatment and prevention (23, 24).

Previous studies showed that some of the above intestinal flora are related to liver metabolism and function. *Anaerostipes* is closely related to bile acid metabolism, and its abundance is inversely correlated with bile acid levels in UDCA-treated patients with PBC (25). Ruminococcaceae is closely related to bile acid metabolism, functions in 7α-dehydroxylation, and is important for bile-acid synthesis (26). Leclercq et al. reported that Lachnospiraceae and *Clostridiales cluster XIV* were present and specifically increased in the stools of patients with mild hepatic fibrosis (27). Accumulation of partially undegraded glycosaminoglycans causes severe and chronic disturbances in liver function (28).

We also correlated different flora with biochemical indicators and pathways. Differentially expressed microbiota in patients with high-risk HLA DRB1 alleles may influence the pathway related to glycosaminoglycan metabolism. *Lachnospiraceae_incertae_sedis* was positively correlated with serum IgG levels. Disrupted microbial balances in both short- and long-term conditions can severely reduce microbial community richness and diversity (17). Genetic variations of major histocompatibility complex (MHC) genes among individuals mediate disease susceptibility by controlling microbiota diversity. An individual MHC genotype against the microbiota may result in compositional differences (29). HLA likely influences specific bacterial abundances because differences in community member overlap vary significantly by genetic risk (9). Our results indicated that the HLA genes were related to PBC pathogenesis and disease progression, and pathways corresponding to different intestinal flora may mediate this process.

Our results indicate that the HLA class II genes may influence the gut microbiome composition in patients with PBC. However, bacterial associations with HLA and clinical features require confirmation in a larger sample of patients. Personalized medicine is an emerging practice in modern medicine, which is committed to surveying, monitoring, and diagnosing risks to provide patients with a specific treatment, taking into account their particular genetic profile, molecular phenotype, and intestinal flora. Our study will be helpful for the personalized-medicine approach for patients with PBC.

## Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA875394.

## Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Ethics Review Board of Beijing YouAn Hospital, Capital Medical University (Ethics: No. [2012] 44 and No. LL-2018-044-K). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

Conception and design: H-PY, Y-ML, C-YH, and H-PZ. Patient's follow up and clinical data collection: Y-ML, C-YH, W-JH, H-YL, BX, YH, X-DZ, JZ, and W-JL. Sample collection, detection, and analysis: H-PZ, D-TZ, Y-XM, L-JL, X-HL, QW, and J-LL. Data analysis and manuscript writing: C-YH, W-JH, H-PZ, Y-ML, and H-PY. Final approval of the manuscript: All authors.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.984697/full#supplementary-material

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# Type 2 autoimmune hepatitis: Genetic susceptibility

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Two types of autoimmune hepatitis (AIH) are recognized; AIH-1 is characterized by the presence of anti-nuclear and/or anti-smooth muscle autoantibodies, while AIH-2 is associated with the presence of anti-Liver kidney microsome and/or anti-Liver Cytosol antibodies. The autoantigens targeted by AIH-2 autoantibodies are the cytochrome P450 2D6 and Formiminotransferase-cyclodeaminase for anti-LKM1 and anti-LC1 respectively. Both autoantigens are expressed in hepatocytes at higher levels than in any other cell type. Therefore, compared to AIH-1, the autoantigens targeted in AIH-2 are predominantly tissue-specific. Distinct clinical features are specific to AIH-2 compared to AIH-1, including diagnosis in younger patients (mean age 6.6 years), onset as fulminant hepatitis in very young patients (3 years of age or less), higher frequency in children than in adults and is frequently associated with extrahepatic T cell-mediated autoimmune diseases. AIH-2 is also often diagnosed in patients with primary immunodeficiency. AIH-2 is associated with specific HLA class II susceptibility alleles; DQB1*0201 is considered the main determinant of susceptibility while DRB1*07/DRB1*03 is associated with the type of autoantibody present. HLA DQB1*0201 is in strong linkage disequilibrium with both HLA DRB1*03 and DRB1*07. Interestingly, as in humans, MHC and non-MHC genes strongly influence the development of the disease in an animal model of AIH-2. Altogether, these findings suggest that AIH-2 incidence is likely dependent on specific genetic susceptibility factors combined with distinct environmental triggers.

KEYWORDS

liver, autoimmunity, genetic, HLA, MHC

## Introduction

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease with a fluctuating course, that progresses to cirrhosis and liver failure if not adequately treated. AIH shows a non-Mendelian inheritance; therefore, a single genetic locus cannot be associated with the development of the disease. However, it is believed that one or several genes, acting alone or in concert, can influence the risk of developing AIH.

Pediatric AIH has an annual incidence of 0.23 per 100000 children, and in North America, AIH-1 is 5 to 6 times more frequently diagnosed than AIH-2 (1). AIH etiology is unknown, however, it has been proposed that environmental triggers, like drugs or viruses, could be responsible for immune activation, possibly through molecular mimicry or by modification of potential autoantigens. Epitopes shared between viruses and autoantigens could be processed and presented to immune system cells in the context of specific HLAs, mainly class II molecules. Interestingly, some viruses, such as the Hepatitis A virus, can induce protracted hepatitis in patients expressing HLA class II DRB1*1301, responsible for susceptibility to the development of AIH in Argentine and Venezuelan populations (2, 3). In patients expressing predisposing HLA class II, an acute infection could trigger an immune response against liver antigens. This has led to the hypothesis that a specific susceptibility HLA class II could initiate an autoimmune response while susceptibility genes outside the HLA locus could assure the perpetuation of it, thus allowing the development of an autoimmune disease (4).

Importantly, HLA class II alleles of protection have also been identified in AIH and other autoimmune diseases. Those HLA class II would present specific epitopes preferentially stimulating regulatory T cells i.e., MHC molecules present self-epitopes that specifically activate Tregs (5).

## Autoimmune hepatitis

Autoimmune hepatitis is a disease of unknown pathogenesis where the infiltration of the liver by self-reactive lymphocytes leads to the progressive destruction of the hepatic parenchyma (6). In absence of treatment, AIH progresses to cirrhosis with a median survival time of 3.3 years (7). Clinical observations and the study of AIH etiology have led researchers to hypothesize that this disease, like the majority of autoimmune diseases, is multifactorial.

AIH is classified into two types according to the type of autoantibodies present (8). AIH-1 is defined by the presence of *anti-smooth muscle antibodies* (SMA) and/or *anti-nuclear antibodies* (ANA). AIH-2 is characterized by the presence of autoantibodies directed against cytochrome P450 2D6 (LKM1 for *anti-liver-kidney microsome type 1 antibody*) and/or against formiminotransferase-cyclodeaminase (FTCD) (LC1 for *liver-cytosol type 1 antibody*).

Type 1 and type 2 AIH show similar features such as the form of presentation, clinical signs of liver disease, presence of other autoimmune diseases in patients or first-degree relatives, an increase of immunoglobulin G in serum, female predominance, histological findings, and a favorable response to immunosuppressive drugs. Nevertheless, in a careful analysis of both types, some significant differences have been uncovered.

AIH-2 is more frequent in pediatric than in adult patients, a peak of incidence is found well before puberty, in contrast, patients with AIH-1 are usually older. In children, the mean age for onset of AIH-2 is 6.6 years and for AIH-1 10.6 years (6). The median age at presentation for AIH-1 is 12 years with an interquartile range of 11 to 14 years compared to 10 years for AIH-2 with an interquartile range of 4.5 to 13 years (1). Cases of AIH-2 presenting as an acute liver failure have a mean age of around 2 to 3 years, but of 13 to 14 years for patients with AIH-1. Female incidence is 9:1 for AIH-2 and 3:1 for AIH-1, thus AIH-2 is largely prevalent in girls (4). Extra-hepatic autoimmune diseases in AIH-2 patients are almost exclusively associated with T cell-mediated diseases; this is not the case for AIH-1 (6) (Table 1). In addition, AIH-2 is more frequently diagnosed than AIH-1 in patients with inherited immune deficiencies (9).

## Genes of the major histocompatibility complex and autoimmune hepatitis

The strongest genetic association with AIH was found with genes of the major histocompatibility complex (MHC). This 3.6Mb region of chromosome 6 contains nearly 260 genes involved in the immune response such as MHC class I and II genes, genes responsible for antigenic presentation, complement genes, and several cytokines.

Several susceptibility alleles for AIH have been identified (Table 2). In North America and Europe, MHC alleles HLA-A1-B8, HLA-DRB1*0301 (DR3), and HLA-DRB1*0401 (DR4) have been found in association with AIH (10, 11). In a linkage disequilibrium study conducted by our team in families of pediatric patients with AIH-1 and 2, we found that compared to their unaffected siblings, HLA-DRB1*0301 (DR3) and DRB1*1301 (DR13) were preferentially transmitted to patients with AIH-1, while HLA-DQB1*0201 was preferentially transmitted to children with AIH-2, compared to the randomly expected frequency or unaffected siblings (12). In a separate study, HLA-DR13 was suggested to be a risk factor in absence of HLA-DR3 or HLA-DR4 alleles (13). However, the size of the study population precluded the authors from reaching statistically significant conclusions (13).

Other HLA alleles have been described in association with AIH in other populations. HLA-DRB1*0404 is predominant in Mexican adult patients with AIH (14) while HLA-DRB1*0405 has been associated with Argentine and Japanese AIH patients (15, 16). In Brazil, HLA-DRB1*1301 and DRB3*01 have been found in association with AIH (17). In AIH-2, an association with HLA-DRB1*07 was found in German, Brazilian and British patients, while the HLA-DRB1*03 allele was identified as a risk factor in Spanish patient (18–20). Recently, Ma *et al.* described a unique genetic profile in juvenile patients of European descent for AIH-1 (DRB1*03), AIH-2 (DRB1*07), and autoimmune

TABLE 1 Extrahepatic autoimmune diseases and Primary Immune deficiencies most frequently found in children with type 1 and 2 autoimmune hepatitis.

| Type 1 AIH | Type 2 AIH |
|---|---|
| **T cell-dependent extrahepatic autoimmune diseases** | |
| Ulcerative colitis | Autoimmune enteropathy |
| Crohn's disease | Thyroiditis[a] |
| Vasculitis | Vitiligo |
| Arthritis | Alopecia |
| Thrombocytopenia | Diabetes[a] |
| Fibrosing alveolitis | |
| Hemolytic anemia | |
| Glomerulonephritis | |
| **Primary Immune Deficiencies** | |
| APECED | |
| Autoimmune lymphoproliferative syndrome | |
| Immunodysregulation polyendocrinopathy enteropathy x-linked (IPEX) syndrome | |
| STAT1 deficiencies | |
| Chronic mucocutaneous candidiasis | |

AIH, autoimmune hepatitis; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome.
[a]These autoimmune diseases are also found in patients with type 1 AIH.

sclerosing cholangitis (DRB1*13) in addition to HLA-B*08, HLA-DRB1*03, and A1-B8-DR3 haplotype that predisposed to all three forms of juvenile autoimmune liver disease (21). Interestingly, homozygosity for DRB1*03 or DRB1*13 was associated with fibrosis at disease presentation while possession of DRB1*03, DRB1*13, and DRB1*07 alleles was associated with a more severe disease for all three forms of juvenile autoimmune liver disease (21). This diversity of susceptibility alleles found in these different ethnic groups for AIH could be explained by the "common motifs" hypothesis that proposes that several alleles of Class II HLA could code for similar motifs (22).

These associations of class II HLA alleles and AIH susceptibility may be directly related to disease pathogenesis. For example, it was found that in 94% of patients with AIH-1, susceptibility alleles encoded for the LLEQKR or LLEQRR motifs at positions 67 to 72 of the HLA class II molecule (11, 23). Interestingly, HLA-DB1*1501, which is associated with a lower risk of developing AIH-1, encodes for ILEQAR at these same positions (11, 23). The substitution at position 71 of lysine with arginine or alanine which alters both the polarity and charge of the amino acid, could cause a change in the orientation and/or binding of peptides within the MHC class II molecule. These changes could influence autoantigens' presentation to T cells and thus modify the development of the disease.

Studying the relationship between HLA alleles and humoral response in AIH-2, our group described the strong influence that class II alleles could have on AIH patients' autoimmune B cell responses (24). HLA-DRB1*03 was strongly associated with AIH-2 patients with circulating anti-LKM1 and anti-LC1 autoantibodies, while the HLA-DRB1*07 allele was predominant in patients with AIH-2 for which anti-LKM1 were the only serological markers present (24). In addition, patients with the HLA-DRB1*07 allele have anti-LKM1 autoantibodies against a smaller repertoire of autoepitopes compared to patients with the HLA-DRB1*03 allele (21).

HLA class II DQ2 in AIH-2 is in strong linkage disequilibrium with HLA DRB1*03 and DRB1*07 and associated with circulating LKM1 autoantibodies (18). An extremely strong association was also found between LKM1 positivity and HLA class II DR7 in patients chronically infected with HCV (18). In individuals with AIH-2 and HCV-, 7 and 9 out of 15 expressed DR7 or DR3 respectively. Additionally, among the 33 HCV+ patients with LKM1 autoantibodies studied, 28 expressed DR7 in a heterozygous or homozygous form (18). This suggests that DR7 is strongly associated with susceptibility to the development of LKM1 autoantibodies (18). Interestingly, it has already been proposed that the production of LKM1 in HCV-positive patients could be due to a molecular mimicry mechanism (25). The development of specific type 2 autoantibodies based on HLA class II alleles suggests that different environmental triggers associated with specific HLA class II susceptibility alleles could be responsible for the development of AIH-2. Interestingly, HLA class II susceptibility alleles for AIH-1 vary significantly in different regions of the world while those for AIH-2 are very similar. For example, HLA-DR7 is carried by almost 70% of patients with AIH-2 in Brazil, the other 30% expressing DRB1*03 (26).

In an interesting study, the T-cell reactivity against CYP2D6 was analyzed in relation to the patient's HLA class II alleles to characterize their association with disease activity, cytokine production profile, and AIH clinical course (27). Analysis

TABLE 2 Susceptibility alleles present in patients with type 1 and 2 AIH.

| Genes | Population | AIH | References |
|---|---|---|---|
| **MHC genes** | | | |
| HLA-A1-B8 | North America, Europe | Type 1 | Manns et al., Gastroenterology, 1994; Doherty et al., Hepatology, 1994, Ma et al., Hepatology, 2021 |
| HLA-DRB1*0401 | North America, Europe, Netherlands | Type 1 | Manns et al., Gastroenterology, 1994; Doherty et al., Hepatology, 1994, De Boer et al. Gastroenterology, 2014 |
| HLA-DRB1*0404 | Mexico | Type 1 | Vasquez-Garcia et al., J hepatol, 1998 |
| HLA-DRB1*0405 | Argentina, Japan | Type 1 | Pando M, Larriba et al. Hepatology, 1999; Seki et al., Gastroenterology, 1992, Yoshizawa et al. J Hepatol, 2005 |
| HLA-DRB1*1301 | North America, Europe, Brazil, Argentina | Type 1 | Djilali-Saiah et al., J Hepatol, 2004; Fainboim et al., Hum Immunol, 1994 |
| HLA-B | China | Type 1 | Liu et al. Hepatology, 2022 |
| HLA-DRB1*0301 | North America, Great Britain, Spain, Argentina, Netherlands | Type 1 and 2 | Manns et al., Gastroenterology, 1994; Doherty et al., Hepatology, 1994; Czaja et al., Am J Gastroenterol, 1999, De Boer et al. Gastroenterology, 2014, Ma et al., Hepatology, 2021 |
| HLA-DRB1-07 | Germany, Brazil, Great Britain | Type 2 | Jurado et al. J hepatol, 2002; Bittencourt et al., Am J Gastroenterol, 1999, Ma et al., Hepatology, 2021. |
| HLA-DRB3*01 | Brazil | | Czaja et al., J Hepatol, 2002; Jurado et al., J hepatol, 1997 |
| HLA-DQB1*0201 | North America, Europe | Type 2 | Djilali-Saiah et al., J hepatol, 2004 |
| HLA-DQB1*0603 | North America, Europe | Type 2 | Djilali-Saiah et al., J hepatol, 2004 |
| **Non-MHC genes** | | | |
| IgA | Europe | Type 1 | De la Concha et al., J Immunol, 2002; Vorechovski et al., Am J Hum Genet, 1999 |
| TNFa*2 | North America, Great Britain | Type 1 | Cookson et al., Hepatology, 1999; Czaja et al. Gastroenterology, 1999 |
| CTLA4 | North America, Europe | Type 1 | Agarwal et al. Hepatology, 2000 |
| Fas | Japan, North America | Type 1 | Hiraide et al., Am J Gastroenterol, 2005 |
| CD28-CTLA4-ICOS | China | Type 1 | Liu et al. Hepatology, 2022 |
| SYNPR | China | Type 1 | Liu et al. Hepatology, 2022 |
| Vitamin D receptor | Germany | Type 1 and 2 | Vogel et al., Hepatology,2002 |
| C4A | Europe, North America | Type 1 and 2 | Vergani et al., Lancet, 1985; Scully et al., Gastroenterology, 1993 |

showed that stimulation with CYP2D6 favored a Th1 response, with the CYP2D6 peptide aa 305-324 inducing the highest levels of interferon production. This peptide is recognized by HLA DR7 and non-DR7 individuals and can be considered the T-Cell's dominant epitope. Other epitopes, mainly recognized by HLA DR7 patients were between aa 73-124, 177-2112, and 217-260 (27). It can be concluded from these studies that an extensive overlap exists between the B and the T cell immune response, both controlled by the presentation within a particular HLA context (24, 27).

In a Genome-Wide Association Study (GWAS) of 649 adult patients with AIH-1, a significant association was found between AIH and SH2B3 and CARD10 variants in the 6p21 region of the major histocompatibility complex (28). Other genes involved in the activation of T cells, by regulating cytokines signaling or inducing cell maturation and proliferation have also been associated, however, without reaching statistical levels of significance.

Recently, in another Genome-Wide Association study of 1622 AIH-1 patients, Li *et al.* confirmed the previous association of AIH with HLA, in this case, SNP RS6932730 located in the intronic region of the HLA-B gene, but also found associations with two novel loci, CD28-CTLA4-ICOS and SYNPR (29). Interestingly, CD28-CTLA4-ICOS is a co-stimulatory receptor gene cluster located on chromosome 2q33 that encodes both the positive (CD28 and ICOS) and negative (CTLA-4) T-cell regulators (29). Unfortunately, similar Genome-Wide Association studies have not been carried out in patients with AIH-2 since their numbers are much lower (28).

## Non-MHC susceptibility genes

A long list of susceptibility genes for autoimmune diseases in humans has been identified, including genes related to lymphocyte activation and intracellular signaling, the major histocompatibility complex, cytokines, and cytokine receptors, innate immunity, microbial recognition, transcription factors, and several other pathways or mechanisms (30).

Other HLA genes have been found in association with susceptibility to AIH, such as the IgA and complement factor 4A (C4a) genes (Table 2) (31). IgA deficiency is frequent in patients with AIH and is genetically related to the MHC locus, specifically to HLA susceptibility alleles HLA-DR1 and HLA-

DR7 (32, 33). In addition, low levels of C4a are found in 69% of children with AIH (34). This deficiency may be related to the pathogenesis of the disease: indeed, several deletions in the C4a gene have been described in patients developing the disease at a young age (35). However, it is difficult to isolate the effect of this complement gene on AIH, as this gene is in strong linkage disequilibrium with the HLA A1-B8-DR3-DQ2 susceptibility haplotype (36).

Despite their odds ratios for AIH susceptibility being significantly lower than those for HLA alleles, several genes outside the MHC locus have been linked to AIH. These genes encode proteins that can regulate innate and/or adaptive immune responses, as is particularly the case in Graves' disease (37), multiple sclerosis (38), or celiac disease (39) where polymorphisms of the CTLA-4 gene have been found in adult and pediatric patients with AIH-1 (40, 41). CTLA-4 is a negative regulator of immune responses therefore, loss of this molecule could lead to a complex immune dysregulation syndrome, affecting several organs, including inflammatory infiltration of the liver (42). Linkage disequilibrium for this gene has also been found in affected children compared to unaffected siblings (41) Indeed, there is an increased transmission of alleles $(AT)_8$ and (A) of exon 1 of the CTLA-4 gene from heterozygous parents to their child with AIH-1 (87.5% and 83.5%, respectively) compared to unaffected children (50.0% for both alleles) (41). In contrast, no difference in the transmission of these alleles was found for patients with AIH-2 and their unaffected siblings (41).

A polymorphism in the promoter region of the FAS gene at position -670 has also been found in association with the development and progression of AIH, leading to an aggressive disease with the early development of cirrhosis (43, 44).

An association between vitamin D receptor gene polymorphisms and the development of primary biliary cholangitis and AIH, two autoimmune liver diseases, has also been found (45). The vitamin D receptor is thought to have a role in several functions of the immune system such as activation of macrophages and monocytes, specific inhibition of the effector functions of CD4+ Th1 cells, and inhibition of dendritic cell differentiation (46–48). Therefore, vitamin D receptor polymorphisms could alter the immunological response to an autoantigen and potentially influence the development of an autoimmune disease. In addition, tumor necrosis factor-alpha (TNF-α) gene polymorphisms can also confer susceptibility to AIH and influence its course. Substitution of G to A at the -308 position could influence gene transcription resulting in increased constitutive and induced circulating levels (49, 50). AIH patients with this polymorphism are susceptible to early disease development, are less likely to go into remission, and are more likely to develop cirrhosis (50).

Mutations in the *autoimmune regulator* gene (AIRE), which are responsible for the development of the Autoimmune Polyendocrinopathy syndrome (APECED), can also lead to the development of AIH in 10% to 20% of cases (38, 51). The AIRE gene is a transcription factor that is central in the thymic negative selection of self-reactive T cells. Thus, mutations that impair its function can lead to multiple autoimmune manifestations by increasing the number of self-reactive T cells. Autoantibodies in those patients are usually against CYP1A2, and less frequently against CYP2D6, the autoantigen recognized by LKM1 autoantibodies (52). AIH can be an early and severe complication in these patients. An apparent association between AIRE mutations and HLA DRB1*0301-DQB1*0201 and LKM1 autoantibodies against CYP1A2 was found in individuals developing an AIH (53). A case report has also shown LKM1 autoantibodies in APECED patients with AIRE mutations and AIH that respond to immunosuppression (54).

Heterozygous AIRE gene mutations have also been found in a few patients with AIH, one with AIH-2 and another with AIH-1 (55). However, studies of known mutations in the AIRE gene in patients with autoimmune liver diseases have shown that mutations in this gene probably do not play a significant role in the pathogenesis of AIH (37, 55).

## Animal models and genetic susceptibility to autoimmune hepatitis

Animal models of AIH can be useful to study the influence of genetic background or specific mutations on the pathogenesis of AIH. An animal model of AIH-2 was developed by our group using xenoimmunization with human CYP2D6 and FTCD proteins of C57BL/6 mice (56). These mice show circulating anti-LKM1 and anti-LC1 autoantibodies, increased serum alanine aminotransferase (ALT) levels, and significant lymphocyte infiltration of the liver (56). In addition, this AIH-2 experimental model shows the same female preponderance as seen in human AIH; xenoimmunized female animals developed severe AIH while xenoimmunization of male mice resulted in minimal liver inflammation (57). Interestingly, this was associated with the development of increased levels of regulatory T cells in males compared to females (57).

Using this non-transgenic mouse model, the susceptibility to develop AIH-2 of three mouse strains (C57BL/6, 129/Sv, and BALB/c) was compared. 129S/v mice share the same class I and II MHC genes as C57BL/6 but have different non-MHC genes while BALB/c mice have different MHC and non-MHC genes. Following xenoimmunization, C57BL/6 mice developed AIH while 129s/v mice showed sparse liver lobular infiltrate and slightly elevated ALT levels while BALB/c mice showed no signs of liver inflammation (56). This experiment showed that both MHC and non-MHC genes can influence the development of experimental AIH-2 and suggests that a class II MHC haplotype

($H\text{-}2^b$ in this case) is permissive but not sufficient for the development of AIH in this model (56).

An animal model of Autoimmune Polyendocrinopathy type 1 was also produced by truncating exon 2 of the *AIRE* gene. A quarter of those mice developed an AIH of intensity dependent on the *AIRE* mutation and the mouse genetic background (58). This study also suggests that genetic susceptibility to AIH is rarely linked with a single genetic locus and that a combination of susceptibility alleles is involved in the development of AIH.

A humanized mouse model expressing the DR3 HLA allele was studied after injection of the DNA plasmid coding for CYP2D6/FTCD to induce the development of AIH-2 (59). Immunized mice showed a sustained elevation of aminotransferases, LKM1/LC1 autoantibodies production, chronic inflammation, and fibrosis in the liver (59). The authors also observed an enhanced Th1 response and decreased levels of liver infiltrating Treg cells (59). These experiments using the AIH-2 animal model confirm the relevance of specific class II HLA alleles in the development of AIH and could allow the study of specific environmental triggers.

The role of Programmed cell death 1 (PD-1) on AIH susceptibility, an immunoreceptor of the CD28/cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) family that provides negative co-stimulation, has also been explored (60). $PD\text{-}1^{-/-}$ mice do not develop spontaneous AIH despite the lack of PD-1-mediated peripheral tolerance (60). However, when a neonatal thymectomy was also performed on these mice, mice developed fatal hepatitis (60). This suggests that in addition to defects in peripheral tolerance, an impaired central tolerance and the presence of circulating autoreactive T cells are required to induce an AIH (60).

Other mouse models have also shown links between AIH susceptibility and several other genes involved in immune tolerance including the TAM subfamily of receptor tyrosine kinases (Tyro3, Axl, and Mer) (61) and TRAF6, an E3 ubiquitin-protein ligase, that influences T cell tolerance through regulation of medullary thymic epithelial cell development (62).

## Conclusion

As with most autoimmune diseases, the main genetic associations in AIH involve genes of the major histocompatibility complex and in the case of type 1 and 2 AIH specifically, HLA class II genes and the HLA-DR locus. AIH is also linked with non-HLA genes, but their odds ratios for AIH susceptibility are far lower than those for HLA alleles. AIH susceptibility has been associated with SNPs in several genes including CTLA-4, TNF-α, vitamin D receptor, and AIRE. Genetic risk factors for AIH have been made with genes involved in central and peripheral immunological tolerance that regulate the proliferation and fate of autoreactive B and T cells, cytokine production, and inflammatory and immune responses in general. Animal models have been useful to pinpoint specific genes or groups of genes however, work remains to be done to elucidate the link between genetic predisposition, the break of immunological tolerance, and the development of AIH.

## Author contributions

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# HLA Associations in pediatric autoimmune liver diseases: Current state and future research initiatives

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The strongest genetic association with autoimmunity is within chromosome 6p21, where the human leukocyte antigen (HLA) complex resides. This review will focus on the HLA associations within pediatric autoimmune hepatitis, autoimmune sclerosing cholangitis and primary sclerosing cholangitis. In general, there is considerable overlap in HLA genotypes conferring susceptibility to pediatric autoimmune liver diseases, however unique HLA associations and protective HLA genotypes exist. There are numerous areas for future research initiatives in pediatric autoimmune liver diseases and HLA associations with clinical outcomes, autoantigen discovery and novel therapeutics targeting the HLA- autoantigen- T cell pathway will be highlighted.

KEYWORDS

**autoimmune hepatitis, autoimmune sclerosing cholangitis, major histocompatibility complex (MHC), primary sclerosing cholangitis, T cell activation**

## Overview of the role of the human leukocyte antigen complex in autoimmunity

A unifying theory on the pathogenesis of autoimmune liver diseases includes an infectious or environmental trigger in the genetically predisposed individual that is associated with an aberrant chronic autoimmune response targeting the hepatocyte or cholangiocyte. The strongest genetic association with autoimmunity is within chromosome 6p21, where the HLA complex resides. The HLA nomenclature is specific to humans, and is inclusive of the broader terminology for other animals, namely the major histocompatibility complex (MHC). There are two classes of HLA molecules- MHC

**Abbreviations:** AIH, autoimmune hepatitis; AILD, autoimmune liver disease; APC, antigen presenting cell; ASC, autoimmune sclerosing cholangitis; HLA, human leukocyte antigen; PSC, primary sclerosing cholangitis.

class I (HLA *A, -B, -C*) which are present on most nucleated cells and MHC class II (HLA *DP, -DQ, -DR*) that are present predominantly on professional antigen presenting cells (APCs) (dendritic cells, B cells, macrophages). MHC class II molecules are a heterodimer of an alpha (α) and beta (β) chain. In the example of HLA *DR*, the α- chain is encoded by the HLA *DRA* locus and the β- chain is coded by either HLA *DRB1, ;-DRB3, -DRB4* or *-DRB5* loci. Furthermore, phenotyping of the HLA molecule of interest may be denoted numerically, reflecting a specific allele within the locus (i.e. HLA *DRB1*0301*) (1).

The T cell activation that predominates in many autoimmune diseases and contributes to disease pathology is dependent on the APC presenting the antigenic peptide complexed to MHC to the T cell receptor ("signal 1"), as well as the APC providing co-stimulation to the T cell ("signal 2"). Certain HLA types are associated with outcomes in specific autoimmune diseases such as type 1 diabetes (2) and celiac disease (3), suggesting a direct link of HLA with disease pathogenesis. This review will focus on the HLA associations of autoimmune liver diseases (AILD) in children, namely autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC) and autoimmune sclerosing cholangitis (ASC; also known as AIH-PSC overlap syndrome). ASC is defined as meeting criteria for both AIH and having radiographic and/or histologic evidence of PSC (4). Unique HLA associations reported in distinct geographical areas and pediatric populations will be highlighted.

## HLA associations in pediatric AILD

### AIH and ASC

The majority of reports of HLA associations with AILD are from adults and include HLA class I (*A*01, B*08*) and HLA class II alleles (*DRB1*03, -04, -07 and/or -13*) (5). Children with AILD are unique compared to their adult counterparts based on higher incidences of type 2 AIH [anti-liver kidney microsomal type 1 (LKM1) and/or anti-liver cytosol type 1 (LC1) antibody positivity] and ASC (6). The largest cohort of children with AIH or ASC that have had detailed HLA association analyses are from King's College Hospital in London, England (7). A current report from this institution entailed outcome analyses of 236 European children with type 1 AIH (ANA and/or anti-actin positivity), type 2 AIH or ASC. With respect to MHC class I alleles, significantly higher frequencies of HLA *A*01* occurred in type 1 AIH and HLA *B*8* in all groups compared to healthy controls. MHC class II alleles that were more predominant in type 1 AIH included HLA *DRB1*03* and *HLA DQ*02*. The presence of homozygosity for HLA *DRB1*03* conferred the highest risk for AIH and ASC compared to healthy controls. Linking MHC class I and II expression, the HLA *A*01-B*8-DRB1*03* phenotype frequency was significantly higher in all groups compared to healthy controls. ASC susceptibility was further delineated based on having both HLA *B*8* and homozygous *DRB1*03* phenotype or being HLA *DRB1*13* positive/*DRB1*03* negative. Unique to type 2 AIH patients was a significantly higher frequency of HLA *DRB1*07* compared to all other groups.

HLA associations with clinical presentation and outcomes in the King's College cohort revealed that baseline AST levels were highest in those expressing HLA *DRB1*03 and DRB1*07* (type 1 and 2 AIH susceptibilities, respectively) compared to HLA *DRB1*13* (ASC susceptibility), while those with HLA *DRB1*13* had higher baseline levels of alkaline phosphatase and GGT. This provides indirect evidence that HLA genotypes contribute to disease pathogenesis, as the marker of hepatocytic injury (AST) was highest in AIH-associated HLA genotypes and cholestatic enzymes reflecting ASC were highest in the HLA *DRB1*13* cohort. Further evidence of HLA contribution to disease is based on the findings that more significant histological inflammation and fibrosis occurred in those children with either HLA *DRB1*03* (homozygous) or *DRB1*13* compared to other HLA DR genotypes.

With regard to outcomes, patients heterozygous for *DRB1*03* had a shorter time to remission, a higher "good responder" status and a lower frequency of end stage liver disease compared to those with *DRB1*07* or *DRB1*13*. Despite the fact that *DRB1*03* was associated with better outcomes, there was no HLA risk allele associated with need for liver transplant. Since liver transplant occurs in only 15-20% of children with AIH (8), a much larger cohort of patients would be needed to determine if a given HLA genotype is an independent risk factor for need for liver transplant.

Junge et al. (9) performed targeted high-resolution genotyping of HLA *DRB1* in 67 children with AILD from Germany. The frequency of HLA *DRB1*0301* (heterozygous or homozygous) was significantly higher in type 1 AIH and ASC compared to healthy controls. However, HLA *DRB1*1301* frequency was significantly higher only in the type 1 AIH, unlike the King's College experience where it predominated in ASC. The AIH group from Germany did not undergo routine screening for ASC and therefore it is unclear if those with HLA *DRB1*1301* were actually AIH versus ASC. Finally, type 2 AIH had a high frequency of HLA *DRB1*0701* compared to type 1 AIH, comparable to the King's College report.

In South America, a recent study from Brazil performed targeted genotyping of HLA *DRB1* in 43 children with either type 1 AIH or ASC (10). When compared to healthy controls, the frequency of HLA *DRB1*03* and HLA *DRB1*13* were significantly higher in the both groups compared to controls. Furthermore, the presence of HLA *DRB1*13* conferred a 3-fold increase risk for ASC. A landmark study from Argentina in 1999 described MHC class II genotyping in 122 children with type 1 AIH (11). HLA *DRB1*1301* was identified as the primary susceptibility allele for AIH. However, there was no description of screening for ASC in this population and therefore accurate differentiation of the HLA *DRB1*1301*

association with AIH versus ASC was not possible. The exclusion of children who were HLA *DRB1*1301* positive uncovered a second association of AIH with HLA *DRB1*0301*. Two other HLA genes that map close to HLA *DRB1* are HLA *DQA1* and *DQB1*. Both HLA *DQA1*0103* and *DQB1*0603* were found in strong linkage disequilibrium with *DRB1**1301 in AIH; thus the risk haplotype was defined as HLA *DRB1*1301-DQA1*0103-DQB1*0603*. A summary of HLA susceptibility genes in AIH and ASC are provided in Figure 1.

## PSC

In contrast to the robust HLA mapping in pediatric AIH, there is a paucity of information on HLA associations in children with PSC. In adults, some of the HLA associations with PSC include HLA *B*07, B*08, and DRB1*1301* (12, 13). One of the first reports of HLA associations in pediatric PSC by Wilschanski et al. (14) from Canada showed that the frequency of HLA *B*8* and *DRB1*1501* in PSC was significantly greater than in controls. Brazil reported on 27 children and 36 adults with PSC and found that, similar to adults, *DRB1*1301* inferred susceptibility in children (15). Ylinen et al. (16) performed HLA genotyping in 19 children with AILD from Finland, including 7 with PSC, compared to 19,807 controls. Higher frequencies of HLA *B*08, DRB1*03* and *DRB1*13* occurred in AILD, however there was no difference in these frequencies when comparing AIH to PSC. The German study by Junge et al. detailed above for AIH and ASC also included 20 children with PSC (9). Significantly higher

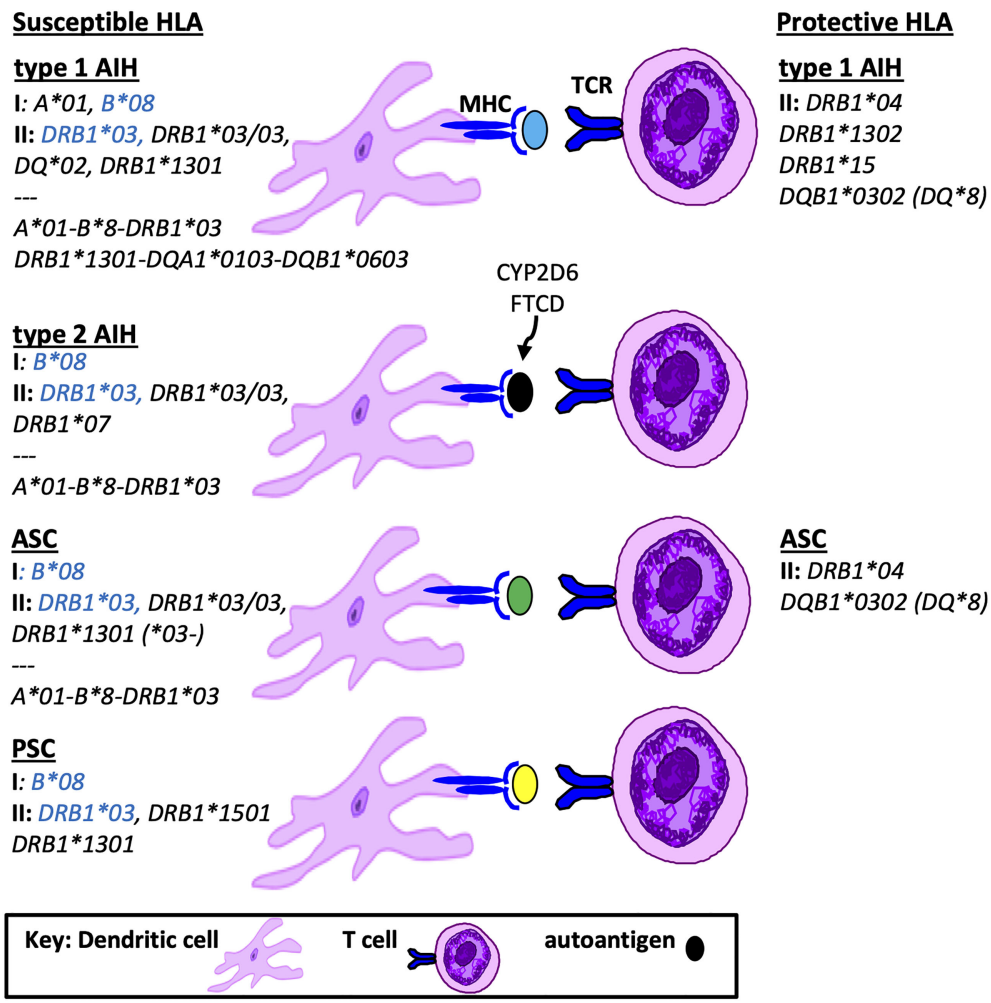


FIGURE 1
Susceptible and Protective HLA genotypes in Pediatric Autoimmune Liver Diseases. Shown is a summary of HLA genotypes associated with pathogenesis or protection from autoimmune liver diseases in children, including MHC class I, class II and grouped haplotypes based on linkage disequilibrium. Highlighted in blue are susceptibility HLA genotypes found in all pediatric AILD.

frequencies of HLA *DRB1*0301* and DRB1**1301* were found in PSC compared to controls.

## HLA genotypes that are protective from AILD

HLA genotypes that are lower in frequency in a disease state compared to the general population implies that those genotypes may actually protect from developing an autoimmune disease. In the King's College study, protective HLA associations in AIH were inferred based on significantly lower frequencies of HLA *DRB1*15* in children with type 1 AIH (7). Similarly, in adults with AIH, HLA *DRB1*1501* was associated with protection from disease (17). The peptide binding pocket of HLA *DRB1*1501* differs from the susceptibility HLA *DRB1*0301* by one amino acid at position 71 (alanine (17) versus lysine (18)), suggesting that this is a key HLA position associated with the risk of developing AIH.

The pediatric King's College study also identified *DRB1*04* and *DQB1*0302 (DQ*8)* as protective in type 1 AIH and ASC compared to healthy controls (7). With regard to HLA *DRB1*04*, the aforementioned pediatric Finnish report likewise showed no HLA *DRB1*04* positivity in those with either AIH, ASC or PSC (16). In contrast, HLA *DRB1*04* is considered a disease susceptibility HLA genotype in adults with AIH (5). The *DRB1*04* risk allele in adult AIH is associated with a later age of onset of AIH (19) and likely represents a novel subtype of AIH not found in children.

Another HLA genotype that was found to be protective for AIH in the Argentinian pediatric cohort was HLA *DRB1*1302 (*11*)*. Interestingly, HLA *DRB1*1302* differs by only one amino acid (valine) from the susceptibility HLA *DRB1*1301* (glycine) in position 86 of the peptide binding pocket, suggesting that this position is key to antigen presentation. With regard to protective HLA genotypes in PSC, there is no data in children. However in adults with PSC the haplotype HLA *DRB1*04-DQB1*03* represents the most consistent protective HLA association (13). A summary of susceptible and protective HLA associations in children is provided in Figure 1.

## Future research initiatives focused on HLA associations in pediatric AILD

In general there is considerable overlap in HLA associations between pediatric AIH, ASC and PSC, with all three AILD types reporting associations with either HLA *B*08, DRB1*03* and/or *DRB1*13*. Type 2 AIH has a unique HLA *DRB1*0701* predominance and PSC has an additional HLA *DRB1*1501* susceptibility. The accuracy of these associations as it relates to AIH versus ASC is limited in the cohorts that were not systematically screened for ASC. Protective HLA genotypes in children include HLA *DRB1*04* (AIH, ASC, PSC), *DRB1*1302* (type 1 AIH), *DRB1*15* (type 1 AIH*)* and *DQB1*0302* (AIH, ASC). In comparison to adult AILD HLA associations, there are both redundant and unique susceptibility and protective HLA genotype associations in children.

In adults with AIH, racial and ethnic differences in outcomes have been reported (20), however little is known about these racial/ethnic groups and HLA associations in pediatric AILD. In addition, in many countries such as North America and Asia, rigorous analyses of HLA associations in pediatric AILD have not performed. Only the recent King's College report analyzed clinical presentation and outcome correlations with HLA genotypes. Herein lies the first future research initiative, whereby HLA association studies worldwide should include sub-analyses based on race/ethnicity and outcomes analyses in order to provide evidence of HLA-associations with prognosis (Table 1).

The second understudied area relates to pediatric AILD pathogenesis. There is a paucity of data on the autoantigens that are being presented to the T cell in an MHC- restricted fashion. Only in type 2 AIH have the autoantigens been discovered, specifically cytochrome P450-2D6 (CYP2D6) (21,

TABLE 1 Future Research Initiatives in Pediatric AILD.

| | |
|---|---|
| **Worldwide Epidemiology & Health Outcomes Research** | |
| AIH, ASC | Determine HLA associations with clinical outcomes (e.g. disease severity at presentation, need for liver transplant, etc.) |
| PSC | Define HLA associations and determine associations with clinical outcomes |
| AIH, ASC, PSC | Determine HLA associations with race/ethnicity |
| *Pediatric AILD Pathogenesis Research* | |
| AIH (type 1), ASC, PSC | Determine autoantigen(s) involved in MHC- restricted T cell activation |
| AIH (type 1), ASC, PSC | Create novel mouse models of disease to study mechanisms of MHC- restricted T cell activation |
| AIH (type 1), ASC, PSC | Utilize the HLA3D toolkit for autoantigen discovery |
| *Novel therapies targeting the MHC- peptide- T cell pathway* | |
| AIH, ASC, PSC | Implement nanoparticle technology to enhance immunoregulation of autoimmunity |

22) and formiminotransferase-cyclodeaminase (FTCD) (23). FTCD is the autoantigen recognized by anti-LC1 autoantibodies that are found in a subset of type 2 AIH patients. CYP2D6 is the antigenic target of the LKM1 autoantibody, which is the hallmark autoantibody in type 2 AIH. Longhi et al. utilized MHC-peptide tetramer technology and found that HLA *A*0201* CYP2D6-specific CD8 T cells in children with type 2 AIH were highest in frequency at the time of diagnosis, decreased in response to immunosuppressive therapy and correlated with hepatocyte injury (22). Recently, a new mouse model of type 2 AIH was described, whereby BALB/c mice were immunized with CYP2D6 in complete Freund's adjuvant (to activate innate immunity) (24). These mice produced high levels of anti-LKM antibodies and CYP2D6-specific T cells and liver histology mimicked the human disease. This new model will be instrumental in discovering novel immune pathways associated with hepatocyte injury and fibrosis in type 2 AIH. Recently, a new technique for MHC-restricted autoantigen detection known as "HLA3D" was reported (25). HLA3D is a comprehensive platform that directly analyzes the interactions between HLA molecules and peptides based on the HLA conformational structure. The HLA3D toolkit integrates HLA conformational differences with antigenic peptide prediction and will likely be a powerful tool for uncovering MHC-restricted autoantigens in AILD (Table 1).

The third area for future research entails the discovery of therapies that specifically target the MHC- peptide (autoantigen)-T cell pathway, thus providing a powerful therapeutic approach to induce remission of AILD. Exciting work from Umeshappa et al. used nanoparticle technology to coat nanoparticles with disease associated, liver autoantigen peptides that were coupled to disease-susceptibility MHC class II molecules (26). The nanoparticles were tested in a spontaneous mouse model of primary biliary cholangitis (PBC) (the NOD.*c3c4* mouse), as well as a humanized mouse model of PBC that utilized human PBC HLA susceptibility genotypes. In both models, administration of the nanoparticle coated with the PBC autoantigen pyruvate dehydrogenase complex-E2 (PDC-E2) coupled to MHC class II resulted in protection from disease. This protection was attributed in part to the expansion of autoantigen-specific regulatory T cells that inhibited pathogenic T cell mediated liver injury. Strikingly, this response was not unique to the PBC mouse model, and similar protection of disease was demonstrated in PSC (*abcb4* $^{-/-}$) and AIH mouse models in response to administration of the PDC-E2-MHCII nanoparticles. The authors clearly demonstrated the ability of ubiquitous autoantigen-based-MHC nanomedicines to blunt liver autoimmunity in an organ-specific rather than disease-specific manner. It was theorized that the initial liver tissue injury in AILD has the potential to trigger activation of autoreactive T cells that recognize many additional autoantigens, thus leading to the pervasive regulatory T cell responses capable of diminishing pathology. This and other novel approaches (27) that manipulate the MHC- peptide- T cell pathway will provide powerful therapies for autoimmune liver diseases.

## Author contributions

CM researched and wrote the entire manuscript and created the figure independently.

## Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# HLA-related genetic susceptibility in autoimmune hepatitis according to autoantibody profile

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Although the prevalence of autoimmune hepatitis in first-degree relatives is small, the relationship between genetic markers, especially human leucocyte antigens (HLA), and susceptibility to this disease, has been studied for over three decades. The genetic susceptibility to AIH is believed to be different in the two subtypes of the disease, AIH type 1 and AIH type 2. Type 1 AIH has anti-smooth muscle and anti-nuclear antibodies as its main markers, while those of type 2 AIH are the anti-liver/kidney microsome type 1 and anti-liver cytosol type 1 antibodies. The anti-soluble liver antigen/liver-pancreas antibodies, which, in addition to being present in both subtypes, mark an important number of patients without serological markers. Therefore, a third type of disease is questionable. The vast majority of immunogenetic studies compare the differences between the two main types and make no difference between which antibodies are present to define the subtype. This review seeks to analyze what was most important published in the AIH in this context, trying to relate the HLA alleles according to the AIH marker autoantibodies.

KEYWORDS

**autoimmune hepatitis, autoantibodies, human leucocyte antigens (HLA), antismooth muscle antibodies, antinuclear antibodes, antiliver kidney type 1 microsome antibodies, antiliver cytosol type 1 antibodies, anti-soluble liver antigen antibodies**

**Abbreviations:** HLA, human leucocyte antigen; AIH, autoimmune hepatitis; MHC, Major histocompatibility complex; ANA, antinuclear antibodies; ASMA, anti-smooth muscle antibodies; anti-LKM1, anti-liver kidney microsome type 1 antibodies; anti-SLA/LP, anti-soluble liver antigen/liver pancreas; SSA, syndrome Sjögren antigen.

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## Introduction

Although autoimmune hepatitis (AIH) has very little familial aggregation, several markers of genetic susceptibility are investigated in an attempt to help understand the pathogenesis of the disease. The most studied markers, which demonstrated the greatest relationship between these variables, are undoubtedly the human histocompatibility antigen (HLA) alleles encoded by highly polymorphic genes linked to the major histocompatibility complex (MHC), on the short arm of chromosome 6. In addition to genetic markers, AIH has important serological autoantibodies that help in diagnosis, classification and, in a way, are related to the severity of the disease and the therapeutic response.

Several studies, over the decades, sought to report the types of AIH according to the genetic profile of HLA antigens. However, the geographic variation of these susceptibility markers is very large and this relationship with the classification of AIH has not been an easy task.

Following the revised diagnostic criteria of the international AIH group, from 1999, the reactivity of antinuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA) and anti-liver kidney microsome antibodies type 1 (anti-LKM1) at titers >1/80, in adults, would be one of the most important criteria for the diagnosis. The presence of DRB1*03 or DRB1*04 should only be scored in case of autoantibody negativity. In these criteria, HLA reactivity was extended to other alleles, outside DR3 and DR4, according to geographic variations (1). This requirement was not strictly followed and in several publications, the HLA score was an additional parameter, regardless of the autoantibody reactivity (2).

While the serological markers of AIH were restricted to ASMA, ANA and anti-LKM1, the aggregation of HLA alleles according to autoantibody reactivity appeared to be a relatively easy job. However, after further investigation of the target antigens for the classic autoantibodies of the disease and the description of new serological markers, this correlation has become even more difficult than expected, since there are often two or even three simultaneously reactive autoantibodies that could have a different or similar relationship with the HLA alleles.

Most studies on AIH analyze the susceptibility relationship of HLA alleles to AIH subtypes and not to a specific autoantibody. (Tables 1, 2 Supplementary Material). They also look at the relationship between different clinical parameters, such as age at disease onset, IgG levels, therapeutic response, and prognosis. The aim of this review was to analyze the relationship between genetic markers of susceptibility linked to HLA according to AIH subtypes and with individual serological markers of the disease.

## Type 1 autoimmune hepatitis

Initially, studies of genetic susceptibility in AIH performed in European and North American Caucasian patients without distinctions regarding AIH subtypes. As in most of the studies, the patients had ANA and/or ASMA as markers, from the moment the reactivity of these autoantibodies began to be established, the results did not differ significantly for type 1 AIH. Thus, genetic susceptibility related to HLA alleles was associated with class II alleles, DRB1*03:01,*04:01, DRB3*01:01 that encode HLA molecules DR3, DR4 and DR52 respectively, in European and North American Caucasian populations (3, 4). When extending these studies to other Caucasian and non-Caucasian populations, for example, for mixed-race Mexican patients, susceptibility was associated with the DRB1*04:04 allele, although these results were not confirmed in a later study (5, 6). In Japanese, the susceptibility ratio was related to the alleles DRB1*04:05 and DQB1*DRB1*04:01, DQB1*04:01 haplotype in addition to the heterozygous genotype DR4/DR8 (7, 8).

In South America, the genetic predisposition to AIH acquired a different pattern. While in Argentina and Venezuela the primary association with DRB1*13:01 was observed in children (9, 10), in Brazil, although this primary association was stronger in younger patients, it was also observed in in all age groups (11). The frequency of DQB1*06 in these three South American countries, in linkage disequilibrium with DRB1*13, was also increased, but in Brazil, five patents with this DR allele carried different DQB1 alleles, conferring to DR13 higher odds ratio. In these three countries, DRB1*03 had a secondary association in patients who were negative for DRB1*13:01. Unlike Venezuela and Argentina, in Brazil this association was observed in patients who were positive for ASMA with specificity for F-actin. In Brazil, there was no association with the DRB1*04, but in Argentinean adult patients, a greater susceptibility was observed in those with DRB1*04:05. However, the ASMA and ANA reactivity was more frequently observed in children and adults with AIH respectively. Maybe this reactivity could also be related to those predisposing HLA alleles.

Although the susceptibility relationship with DRB1*03:01 and DRB1*04:01 was observed in North American and Europeans Caucasians with type 1 AIH, when analyzing whether this relationship held with ASMA or ANA reactivity, the results are, indeed, very contradictory. In 1993, results from North American patients revealed that ASMA reactivity and high ANA titers were found in patients who had DRB1*04. In 1996, there was no relationship between ASMA reactivity and HLA alleles, but when studying antibody reactivity against F actin, the relationship with HLA B*08 and DRB1*03:01 was

verified in younger patients and with worse prognosis (4, 12). All patients who died with liver failure had reactivity for antibodies against microfilaments, as did almost all who required liver transplantation when compared with those antiactin negative/ANA positive patients. In other populations, a greater predisposition to AIH has also been reported in patients who carry the DRB1*13 allele, such as in Indian patients (13). North American patients with DRB1*13 as the sole marker of susceptibility, excluding patients DRB1*03 and DRB1*04, had a lower frequency of relapse and a higher occurrence of sustained remission after treatment withdrawal than patients with DRB1*03 as a single susceptibility marker (14). However, no relationship with serological markers was characterized in these studies.

In British Caucasian children, the DRB1*13 allele was more prevalent in children with type 1 AIH with biliary alterations, also named as having autoimmune sclerosing cholangitis in relation to healthy controls and type 2 AIH. However, when comparing these children with those with genuine type 1 AIH, who more frequently had DRB1*03, there was no significant difference in relation to DRB1*13. In this study, there was no stratification according to type 1 AIH serological markers, but the predominance of ASMA can be expected, since ANA reactivity alone in children is less frequent. In addition, the profile of autoantibodies in classical type 1 AIH and in its variant with more evident biliary injury was quite similar (15). Results from different pediatric centers of Europe, North and South America reinforced those obtained by family-based association studies in Canadian-French patients by the transmission disequilibrium test that susceptibility to type 1 AIH is linked to DRB1*03 and/or DRB1*13 (16).

The results of the association of the DRB1*04 allele with autoantibodies are also difficult to interpret. Initially, this class II allele was related to ASMA reactivity and high ANA titers. However, this relationship was no further confirmed in patients from the same population (12, 17). In Japanese patients, the DRB1*04:05/DQB1*04:01 haplotype was related to reactivity for ASMA (7). One of the difficulties in interpreting and studying the relationship between HLA alleles and ANA reactivity is due to the lack of characterization of ANA patterns in studies that evaluate this disease. The most common ANA patterns related to AIH are the homogeneous and the fine speckled. Despite the lack of studies analyzing this stratification, there are investigations in which the relationship between the HLA profile and reactivity for antibodies against specific nuclear antigens was analyzed. No susceptibility relationship was observed with anti-histone, anti-chromatin and anti-single-stranded DNA antibodies (18–20). The only antibody reactivity that was related to DRB1*04 was that to double-stranded anti-DNA searched by ELISA and indirect immunofluorescence (20).

On the other hand, there is also a protective relationship between a given HLA and susceptibility to the disease, although with less evident and less concordant results in different populations. This relationship with DRB1*13:02 deserves attention, because this allele differs from DRB1*13:01 by only one amino acid at position 86, glycine for valine respectively (7). It is possible that valine at position 86 of DRB1*13:01 may modulate the immune response to a putative auto-antigen. This protective action of the DRB1*13:02 was observed in Latin America and Japan and in several autoimmune diseases such as rheumatoid arthritis, primary biliary cholangitis, Graves' disease, Hashimoto's thyroiditis and psoriasis (21). Other alleles that were also discriminated as protective for the development of type 1 AIH were DQB1*04, DRB1*11 and the DRB1*15:01/DQB1*06:02 haplotype (7, 10, 22). This protective action was generally evaluated in relation to type 1 AIH and very little analyzed in type 2 AIH and, even in type 1 AIH, there is no assessment as to whether it would have any specificity for a particular autoantibody.

In immunogenetic studies, other HLA alleles were related to AIH susceptibility, including class I and class II antigens, usually not in isolation but as haplotypes, such as A*01-B*08-C*07-DRB3*01:01 (DRB1*52) whose alleles are in linkage disequilibrium with DRB1*03, just as the DRB4*01:03 is with DRB1*04:01. On the other hand, the DQA1*01:03 and DQB1*06:03 were also found to be in strong linkage disequilibrium with DRB1*13:01 according to the haplotype HLA-DRB1*13:01-DQA1*01:03-DQB1*06:03 (3, 8, 11, 22). However, there is, in general, no relationship of these haplotypes with a specific profile of autoantibodies.

## Type 2 autoimmune hepatitis

Few immunogenetic studies have focused on studying the relationship between HLA alleles and reactivity for type 2 AIH markers. The biggest obstacle in carrying out these studies is the low prevalence of this type of autoimmune in different geographic regions, for example, its rarity in North American studies. Unlike the AIH-1 markers, commercial kits with specific antigens for the determination of both, anti-LKM1 and anti-liver cytosol type 1, are commercially available and better homogenization of patients in relation to the autoantibody profile is possible.

In a 1997 study, when comparing German patients with type 2 AIH with North American controls and patients with type 1 AIH, it was observed a higher frequency of alleles DRB1*07, DRB1*15, DRB4*01 and DQB1*06 alleles (23). However, no results remained significant after the p correction. Until then, the immunogenetic studies performed did not suggest a susceptibility relationship of type 2 AI with MHC class II antigens (24). That same year, in Spanish patients, a higher frequency of DQB1*02 was found in carriers of type 2 AIH, an antigen that is in linkage disequilibrium with DRB1*07. The relationship with DRB1*07 was recorded more frequently in patients with Hepatitis C, who also had anti-LKM1 reactivity (25). This concomitance of hepatitis C with anti-LKM1 reactivity with DRB1*07 was also reported in Italians (26).

In 1999, in Brazilian patients, both alleles mentioned above plus DRB4 were significantly more frequent in patients with type 2 AIH, almost entirely with anti-LKM1 (25 of 28 patients). As two patients, who were DRB1*07 positive, were not DQB1*02, the Odds Ratio for DRB1*07 was higher suggesting that this allele was related to increased susceptibility (11). In a study with a greater number of patients with type 2 AIH and with better stratification of anti-LKM1 and anti-liver cytosol type 1 antibodies, Canadian and French Caucasian children presented the DQB1*02:01 allele as the main genetic determinant of susceptibility. The DRB1*03 allele was significantly increased among patients with simultaneous reactivity to anti-LKM1 and anti-liver cytosol type 1 antibodies, as well as those with isolated reactivity to anti-liver cytosol type 1 compared to those with isolated reactivity to anti-LKM1. The DRB1*07 was the most representative allele in patients with isolated reactivity to anti-LKM1 (27). All patients with DRB1*03 and DRB1*07 carried DQB1*02, and for this reason the latter HLA allele conferred the highest odds ratio. The result of this study corroborated the conclusion of a previous family-based association study by the transmission disequilibrium test from the same Canadian-French research center that type 2 AIH is associated with DQB1*02 (16). As these three alleles are present with strong linkage disequilibrium, the discordant results are understandable and demonstrate that there is a need for additional studies with a larger number of patients and with well-defined autoantibody markers. In patients outside Europe and North and South America, few publications, without stratifying the two different markers, recorded the susceptibility of type 2 AIH with the HLA alleles. In India, DRB1*14 have been linked to this AIH subtype analyzed in 13 patients (13).

## Autoimmune hepatitis with anti-soluble liver antigen/liver pancreas antibody reactivity

Some studies have linked anti-SLA/LP reactivity with genetic susceptibility to HLA alleles. In a meta-analysis study conducted in 2015, 195 patients with anti-SLA/LP were cataloged and the DRB1*03 allotype was positively associated with anti-SLA/LP reactivity (28). A bias that hinders the analysis of these results is the concomitance of the reactivity of this marker with ASMA, ANA and, to a lesser extent, with anti-LKM1/anti-liver cytosol type 1, which makes it difficult to interpret the relationship with DRB1*03. Another important factor that can make interpretation more difficult is the almost universal concomitant reactivity of anti-Ro52 with anti-SLA/LP (29). From publications on rheumatologic diseases, the correspondence of anti-Ro/SS-A with DRB1*03 has already been characterized (30). Patients with AIH and reactivity to anti-Ro52 and anti-SLA/LP antibodies had a higher frequency of DRB1*03 and a lower occurrence of DRB1*04 than patients with anti-Ro52 (29). However, in an analysis of Greek patients with AIH, when patients were stratified for anti-Ro52 and anti-SLA/LP reactivity, the frequency of DRB1*03 was higher only for patients positive for the former marker (31).

In a 2021 study, the immunogenetic analysis of 62 Chinese patients with AIH and anti-SLA/LP reactivity was compared with that of 500 healthy controls. A concomitant ANA reactivity was very high and, surprisingly, the class I alleles, HLA B*35:01 and C*08:01, were the only ones significantly more frequent in this set of patients (32). Other class I HLA alleles and class II alleles HLA-B*08:01, B*40:02, DRB1*04: 01, DRB1*04: 05, DRB1*14: 01 and DRB1*16: 02 also had a clear trend of greater frequency, while DRB1*15:01 of lower frequency, but did not reach statistical significance after Bonferroni's correction, probably because of the small sample size. In our experience, anti-Ro52 is present in about 90% of patients with anti-SLA/LP reactivity, in 25% of patients with ASMA with F actin specificity without anti-SLA/LP reactivity and 23% of anti-LKM1 patients (unpublished data). Therefore, anti-Ro52 reactivity is yet another bias in the interpretation of the relationship of HLA alleles with autoantibody reactivity for not only patients with anti T SLA/LP reactivity but also for patients with type 1 and type 2 AIH.

## Final considerations

Few studies have studied the relationship between HLA alleles and the reactivity of AIH autoantibodies. In part, the obstacles faced in this regard are related to the genetic differences inherent in different populations. However, there are also shortcomings related to the methodology of the studies, such as the small number of patients, because it is a relatively uncommon disease. Another obstacle also lies in the patient selection criteria in relation to autoantibody reactivity. Comparison between the results of different cohorts of patients is greatly impaired as the frequency of autoantibody reactivity is extremely variable between series. For example, there are studies with a predominance of patients with reactivity for ANA and little reactivity for ASMA and vice versa. In other series, patients without autoantibody markers and patients with reactivity for antimitochondrial antibodies are included.

The ASMA reactivity may correspond to antibodies against antigens present on microfilaments, intermediate filaments, and microtubules, but the target antigen of this pattern in AIH is located in the polymerized form of actin (F-actin) of microfilaments and

few studies adopt this criterion. Regarding ANA, little is known which would be their true target antigen(s) in AIH. Histones may potentially be the target antigen of ANA with the homogeneous pattern, but there are five types of histones and when testing the relationship between HLA alleles and anti-histone antibodies, no positive results were found. Regarding the fine speckled pattern of ANA, the target antigen is also still not identified. The rare studies that were more rigorous in selecting patients with ASMA/F-actin antibodies suggested a more important relationship with the DRB1*03 and DRB1*13 alleles. Regarding type 2 AIH, as the target antigens are better defined, the DRB1*07 and DRB1*03 alleles seem to be more related to anti-cytochrome P450 IID6 and anti-forminotransferase cyclodeaminase, respectively. Likewise, anti-SLA/LP, whose target antigen is the synthase converting O-phosphoseryl-tRNA to selenocysteinyl-tRNA, have a very close relationship with DRB1*03.

Celiac disease is a typical example of an autoimmune disease in which the genetic component (DQB1*02 and DQB1*08), the involved auto-antigen (tissue transglutaminase), the environmental trigger (gliadin) have been identified. Tissue transglutaminase-deaminated gliadin peptides that are recognized by DQ2/DQ8+ antigen presenting cells to T helper cells start the activation and maturation of B-lymphocytes that culminate in the production of anti-tissue transglutaminase antibodies. In addition, there is release of pro-inflammatory cytokines that, together with T killer cells, initiates the typical lesion of the intestinal mucosa (33). Studies in AIH are necessary with strict criteria in choosing patients with a well-defined diagnosis, who have a unified profile of autoantibodies that allows the characterization of specific target antigens. Perhaps this way we can identify, as in celiac disease, the environmental agent that triggers AIH and a more precise relationship between HLA alleles and autoantibody reactivity, finally developing specific curative treatment strategies.

## Author contributions

EC: manuscript design, reading the references, write the manuscript. JC: survey of bibliographic references, reading the references and obtaining data of relevance to the manuscript, revision. DT: survey of bibliographic references obtaining data of relevance to the manuscript, co-write the manuscript. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1032591/full#supplementary-material

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# Standard immunosuppressive treatment reduces regulatory B cells in children with autoimmune liver disease

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**Introduction:** Autoimmune hepatitis (AIH) is a chronic liver disease caused by a perturbed immune system. The scarcity of short- and long-term immune monitoring of AIH hampered us to comprehend the interaction between immunosuppressive medication and immune homeostasis.

**Methods and patients:** We recruited children with AIH at the time of diagnosis and at the 1st, 3rd, 6th, 12th, 18th, and 24th months of immunosuppression (IS). We also enrolled children with AIH being on IS for >2 years. Children with drug-induced liver injury (DILI), and those receiving tacrolimus after liver transplantation (LT), were enrolled as disease/IS control subjects. Healthy children (HC) were also recruited. Peripheral blood mononuclear cells (PBMCs) were isolated from all participants. Healthy liver tissue from adult donors and from livers without inflammation were obtained from children with hepatoblastoma. By using flow cytometry, we performed multi-parametric immune profiling of PBMCs and intrahepatic lymphocytes. Additionally, after IS with prednisolone, tacrolimus, rapamycin, or 6-mercaptopurine, we carried out an *in vitro* cytokine stimulation assay. Finally, a Lifecodes SSO typing kit was used to type HLA-DRB1 and Luminex was used to analyze the results.

**Results:** Untreated AIH patients had lower total CD8 T-cell frequencies than HC, but these cells were more naïve. While the percentage of naïve regulatory T cells (Tregs) ($CD4^+FOXP3^{low}CD45RA^+$) and regulatory B cells (Bregs, $CD20^+CD24^+CD38^+$) was similar, AIH patients had fewer activated Tregs ($CD4^+FOXP3^{high}CD45RA^-$) compared to HC. Mucosal-associated-invariant-T-cells (MAIT) were also lower in these patients. Following the initiation of IS, the immune profiles demonstrated fluctuations. Bregs frequency decreased substantially at 1 month and did not recover anymore. Additionally, the

frequency of intrahepatic Bregs in treated AIH patients was lower, compared to control livers, DILI, and LT patients. Following *in vitro* IS drugs incubation, only the frequency of IL-10-producing total B-cells increased with tacrolimus and 6MP. Lastly, 70% of AIH patients possessed HLA-DR11, whereas HLA-DR03/DR07/DR13 was present in only some patients.

**Conclusion:** HLA-DR11 was prominent in our AIH cohort. Activated Tregs and MAIT cell frequencies were lower before IS. Importantly, we discovered a previously unrecognized and long-lasting Bregs scarcity in AIH patients after IS. Tacrolimus and 6MP increased IL-10+ B-cells *in vitro*.

KEYWORDS

**bregs, autoimmunity, liver, children, HLA**

# Introduction

Autoimmune hepatitis (AIH) is a fatal immune-mediated liver disorder if left untreated (1). It is characterized by the presence of hypergammaglobulinemia, periportal inflammation with interface hepatitis, and organ (non-)specific autoantibodies. AIH type-1 is defined by the presence of anti-smooth muscle (SMA) and/or anti-nuclear antibodies (ANA), whereas type-2 AIH is diagnosed when liver-specific autoantibodies against Cytochrome P450 enzyme CYP2D6 (anti-LKM1) and/or formimidoyltransferase cyclodeaminase (anti-LC1), are detected (2). Autoantibodies against soluble liver antigens (SLA) can be seen in both types of AIH (3). AIH patients with evidence of cholangitis on histology are diagnosed with autoimmune sclerosing cholangitis (AISC). For more than a half-century, non-selective immunosuppressive drugs (IS) have been used to treat AIH (4). Standard IS in the induction phase exists out of steroids for the first two weeks, and then stacked with azathioprine (AZA) after the second week. Maintenance therapy, depending on patients' response, side effects, or intolerance, is done with AZA ± steroids, with additional ursodeoxycholic acid in patients with AISC (4). Treatment success is not 100% and is affected by non-adherence, particularly in adolescents, but the type of HLA allele present is also important, as no biochemical remission or frequent relapse (5) was linked with the presence of HLA-DR7 or HLA-DR3 alleles. Furthermore, paediatric AIH is more severe and less controllable compared to adult AIH (6). Hitherto, a detailed systemic and hepatic profile of the activity of innate and adaptive (regulatory) immunity, prior to the start of any IS, has been elusive. The general concept of the AIH immunopathophysiology is that loss of self-tolerance of autoreactive T cells results in T helper (TH) 1, TH2, and TH17 cells, accompanied by regulatory T cell aberrations (2, 7, 8), stimulating CD8 T cells, B cells and NK cells to ultimately damaging hepatocytes *via* cellular, humoral, and granzyme mediated auto-aggression, respectively (9). By virtue, the use of IS is the mainstay of AIH treatment. Although IS improves hepatitis in the majority of patients, it also suppresses Tregs, the very cells that constitute immune tolerance. More importantly, Diestelhorst et al. clearly demonstrated that standard IS treatment reduced inflammation (CD4/CD8 cell infiltration) by 39% (10). Yet, the proportion of Tregs ($CD4^+FOXP3^+$) was disproportionally diminished by more than 50%, which may explain why weaning off IS is not justified given our current and partial comprehension of AIH pathophysiology at baseline and after IS. T cell biology has received a lot of attention, but B cells are also very important because bidirectional B cell-T cell communication is crucial in both homeostasis and disease. For example, regulatory B cells (Bregs) interact with Tregs, causing an increase in their numbers (11). However, little is known about Breg homeostasis in AIH. To that end, our primary aim was to investigate the immunological properties of naïve (untreated) and treated AIH patients using deep immune phenotyping of PBMCs and intrahepatic immune cells. The secondary aim was to understand the B cell dynamics and effects of IS drugs on B cells and Bregs, as well as whether there is a difference between type-1 and type-2 AIH. Lastly, we also investigated the HLA-DRB1 allele frequencies in these patients in order to better understand the pattern of HLA alleles associated with AIH patients.

# Patients and methods

## Study population

The study protocol was approved by Koç University (IRB: 2019.255.IRB2.077 and 2019.262.IRB2.084) and complied with

the Declaration of Helsinki's ethical principles from 1975. Written informed consent was obtained from all parents and/or patients. Between 2019 and 2022, we recruited children for the different study groups. Group-1 consisted of naïve AIH patients with sequential follow-up samples after the standard IS regimen, consisting of Pred and Aza as per the guidelines (4, 12); Group-2 AIH patients are cross-sectional ones under treatment, and Group-3 AISC patients are also cross-sectional ones being treated. Group-4 children are recruited as age-matched healthy controls (with only functional constipation), Group-5 are patients with drug-induced liver injury (DILI), and Group-6 have had a liver transplant >6 months ago, have a protocol liver biopsy, and are treated with tacrolimus. Group-7 includes patients who underwent a partial hepatectomy due to hepatoblastoma, and Group-8 includes donors that had undergone surgery as living-related liver donors for their relatives/next of kin (2019.020.IRB2.019). Table 1 lists the demographics for each group, and Supplementary Figure 1 shows a schematic overview of each group. We collected the following data: gender, age, autoantibodies anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-soluble liver antibody (SLA), anti-mitochondrial antibody (AMA), liver kidney microsomal type-1 antibody (anti-LKM1), and liver cytosol antibody type 1 antibody (anti-LC1), perinuclear-anti neutrophil cytoplasmic antibodies (pANCA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), immunoglobulin G (IgG) (Table 1) from patient charts and electronic patient records. The diagnosis of AIH was made according to EASL AIH guidelines (12).

## Liver biopsy and fine needle aspiration

Children that underwent a liver biopsy, either for diagnostic or routine histological assessment, conform to the guidelines (13). The biopsy and fine needle aspiration (FNA) procedures were both ultrasound guided under general and local anesthesia. As we recently published, FNA was used to obtain intrahepatic lymphocytes (IHL) (14). The aspirate was then filtered through a 40µm filter and collected in cold phosphate-buffered saline (PBS) containing 2% FBS (Biowest) (14).

## Peripheral blood mononuclear cells isolation, cell staining, and gating strategy

To isolate peripheral blood mononuclear cells (PBMC), whole blood was diluted with PBS and layered onto Lymphoprep (GE), followed by density gradient centrifugation (15). Following a PBS wash, cells were resuspended in PBS, stained with viability dye EF780 (BD-eBioscience, ThermoFisher) before being incubated with cell surface antibodies specific for (regulatory) T-cells, mucosal-associated-invariant-T-cells (MAIT), (regulatory) B cells, and NK/NKT cells as previously described (7, 14–16). These antibodies are; CD3 (clone HIT3a), CD161 (clone HP-3G10), CD20 (clone 2H7), CD24 (clone ML5), CD25 (clone M-A251), CD38 (clone HIT2), CD4 (clone A161A1), CD45RA (clone HI100), CD56 (clone 5.1H11), CD8 (clone HIT8a), HLA-DR (clone L243), and TCR Valpha7.2 (clone 3C10) (Figure 1), and gating strategy is shown in Supplementary Figure 2. FOXP3 was determined by intranuclear staining with anti-FOXP3 (clone PCH101, eBioscience) and intracellular CTLA-4 was detected with anti-CTLA-4 (clone BNI3, Biolegend) after FOXP3 cellular fixation/permeabilization buffer (eBioscience) was used, followed by addition of intracellular permeabilization buffer (eBioscience). After washing with PBS, the cell pellet was resuspended in 300µL of PBS and passed through a multicolor flow cytometer (Attune-NxT, ThermoFisher). Analysis was performed using Flowjo (Treestar Inc, USA).

## Cell stimulation and *in vitro* immunosuppression with drugs

The PBMCs were first resuspended in complete cell medium (RPMI-1640 with L-glutamine and 0.5% penicillin/streptomycin (GIBCO), 0.5% amphotericin (Sigma-Aldrich), and 10% heat-inactivated fetal bovine serum) (GIBCO). These cells were then seeded onto a 96-well round-bottom plate at a density of 200,000 cells/200µL/well. The *in vitro* effects of IS drugs were explored by adding prednisolone, the active metabolite of azathioprine (6-MP), rapamycin, or tacrolimus (Sigma-Aldrich), at a final concentration of 10 nM, according to the published protocol (17). After 2 hours of incubation with IS drugs (18), cells with and without IS were stimulated for 6 hours at 37°C in the incubator with a cell activation cocktail (Biolegend) and recombinant human IL-2 (Biolegend) (100U/ml) (2). For intracellular cytokine staining, PBMCs were stained with cell surface antibodies staining B cells (CD20) and Bregs (CD20CD24CD38). Cells were fixated for 20 mins, washed with FBS, and the cell pellet was resuspended with intracellular permeabilization buffer (eBioscience), followed by anti-IL-10 (clone JES3-19F1) antibody incubation for 30 mins at 4°C. Thereafter, cells were washed with PBS and resuspended in 300µL of PBS, prior to flow cytometry.

## HLA determination

The routine tissue typing lab at Koç University Hospital used the Lifecodes HLA-DR eRES SSO typing kit (ref:628925) to

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TABLE 1 Demographics and clinical parameters.

| GROUPS Number (n) | AGE Months (IQR) | GENDER F/M | ALT<41IU (IQR) | AST<52IU (IQR) | GGT<18IU (IQR) | IgG5-15 g/L (IQR) | Presence of Autoantibodies |
|---|---|---|---|---|---|---|---|
| **Group-1: AIH NAIVE PATIENTS (7)** | 103 (50-221) | 4/3 | 180 (61-1119) | 64 (26-697) | 49 (25-116) | 15.8 (10-17) | Anti-ANA/SMA: 28.5% (2) Anti-LKM1/LC1: 57% (4) Seronegative: 14.3% (1) |
| **Group-2: AIH LONGTERM FOLLOW-UP (9)** | 192 (142.5-212) | 5/4 | 34 (32-50) | 38 (34-51) | 22 (13-29) | 13.7 (10.8-14.9) | Anti-ANA/SMA: 89.9% (8) Anti-LKM1/LC1: 0% (0) Seronegative: 11.1% (1) |
| **Group-3: AISC overlap (3)** | 204 (177-213) | 1/2 | 34 (15-63) | 34.5 (18-51) | 51 (28-88) | 10.8 (7.7-14) | Anti-ANA/SMA: 66% (2) pANCA: 100% (3) |
| **Group-4: HC PBMC (7)** | 91 (57-108) | 2/5 | 14 (13-15) | 25 (na) | 15 (na) | 9.56 (7.8-10.6) | na |
| **Group-5: DILI (3)** | 144 (52-204) | 1/2 | 386 (15-1457) | 97 (15-122) | 179 (14-413) | 8.8 (7.6-9.6) | 0% |
| **Group-6: LT (9)** | 72 (24-102) | 5/4 | 41 (25-77) | 46 (37-67) | 25 (13-68) | 12.9 (8.1-15.4) | na |
| **Group-7: HBL (3)** | 35 (26-174) | 2/1 | 32 (27-409 | 50 (50-290) | 24 (20-62) | 7.3 (4.9-13.9) | na |
| **Group-8: HC DONOR LIVER (8)** | 412 (262-444) | 5/3 | 13 (11-23) | 17 (14-22) | 16 (13-24) | na | na |

DILI, drug-induced liver injury; HBL, hepatoblastoma; PBMC, peripheral blood mononuclear cells; LT, liver transplanted patients; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpepdidase; IQR, interquartile range; IgG, immunoglobulin G; anti-ANA, anti-nuclear antibodies; anti-SMA, anti-smooth muscle antibody; anti-LKM1, anti liver kidney microsomal antibodies; anti-LC1, anti liver cytosol antibody; pANCA, perinuclear antineutrophil cytoplasmic antibodies; AIH, autoimmune hepatitis; AISC, autoimmune sclerosing cholangitis; na, not applicable.

genotype HLA DRB antigens. Data were collected on a Luminex platform, and allele allocation was performed by a trained lab technician using MATCH IT! DNA software.

## Statistics

Statistical analysis was performed using GraphPad 5. Normality was assessed with Kolmogorov-Smirnov Test. Comparisons between two groups were performed by student's t-test or Mann-Whitney U test. Multiple comparisons were analyzed by one-way analysis of variance or by Kruskal-Wallis, depending on the normality. For parametric variables, the standard error of the mean ( ± SEM) was provided, whereas, for non-parametric variables, we used the interquartile range (IQR). A p-value of <0.05 was considered significant.

# Results

## Peripheral blood CD8 T cells, activated Tregs, and MAIT cells were lower in treatment-naïve autoimmune hepatitis patients, compared to healthy controls

As depicted in Figure 1 and Supplementary Figure 2, we have investigated 17 cells (and subsets) in the peripheral blood of AIH patients. The frequency of CD3+ T cells, CD20+ B cells, CD4+ T cells, and naïve CD4 T cells was unaltered compared to PBMCs of HCs (Figures 2A, B). Total CD8+ T cells (21.1%) were significantly lower compared to HCs (35%), whereas these cells were more naïve compared to their counterparts (74.9% versus 46%) (Figures 2A, C). CD24+CD38- memory B cells were unaltered (Figure 2D). In terms of Treg homeostasis in

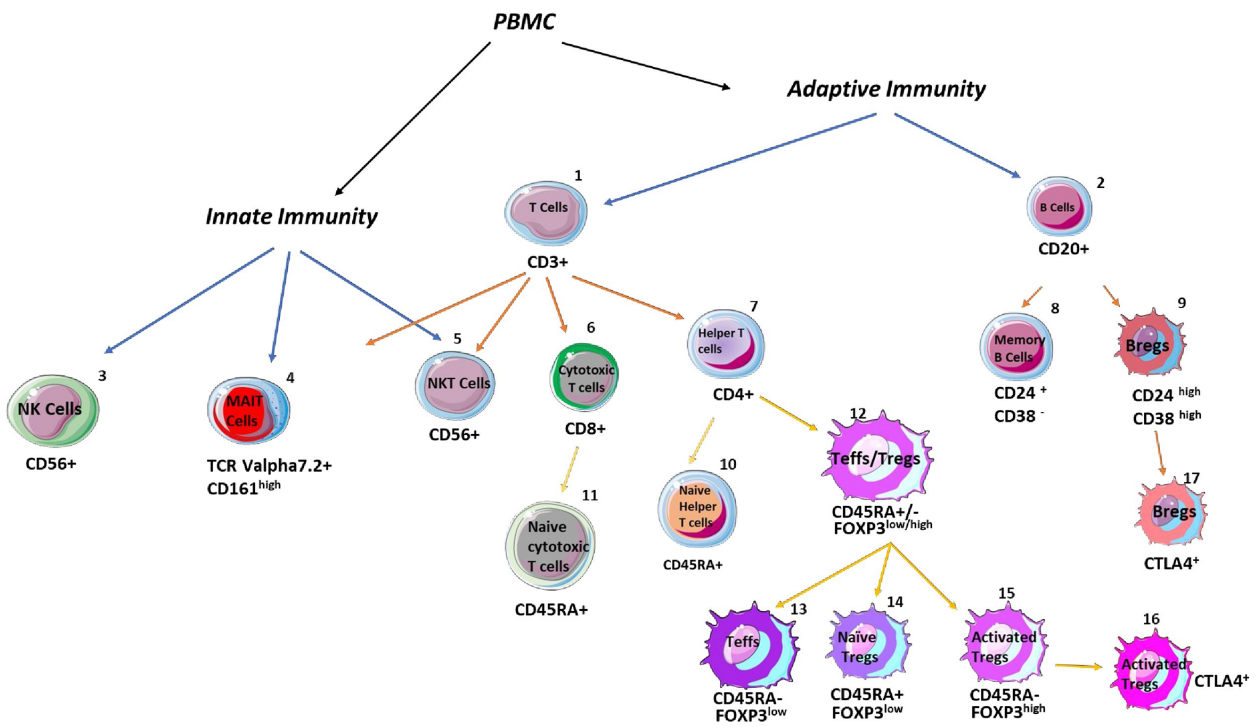


FIGURE 1
Schematic overview of analysed immune cells with their specific markers. The percentages of adaptive and innate immune cells were assessed in the blood and livers. The following CD markers were applied to define total T cells: CD3+CD20-, T helper cells: CD4+CD8-, cytotoxic T cells: CD8+CD4-, naïve T helper cells: CD4+CD45RA+, naïve cytotoxic T cells: CD8+CD45RA+, naïve regulatory T (Tregs) cells: CD4+CD45RA+FOXP3low, activated Tregs: CD4+CD45RA-FOXP3high, CTLA-4 positive Tregs: CD4+CD45RA-FOXP3highCTLA-4+, total B cells: CD3-CD20+, memory B cells: CD20 +CD24+CD38-, regulatory B (Bregs) cells: CD20+CD24highCD38high, CTLA-4 positive Bregs: CD20+CD24highCD38high, NK cells: CD3-CD56+, NKT cells: CD3+CD56+, and mucosal-associated invariant T cells: CD3+CD161+TCRVα7.2+.

peripheral blood, we found fewer activated Tregs ($CD4^+FOXP3^{high}CD45RA^-$) in treatment-naïve AIH patients than in HCs (0.56% versus 1.18%) (Figures 2E, F). Albeit, the proportions of naïve ($CD4^+FOXP3^{low}CD45RA^+$) and total (activated + naïve) Tregs were comparable between AIH and HCs. Furthermore, the ratio of total Tregs to effector T cells (Teffs: $CD4^+FOXP3^{low}CD45RA^-$) were similar between AIH patients (Supplementary Figure 3A) and HCs. Additionally, activated Tregs from treatment-naïve AIH patients expressing CTLA-4 were similar compared to HCs (24.1% versus 12.5%) (Figure 2G).

We also explored the proportions of innate immune cells such as MAITs (CD3+CD161+Vα7.2+), NK (CD3-CD56+), and NKT (CD3+CD56+) cells. Even though NK/NKT cell frequencies were indifferent between treatment-naïve AIH and HCs, MAIT cell frequencies in treatment-naïve AIH were much lower (0.54%) compared to HCs (2.7%) (Figures 2H–J).

Furthermore, treatment-naïve AIH patients had similar proportions of Bregs ($CD20^+CD24^{high}CD38^{high}$) in comparison to HCs (8% versus 6.3%) (Figures 2K–M). A significant proportion of Bregs expresses CTLA-4 (19). CTLA-4 + Bregs from treatment-naïve AIH (18.6%) were equally abundant as HC Bregs expressing CTLA-4 (19.1%) (Figures 2L, M).

## Immunophenotypic monitoring following IS treatments in the prospective group

The peripheral immune composition in the prospective treatment group was assessed after 1, 3, 6, 12, 18, and 24 months of standard IS therapy. We observed that the total T cells (CD3+), total B cells (CD20+) as well as CD4 T – and CD8 T cell frequencies, remained stable over the course of 24 months (Figure 3A). Regarding the activation status (CD45RA expression), the proportion of naïve CD4 T and CD8 T cells remained stable as well (Figures 3B, C). Nonetheless, memory B cells ($CD20^+CD24^+CD38^-$) significantly increased from 11.6% at diagnosis to 18.7% at 1 month and 22.9% at 12 months, remaining somewhat high thereafter (Figure 3D).

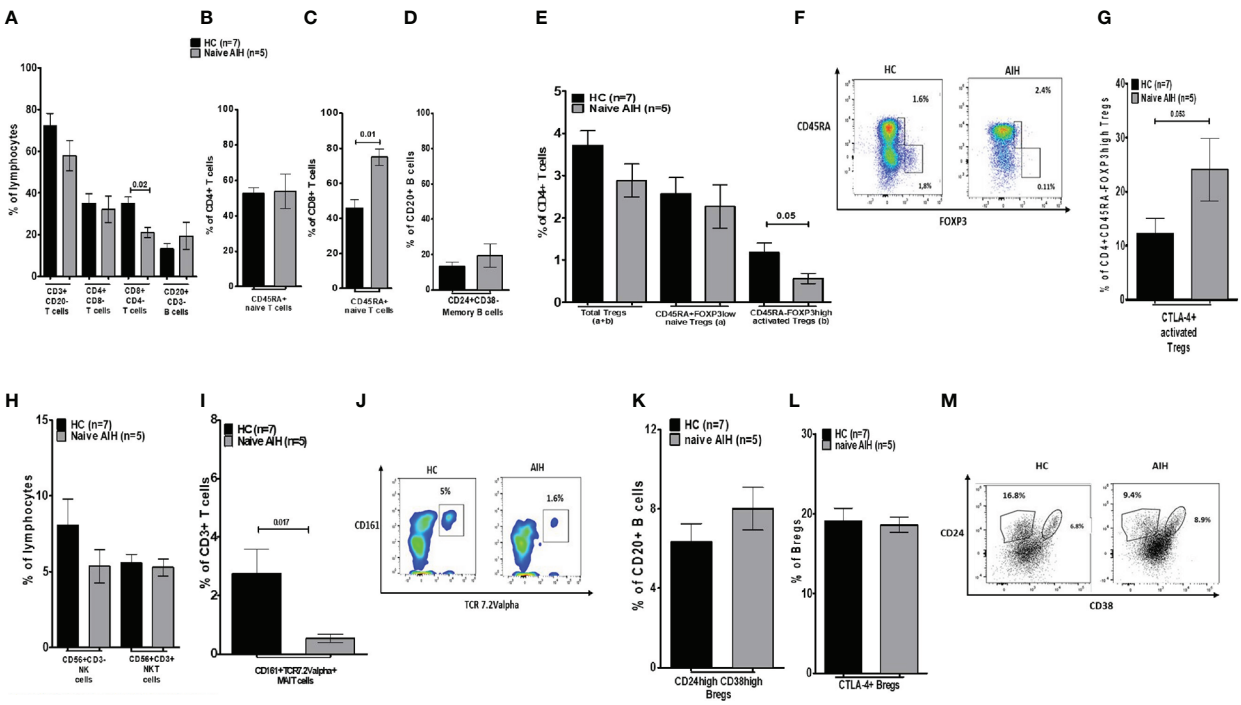


FIGURE 2
Analysis of the distribution of lymphocytes and their subsets in blood, in healthy controls and naïve autoimmune hepatitis patients. **(A)** Is a bar chart of the frequency of peripheral blood immune cells such as CD3, CD4, and CD8 T cells, and total CD20 B cells. **(B, C)** Show naïve CD4 and CD8 T cells. **(D)** Shows memory B cells. **(E)** Demonstrates the proportion of total, naïve, and activated Tregs. **(F)** Is a depiction of gating for Tregs. **(G)** Provides frequencies for CTLA-4+ Tregs. **(H)** Is a depiction of NK/NKT cell proportion. **(I)** Shows MAIT cells and **(J)** is showing the gating for MAIT cells. **(K)** Is a bar chart of Bregs and **(L)** shows CTLA-4 positive sub-group. **(M)** Depicts gating for memory B cells and Bregs. Five naïve AIH patients and seven healthy controls were included for this part of the study.

## Activated Tregs increased in long-term follow-up patients whereas Bregs remained low

The cross-sectional cohort who were >2 years on IS broadly followed the immune phenotypical trends observed in the prospective cohort (Figures 3A–D).

Next, we also investigated whether short/long-term IS affected Treg homeostasis. We demonstrated that the proportion of total Tregs fluctuated non-significantly for several months after IS. Nonetheless, the percentage of total Tregs fell at months 18, 24, and thereafter when compared to earlier time points. This was also observed in the naïve Tregs population, which was significantly less than 1.5% in the last three time points, compared to the first cohort's month 6 (3.7%) of IS treatment (Figure 3E). On the contrary, activated Tregs were significantly higher (1.17%) in the long-term IS group compared to month 3 of IS (Figure 3E). However, the proportion of activated Tregs that expresses CTLA-4 showed a significant reduction in the long-term group (Figure 3F). Furthermore, the ratio of Teffs to total Tregs in AIH patients was significantly higher than in HCs (Supplementary Figure 3A), indicating an unresolved immune dysregulation.

We also investigated the frequencies of NK/NKT cells and MAIT cells (Figures 3G, H). NK cells exhibited significant fluctuations during the different time points. NKT cell proportion was higher in the long-term cohort compared to baseline levels, and MAIT cell frequencies remained unaltered. Although the frequency of Bregs was 8.1% prior to treatment, following IS therapy, we observed a substantial reduction in Bregs to 0.75% at 6 months (Figures 3I, J). In the long-term cohort, Bregs remained significantly low (<2.5%) compared to treatment-naïve patients (Figures 3I, J). The expression of CTLA-4 on the few remaining Bregs could not be assessed due to the low number of events (data not shown).

In terms of comparisons between AIH-1 and AIH-2, we only found that at 3 months of IS, the frequency of NK cells was higher in AIH-2 (2.9%) than in AIH-1 (1.7, p=0.05). All other cell populations were similar (data not shown). The difference was unrelated to IS because all AIH-1 and AIH-2 patients received Aza+Pred at that time. Furthermore, AIH patients on long-term IS were compared to AISC patients and there were no

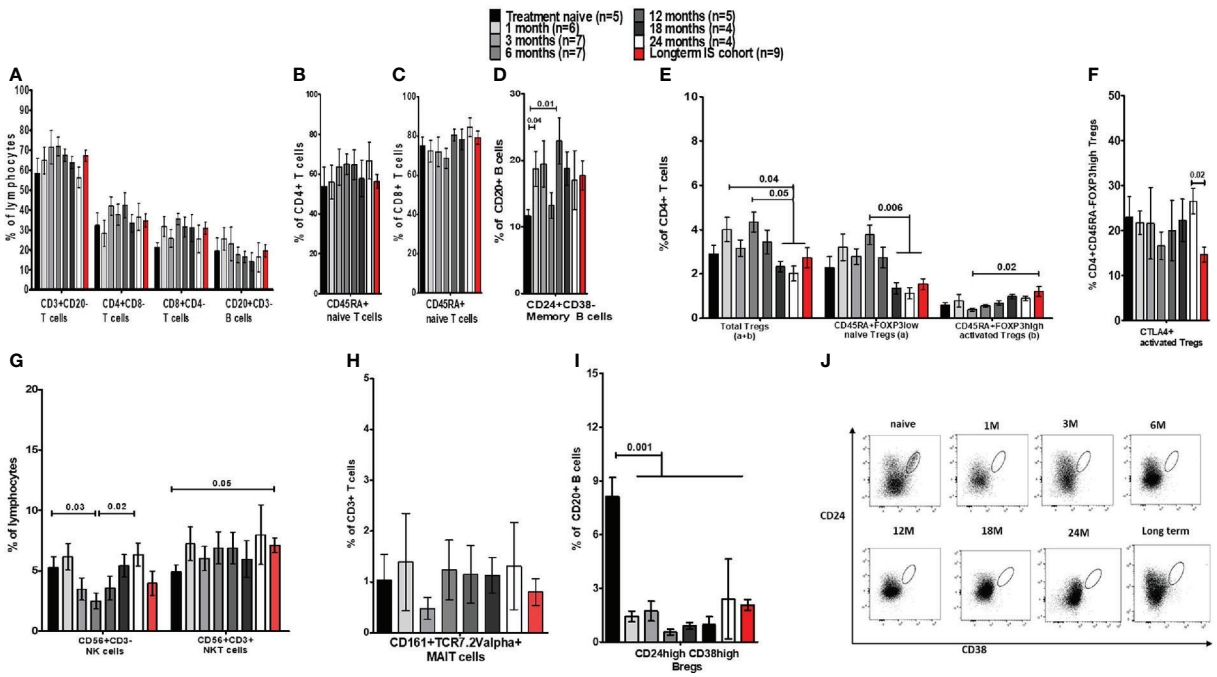


FIGURE 3
Frequency analysis of lymphocytes and their subsets in blood before and during immunosuppressive treatment of autoimmune hepatitis. **(A)** Is a bar chart of the frequency of peripheral blood immune cells such as CD3, CD4, and CD8 T cells, and total CD20 B cells from AIH patients who were either prospectively followed up from prior to IS until 24 months during treatment (prospective cohort) or assessed cross-sectionally (long-term cohort). **(B-D)** Show naïve CD4 and CD8 T cells, and memory B cells in these patients. **(E)** Demonstrates the proportion of total, naïve, and activated Tregs. **(F)** Provides frequencies for CTLA-4+ Tregs. **(G)** Provides frequencies of NK/NKT cells and **(H)** shows MAIT cells. **(I)** Is a bar chart of Bregs in these AIH patients. **(J)** Depicts gating for Bregs analysed at different time points.

differences observed between the two groups (Supplementary Figures 4A–I).

## More B cells, and fewer Tregs and Bregs in autoimmune hepatitis patients' liver

Following the immune profiling of PBMCs, we also aimed at elucidating the intrahepatic immune microenvironment. Intrahepatic lymphocytes were isolated from FNAs, during liver biopsies for the diagnosis of AIH (n=1) or the follow-up of AIH (n=3) or AISC (n=2). In contrast to CD3 T cells, we found that CD20 B cells were significantly enriched in the livers of AIH patients (9.0%) compared to HCs (2%) (Figure 4A). Other cell populations such as CD4 and CD8 T cells, and their CD45RA expressing subsets, as well as memory B cells, were similar (Figures 4A–D).

In terms of the intrahepatic Treg (sub)population, we found that naïve Tregs were more prevalent in AIH than HC. When compared to HCs (2%) and HTs (2.3%), the frequency of activated Tregs was significantly lower (1.0%) (Figure 4E). Furthermore, the Teffs/Tregs ratio in AIH livers was comparable between the groups (Supplementary Figure 5A). Additionally, the proportion of activated Tregs expressing CTLA-4 was significantly lower when compared to HC (Figure 4F). Moreover, the proportions of NK and NKT cells were unaffected, whereas the frequency of MAITs cells was significantly lower in AIH livers (2.9%), compared to HCs (8.5%) and HTs (10.3%) (Figures 4G, H). Complementary to what we saw in peripheral blood, the proportion of intrahepatic Bregs in AIH livers (2.9%) was significantly lower than in HCs (10%) and HTs (22.3%) (Figures 4I, J).

To determine whether the paucity of liver Bregs is caused by disease or treatment, we compared the intrahepatic proportion of AIH Bregs to the intrahepatic proportion of DILI (disease control) and transplanted children on tacrolimus. We found that both DILI and post-transplant patients on tacrolimus had significantly more intrahepatic Bregs, 12.9% and 13.6% respectively, than AIH patients (Figure 5A). Similarly, we wondered if the low proportion of intrahepatic Tregs was unique to AIH, or a result of treatment and on comparison, found out that intrahepatic activated Tregs were in fact similar in AIH and DILI (Figure 5B).

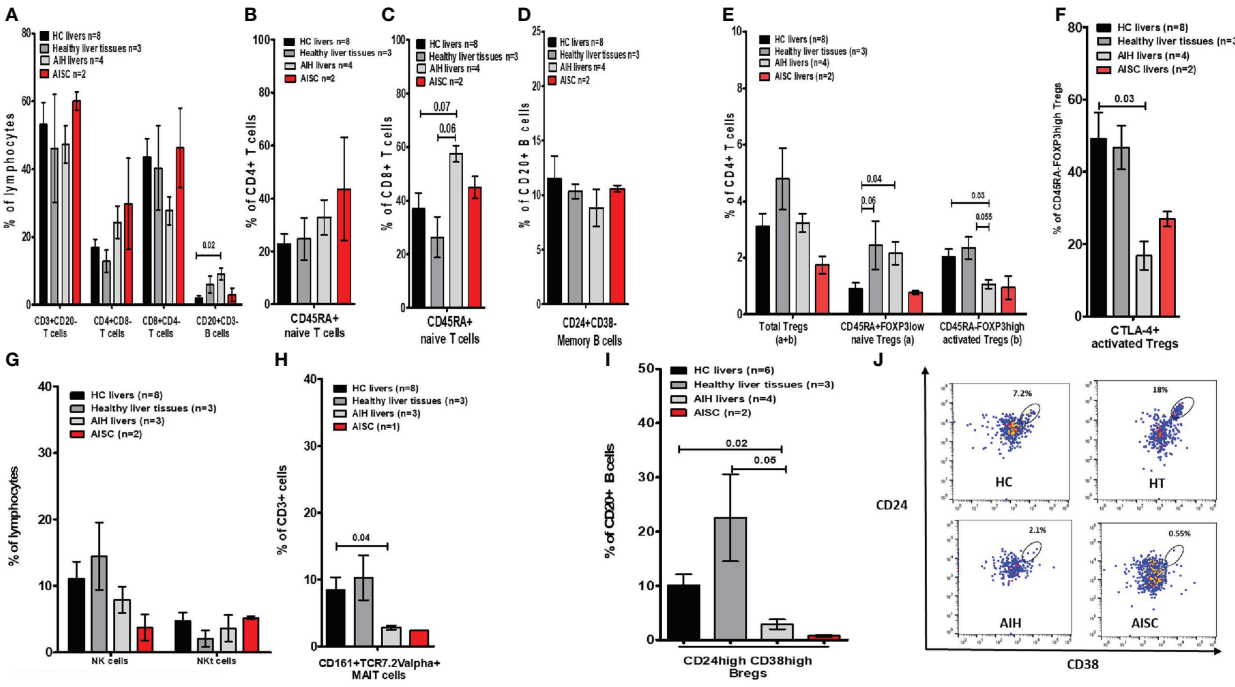


FIGURE 4
Frequency analysis of intrahepatic lymphocytes in autoimmune hepatitis, autoimmune sclerosing cholangitis, healthy donor livers, and age-matched healthy liver tissue. **(A)** Is a bar chart of the frequencies of intrahepatic immune cells such as CD3, CD4, and CD8 T cells, and total CD20 B cells. **(B, C)** Show naïve CD4 and CD8 T cells. **(D)** Shows memory B cells. **(E)** Demonstrates the proportion of total, naïve, and activated Tregs. **(F)** Provides frequencies for CTLA-4+ Tregs. **(G)** Is a depiction of NK/NKT cell proportions. **(H)** Shows MAIT cells. **(I)** Is a bar chart of Bregs and **(J)** shows gating for Bregs.

## Effect of immunosuppressive drugs on regulatory B cells

Finally, we wanted to know which IS drug in particular affected the frequency of Bregs. However, because we were short of PBMCs from naïve AIH patients, we used blood from HCs. We measured the frequency of total B cells, IL-10+ B cells, Bregs, and IL-10+ Bregs in PBMCs after incubating them with various IS drugs followed by a cytokine stimulation cocktail. In these *in vitro* conditions, the normalized ratios of total B cells and Bregs frequencies did not differ from baseline (no IS) levels (Figures 6A, B). Furthermore, when 6MP and Tac were used, the normalized ratio of IL-10 producing Bregs tended to be higher than at baseline (Figure 6C). Nonetheless, we found that 6MP and Tac significantly increased the normalized ratios of IL-10+ total B cells (Figure 6D and Supplementary Figure 6C).

## HLA-DR11 allele was prominent in AIH patients

Lastly, we also explored the frequency of HLA-DRB1 alleles in AIH patients. We found that HLA-DR11 was the most frequent allele in AIH-1 (70%) and AIH-2 (66%) patients (Supplementary Figures 7A, B). Alleles known to be associated with AIH in Caucasian AIH patients (DR3, DR4, DR7, DR13) had lower frequencies (Supplementary Figures 7A, B). We were also able to investigate the presence of the HLA-DRB1 allele in two AISC patients. One was DR3 homozygous, while the other was DR15 homozygous. We also compared ALT, AST, GGT levels, and immune profiles in DR11+/- patients but found no differences (data not shown). Due to the small sample size, it was not possible to assess patients' responses to IS (early remission, good response, or frequent relapse) according to their HLA-DRB1 allele.

# Discussion

The aetiopathology of AIH is only partially understood. It is characterized primarily by the activation of adaptive immunity and an aberration in immunoregulatory mechanisms. While T-cells play a central role in the pathogenesis by producing pro-inflammatory cytokines coinciding with Tregs alterations, data concerning the role of B-cells is elusive. However, their potential role could be important as B cells also contain a variety of

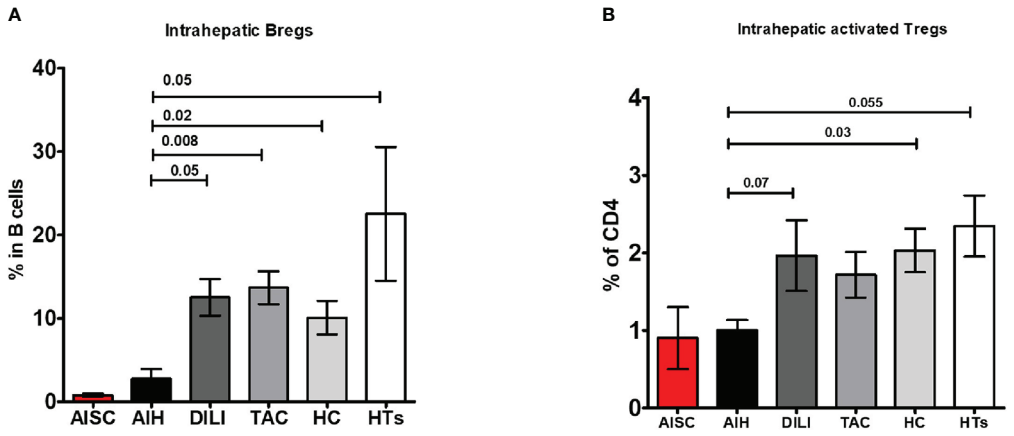


FIGURE 5
Frequency analysis of intrahepatic regulatory B cells and activated regulatory T cells. **(A, B)** Are bar graphs of intrahepatic Bregs and activated Tregs, respectively, in patients with AISC (n=2), AIH (n=4), drug-induced liver injury (DILI) (n=3), liver transplanted patients on tacrolimus monotherapy (n=9), healthy donor livers (n=6) and healthy tissue from hepatoblastoma patients (n=3).

immunoregulatory B cell subtypes such as CD24CD38 double-positive Bregs producing anti-inflammatory cytokines IL-10 and IL-35. Therefore, in this study, we reported on the findings of immune phenotyping of T, NK, NKT, and MAIT cells as well as B cells and their subtypes in treatment naïve, short- or long-term IS drug-treated children with AIH.

In essence, in the peripheral blood of naïve AIH patients, only total CD8 T cells and MAIT cells were significantly lower,

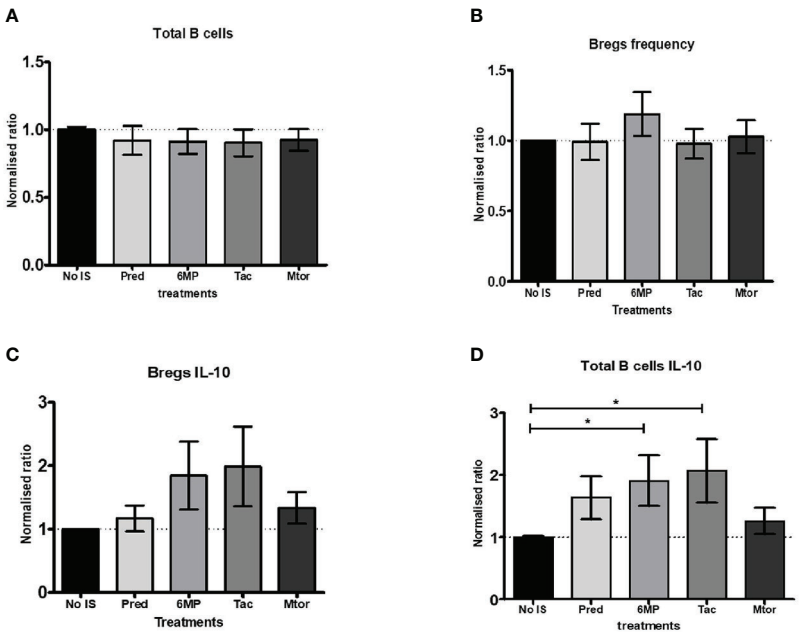


FIGURE 6
Analysis of *in vitro* effects of immunosuppressive drugs on B cells and Bregs producing IL-10. **(A, B)** Show bar charts of normalised total B cells and Bregs frequencies following incubation with medium only or with additional (10 mM) immunosuppressive drugs such as prednisolone, 6MP (active form of azathioprine), tacrolimus, or rapamycin (Mtor-inhibitor). **(C, D)** are bar charts of normalised total B cells and Bregs frequencies producing IL-10 under the same circumstances as in **(A, B)**. The PBMCs from seven (age-matched) healthy controls were included in this part of the study. *, 0.05.

whereas naïve CD8 T cells were more abundant compared to HCs. Regarding the immunoregulatory immune cell compartment, total Tregs and naïve Tregs were equally distributed in AIH and HCs. Yet, activated Tregs were lower in AIH compared to HCs. Importantly, the proportion of Bregs and CTLA-4+ Bregs was unaltered. After beginning IS medications, there were fluctuations, but only the frequency of memory B cells changed significantly from the starting point. Following the start of IS, there were marginally higher Treg frequencies (total and naïve), which subsequently started to decline after 18 months of IS in comparison to earlier time points. However, one cell population did not recover from IS treatment; the proportion of Bregs dropped sharply after IS and remained low throughout the entire investigation. Due to non-adherence, Bregs only returned in one patient (6.9%), which accounts for the significant variation at the 24-month mark. Regarding the hepatic immune microenvironment, naïve CD8 T cells and total B cells were higher in AIH. Furthermore, not total nor naïve but activated Tregs in AIH were lower than HCs. In AIH livers, MAIT cells were also less frequent. Yet again, Bregs were lower in AIH livers.

Few studies have been conducted to date, mostly on adults, to better understand how IS medications affect systemic and/or intrahepatic lymphocytes in patients with AIH (3, 10, 20–22). Most notably, by means of immunohistochemistry (IHC), Diestelhorst et al. noted a sharp decline (>50%) in intrahepatic Tregs proportions, following IS in children with AIH. However, it is unclear how this decline will affect long-term treatment outcomes in these children. The same group also investigated hepatic Treg homeostasis in adult AIH (20). There, they found that following IS, hepatic Tregs were reduced, especially in patients with incomplete remission (IR). Importantly, the baseline (treatment naïve) hepatic Treg proportions in these IR patients were not lower compared to patients that would become biochemical responders (BR) after IS. This demonstrates that IR is not necessarily the consequence of any initial Treg numerical impairment, at least when compared to the BR patients. However, as they used IHC, further delineation of Tregs subtypes was not possible. Our flow cytometric analysis of intrahepatic Tregs demonstrated that the frequency of total Tregs was indifferent compared to HCs and age-matched HTs. Contrarily, naïve (Sakaguchi type I Tregs (16)) Tregs were higher in AIH livers only when compared to adult livers. Yet, Sakaguchi type II Tregs (16) (activated Tregs) were lower in AIH compared to both HC/HT livers and expressed less CTLA-4. This supports the notion that a detailed Tregs subgroup analysis and selecting appropriate control groups are incumbent for a valid comparison. Additionally, this is the first report of CTLA-4 expression on intrahepatic Tregs. Nonetheless, one recent paper did report the intrahepatic CTLA-4 expression on total CD4 T and CD8 T cells within the liver. The proportion of CTLA-4 was similar between normal livers and adult AIH livers, whether they were under treatment or not (22).

Multiparametric flow cytometry also permitted us to investigate unconventional T cells, such as MAIT cells. In blood, the proportion of MAIT cells was lower at baseline and did not recover after IS treatment in pediatric AIH. Hepatic MAIT cell frequency was also low compared to HC. Similar results were noted recently, by two unrelated investigations in adult AIH patients (3, 23). Moreover, they found that these MAIT cells expressed similar or higher levels of granzyme-B, but similar proportions of IFN-γ and TNF-α were detected in another study. In this study, CTLA-4 positivity was also higher in MAIT cells. Combined, it was proposed that MAIT cells were exhausted in AIH (24).

B cells, including memory and plasma cells, are involved in AIH humoral immunity (9). In our study, systemic total B cell frequency was similar at baseline and remained as such following IS. In the liver, B cell proportions were higher in AIH patients compared to controls. However, these samples were from cross-sectionally assessed patients. It was not possible to explore the role of IS on hepatic B cells. Nonetheless, several groups clearly demonstrated that, by IHC, intrahepatic B cell numbers and or proportions decline sharply following IS (10, 20, 22), which was even bigger compared to the CD4 and CD8 cell number reductions. This may cause to question of whether or not B cells are incumbent as a "driver" in the pathogenesis of AIH (25).

One of the novel findings of this study is the alteration in Bregs in the periphery and the liver in AIH/AISC. They are indispensable in the maintenance of tolerance and immune homeostasis, despite representing fewer than 10% of total B cells in the circulation in healthy individuals (26–29). Even more, numerical and/or functional impairments in CD24+CD38+ or CD24+CD27+ Bregs were also implicated in autoimmunity (rheumatoid arthritis, psoriasis, systemic sclerosis), viral infection (hepatitis B virus), and allergy (allergic rhinitis) (30). The involvement of Bregs in AIH is currently unknown (25). Nonetheless, in primary biliary cholangitis (PBC), it was demonstrated that the frequency of peripheral Bregs was significantly elevated in PBC patients compared to HCs, advocating against a numerical impairment (31). These Bregs expressed less T cell immunoglobulin mucin domain-1 (Tim-1), which has immunoregulatory properties. Previously, it was shown that a variant of it, Tim-3, was also downregulated in T cells in AIH (32). In our study, the proportion of peripheral Bregs at diagnosis demonstrated no difference compared to HCs. The CTLA-4+ proportion within Bregs was also unaltered. However, following the initiation of IS treatment, Bregs' proportion decreased sharply in the prospective cohort and remained low in the longitudinal cohort. Hence, Breg homeostasis is deranged. Peripheral

paucity was also reflected in the liver. The frequency of hepatic Bregs was lower compared to HC, HTs, and disease/treatment controls. Actually, this is not the first report of peripheral Bregs scarcity caused by IS treatment in AIH patients. Our group had the unique opportunity to monitor a child with recent onset of AIH who also developed COVID-19 disease (33). As we were screening the immune system for potential changes owing to this infection, we found that starting IS severely reduced Bregs in the blood, which did not recover during the observation period. The current investigation allowed us to confirm this phenomenon. Another study found that recipients of allografts with plasma cell hepatitis who received multiple IS also had significantly fewer Bregs and IL-10+ B cells in their blood than HCs (29). Furthermore, we also wondered whether a particular IS drug was to blame for the Bregs decline. In our Tac treated transplanted cohort, Bregs frequency was not much affected compared to our healthy controls and disease control (DILI). Our *in vitro* results did not particularly show a decline of Bregs frequencies after IS incubation of healthy PBMCs. Actually, the results from our *in vitro* data are in contradiction with our data where treatment with IS clearly caused a drop in Breg frequencies in all patients and at all time points. Bregs (transitional B cells) continue their maturation process in the secondary lymphoid organs such as the spleen and lymph nodes (26, 27, 29, 30). Furthermore, the homing of B cells between secondary lymphoid organs and (inflamed) tissues is regulated by chemokine receptors such as CXCR5, CCR7, CXCR3, CCR1, and CCR5 (34). Similarly, another study about Bregs in plasma cell hepatitis patients found that these cells express integrins (CD11a, CD11b, α1, α4, and β1) (29). It is possible that the expression of all these markers and thereby the homing capacity of Bregs are affected by IS, potentially explaining why Bregs in the blood almost disappeared. A recent study demonstrated that BCR signaling was affected following 4 hours of *in vitro* incubation with methylprednisolone (35). However, whether this also affected homing markers on B cells/Bregs requires further investigation. Additionally, we found that Tac and 6MP increased the frequency of IL-10+ B cells. Similar trends were also noted in IL-10+ Bregs. Yet, the aforementioned study also found that methylprednisolone stimulated the expression of IL-10 mRNA in B cells (35). It seems that IS drugs have an effect on B cell/Breg homeostasis and on their IL-10 production.

Lastly, we also found that not HLA-DR3, DR4, DR7, nor DR13, but HLA-DR11 is the most frequent allele in AIH patients. These results are not compatible with earlier studies in Caucasian AIH patients. However, this is not entirely surprising as the ancestry of the current Turkish population has roots mainly in central Asia. Yet, two Turkish studies investigated the HLA-DRB1 allele frequency in children or adult AIH patients (36, 37). In adults, they found that 58% of patients had DR4 with the DR3 frequency being similar to our population 29%. However, the study in children did find that HLA-DR11 was one of the most common alleles. However, mechanistically, it is still unknown why DR11 may be linked to AIH in Turkish patients. One intriguing avenue of investigation is computational modeling, which could shed light on HLA-DR11 characteristics such as autoantigen binding capacity and stability mediated by HLA-DM (38). For example, peptide binding is regulated by the charge and the three-dimensional structures of amino acids (AA) constituting the antigen-binding pockets in the peptide binding groove. One such pocket is formed by HLA-DRβ chain AAs between positions 67 and 72. These AA encode LLEQKR (DR3) and LLEQRR (DR4) motifs (5, 39, 40). Indeed, we found that HLA-DR11 expresses FLEDRR, which is 100% similar to DR3/DR4 based on the charge of the AAs. Additionally, the AA at position 71, lysine (K) or arginine (R), is paramount in conferring susceptibility to AIH as HLA-DR15 (protective against AIH) differs at AA position 71 encoding alanine (uncharged) (41). HLA-DR11 has also arginine at position 71, potentially explaining why HLA-DR11 is prevalent in our AIH cohort.

There are also limitations in our study. The sample size of the prospective AIH Group-1 is small owing to the fact that AIH is a very rare disease. The serial assessment of AIH livers before and during IS was not always possible because the initial diagnosis and commencement of IS may have already started by the time patients were referred to our tertiary center. Furthermore, children donate a limited amount of blood. By virtue, functional assays such as the baseline and/or *in vitro* IL-10 production in Bregs and total B cells following IS could not be performed in these patients alongside deep peripheral immune phenotyping. Instead, we used age-matched HCs. Lastly, there are several Breg populations such as CD20+CD24$^{hi}$CD27+ memory B cells and CD20$^{low}$CD38+CD27$^{low}$ plasmablasts among others (26). Their individual susceptibility to IS is unknown, which is a matter for further investigation. In adults, they found that 58% of patientshad DR4 with the DR3 frequency being similar to our population 29%. However, the study in children did find that HLA-DR11 was one of the most common alleles.

In conclusion, major findings in the peripheral blood of naïve AIH patients were: fewer activated Tregs and MAIT cells with preserved Breg frequencies. Following IS, most cell frequencies fluctuated, with total and naïve Tregs decreasing over time. Even more, the proportion of Bregs dropped sharply. The paucity of Bregs was also observed in the livers. However, *in vitro* IS did not affect the proportion of Bregs but increased IL-10 + total B cells, instead. Importantly, the current findings of Bregs scarcity need to be investigated to comprehend what the potential clinical implications (good response to IS, survival, and liver transplantation) are. Further research into the role of DR11 in AIH is also warranted in larger AIH cohorts that include local health subjects.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by Koç University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

ÇA and MY participated in the study conception and design. ÇA, MY, FN, EP, and DW were involved in the acquisition of samples, analysis, and interpretation of data. ÇA, MY, FN, and MA drafted the manuscript, and also provided critical revisions. SA overviewed HLA DRB1 allele typing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1053216/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Schematic overview of the different groups, time points, and tissues assessed in this study **(A)** Shows the assessment time points for the prospective cohort of naïve AIH patients. **(B)** Shows the different groups used as disease control, treatment control, or healthy control.

SUPPLEMENTARY FIGURE 2
Gating strategy for all immune cells and their subsets. This figure sets out in detail all parent gates and used CD markers defining the mentioned cells and their subsets, starting from FSC and SSC lymphocyte gating. Specifically, we show gating for total B cells, total T cells, CD4 and CD8 T cells, memory B cells and Bregs. Within the Bregs, we also gate for CTLA-4. Furthermore, naïve CD4 and CD8 T cells are shown. Both naïve and activated Tregs are gated as well with CTLA-4 gating for activated Tregs. Activated Tregs are also gated for their CD25 expression. Lastly, the gating strategy for NK and NKT cells as well as MAIT cells, are provided.

SUPPLEMENTARY FIGURE 3
Analysis of ratios of effector T cells to total Tregs. **(A)** Is a bar chart of the ratio of effector T cells to total Tregs in healthy control and AIH patients before or following treatment.

SUPPLEMENTARY FIGURE 4
Comparisons between autoimmune sclerosing cholangitis and patients with autoimmune hepatitis **(A)** Is a bar chart of the frequency of peripheral blood immune cells such as total T cells, total B cells, CD4 T cells, CD8 T cells, naïve (CD45RA+) CD4 and CD8 T cells, and memory B cells. **(B)** Demonstrates the proportion of total, naïve, and activated (with or without CTLA-4) Tregs. **(C)** Provides frequencies of NK/NKT cells and MAIT cells. **(D)** Is a bar chart of Bregs.

SUPPLEMENTARY FIGURE 5
Analysis of ratios of effector T cells to total Tregs in healthy livers, healthy liver tissue, autoimmune sclerosing cholangitis, and patients with autoimmune hepatitis. **(A)** Is a bar chart of the ratio of effector T cells to total Tregs in the livers.

SUPPLEMENTARY FIGURE 6
Flow cytometry dot plots of total B cells and Bregs producing IL-10. **(A, B)** demonstrates gating for total B cells and Bregs. **(C, D)** shows gating for B cells and Bregs producing IL-10 in healthy controls under various *in vitro* (immunosuppressive) conditions. **(E, F)** are FMOs for total B cells and Bregs, respectively.

SUPPLEMENTARY FIGURE 7
Analysis of HLA-DRB1 allele distribution. **(A)** Demonstrates the HLA-DRB1 allele frequency in AIH-1. **(B)** Demonstrates the HLA-DRB1 allele frequency in AIH-2.

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# HLA and Non-HLA gene polymorphisms in autoimmune hepatitis patients of North Indian adults

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Autoimmune hepatitis (AIH) is a chronic and progressive disease of the liver. This is a multifactorial autoimmune disease with both environmental factors and genetic factors playing a role in its pathogenesis. Certain environmental agents like viruses, drugs, etc., can trigger the disease in a genetically susceptible individual. The present study was aimed to explore the distribution of human leukocyte antigen (HLA)-DRB1, Protein tyrosine phosphatase non-receptor type 22 (PTPN22) and Cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4) polymorphisms in North Indian adult AIH patients and their associations with clinical and pathological characteristics associated with the disease. A total of 147 subjects with 47 cases and 100 healthy controls were enrolled. Diagnosis of AIH was made by Revised International Autoimmune Hepatitis Group scoring system. HLA-DRB1 Typing was done by Luminex-based reverse Sequence-Specific Oligonucleotide Probing (SSOP). Single nucleotide variant (SNV) genotyping for CTLA-4 and PTPN22 was done by simple probe-based SNP arrays. Results indicated SLA positive AIH patients are poor responders to therapy. A significant predispositional association of HLA-DRB1*03 was observed in AIH patients from the North Indian population (p= 0.0001, OR=4.83 (2.30-10.15). The frequency of the GG genotype of CTLA-4 CT 60 was significantly increased in AIH patients compared to controls. Multinomial analysis showed that CTLA-4 CT 60 is an independent predictor for cases.

KEYWORDS

**autoimmune hepatitis, adults, HLA, CTLA-4, PTPN22, polymorphisms, SLA**

# Introduction

Autoimmune hepatitis is a chronic and progressive disease of the liver. Patients present with varied symptoms ranging from asymptomatic to vague symptoms like arthralgia and/or fatigue to acute hepatitis-like presentation. Thus, it is often misdiagnosed initially. In India, all the centres do not have the facility for serological autoimmune workup, and around one-third of the patients have liver cirrhosis at the time of diagnosis (1). AIH patients respond to immunosuppression, emphasizing the importance of its early diagnosis and understanding its immunopathogenesis.

This is a multifactorial autoimmune disease with environmental and genetic factors playing a role in its pathogenesis. Certain environmental agents like viruses, drugs, etc., can trigger the disease in a genetically susceptible individual. Susceptibility to type 1 AIH (AIH-1), which is characterized by anti-nuclear (ANA) and/or smooth muscle antibodies (SMA), has been linked to MHC class II human leukocyte antigen (HLA) DRB1 alleles and an evident ethnic variation is seen in these associations (2–4). The structure of the surface binding groove of the class-II MHC molecule is determined by the HLA allele, which confers susceptibility to certain diseases. Different alleles of HLA play a role as a risk factor or a protective factor in different individuals. The HLA-DRB1 alleles HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB1*04:04 and HLA-DRB1*04:05 confer susceptibility to develop AIH-1 in different populations. These alleles express proteins which have similar 6 amino acid sequences (LLEQR or LLEQRR) at position 67 to 72 in the antigen-binding groove of Class-II MHC molecule (5). Among Asians, different studies have found an association between HLA-DR4 in East Asian and HLA-DR3 in South Asian populations with AIH-1 (6).

Cytotoxic T lymphocyte-associated protein 4 *(*CTLA-4*)*, a widely studied non-HLA susceptibility gene in autoimmune disorders, is mainly expressed on the surface of regulatory T cells and conventional T cells and suppresses self-reactive T cells responses *via* downregulating ligand availability for the costimulatory receptor CD28 to elicit inhibitory signals. Single nucleotide variant (SNV) in CTLA-4 CT60 +6230G/A has been shown to be associated with reduced sCTLA-4 and higher risk for acute rejection in allogenic liver transplant patients (7). While another non-HLA gene, Protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), encodes the protein lymphoid tyrosine phosphatase (LYP), which regulates the activation of protein kinases to modulate intracellular tyrosine phosphorylation incidence and act as a regulator of molecular signal transduction (8). PTPN22 -C1858T is the most studied SNV in the field of autoimmunity (9).

Since AIH is a rare disease and it is quite challenging to enlarge the sample size for the Genome Wide Association Study (GWAS). There has been only one GWAS aimed at AIH in the literature till now. This GWAS, performed on the European population, demonstrate significant associations of SNVs in HLA and some of the non-HLA genes. The three main non-HLA genes in concern were SH2B3 (Src Homology 2 Adapter Protein 3), CARD10 (Caspase Recruitment Domain Family member 10) and ICOS (Inducible co-stimulator). The other genes, such as STAT4, IL-12A, IL-12RB, BACH2 and CTLA-4/CD28, were described as nominally significant (2). After this GWAS, several major attempts have been made to delineate the genetic architecture and its contribution to disease pathogenesis (3, 4). However, only a limited number of studies have explored the non-HLA association with AIH.

From India, there is no study regarding the non-HLA gene associations with the disease, and only a few studies have demonstrated the HLA-DRB1 association in the same disease. Within the same country, studies have shown variability in the association between HLA alleles and AIH (10, 11). Therefore, we conducted a case-control association study examining eight non-HLA candidate loci (3 SNVs for PTPN22 rs2476601, rs1217412, rs2488457 and 5 for CTLA-4 rs3087243, rs231775, rs5742909, rs4553808 and rs733618) in north Indian adult AIH patients. We also performed an HLA-DRB1 association analysis in these patients. We compared HLA and non-HLA associations with disease outcome and treatment response and also compared cases with or without Anti-SLA antibodies (Antibodies against soluble liver antigen/liver-pancreas) with treatment outcomes and if any of the genetic components correlated with the clinical parameters of the disease.

# Materials and methods

## Study population

A total of 147 subjects were recruited, with 47 cases and 100 healthy controls. Patients were recruited from the Department of Hepatology of PGIMER, Chandigarh. Diagnosis of AIH was made by Revised International Autoimmune Hepatitis group scoring system for diagnosis of AIH (12). Patients diagnosed with type-2 AIH who were positive for LKM1 and/or LC1, AIH-PSC overlap, AIH-Autoimmune sclerosing cholangitis and AIH-PBC overlap were excluded from the study.

The controls were age, gender, and ethnicity matched with the patients and were not suffering from any disease. A written informed consent was obtained from all the participants after explaining the aims of the study. The study was approved by institutional ethics committee of PGIMER (INT/IEC/2018/000795). Clinical details were taken from the patients and their Liver clinic outpatient record files.

## Sample collection and autoantibody screening

Five millilitres (ml) of peripheral venous blood sample was obtained from the patients and was divided into two portions: 3 ml in plain vials for serum separation and 2 ml in EDTA vials for DNA extraction. AMA, SMA, ANA, LKM and LC-1 were detected in the serum of patients by indirect immunofluorescence (IIF) and confirmed by Immunoblot (Euroimmune) and ELISA except for SMA. SLA ELISA was performed using QUANTA Lite® SLA ELISA kit by Inova Diagnostics. Patients showing positivity for Anti-LKM/Anti-LC-1 antibody were excluded from our study. We had excluded two such patients from our study (positive for Anti-LKM antibody) as there is different genetic susceptibility for AIH-1 and AIH-2, thus we had enrolled adult Non-type 2 AIH cases (13).

## DNA extraction, HLA and non-HLA genotyping

DNA extraction was done using spin column-based method (QIAmp DNA, Qiagen). HLA-DRB1 typing was done by Luminex-based reverse Sequence-Specific Oligonucleotide Probing (SSOP) (for details, see Supplementary File S1). Due to constraint resources, we had performed low-resolution HLA typing.

SNV genotyping was done by simple probe-based SNP arrays (Roche Diagnostics) for the following genes - CTLA-4 [+49 A/G (rs 231775), -318 C/T (rs 5742909), CT 60 (+6230G/A, rs3087243), -1722 C/T (rs 733618) and -1661 A/G (rs 4553808)] and PTPN22 [+2740 A/G (rs 1217412), -1123 C/G/T (rs 2488457) and +1858 C/T (rs 2476601)] (for details see Supplementary File S1). We had chosen these 8 SNPs based on the GWAS on AIH and other autoimmune diseases. Both of them are negative regulators of T cells and present knowledge underlines the understanding that polymorphisms in these two genes are associated with loss of tolerance, autoimmunity and also susceptibility to cancers (2–4).

## Statistical analysis

Data are expressed as mean with standard deviation and median with interquartile range, as applicable. Allelic and genotypic frequencies were compared between patients and controls using $\chi 2$ test with Yates' correction. Calculations of odds ratios (ORs) and 95% confidence intervals (CIs) for relative risks were performed after the application of Fisher's exact test, if appropriate. Two-sided *P* values less than 0.05 were considered significant. The analysis was conducted using IBM SPSS STATISTICS (version 22.0).

# Results

## Clinical characteristics of the study Participants

There was a female preponderance with female: male ratio of 2.6:1. Patients had a variable presentation of the disease with a mean age at the time of presentation 32.82 years (SD ±13.6). One-third of our patients (n=16) had acute presentation (Acute presentation was categorized as a cut-off of bilirubin >2 mg/dl and ALT or AST levels >10 times the Upper Limit of Normal) (14). The baseline characteristics of AIH patients are given in Table 1. Histopathological assessment of liver biopsy was done in 41 cases. The characteristic histopathological features are shown in Figure 1. In all the 41 cases, significant interface hepatitis was present; in 29 cases, lymphoplasmacytic infiltration in the portal tracts was seen, 25 cases had hepatocyte rosetting, and 3 cases had emperipolesis. None of the cases showed any biliary changes. 42.55% (n=20) of the patients had liver cirrhosis at the time of presentation at our institute. In the cases where liver biopsy was not available or was inadequate for opinion, Fibroscan (Transient Elastography) reports were considered for the status of fibrosis. A cut-off of 12.67 kPa was taken for Cirrhosis (15). The liver biopsy fibrosis scoring was done by the French Metavir scoring system (16). 42.5% (n=20) of our patients had concurrent other autoimmune diseases, with the most common being Hashimotos thyroiditis (n=11, 23.4%) followed by Celiac disease (n=2, 4.25%). The patients of Hashimotos thyroiditis had raised levels of serum TSH and Anti-TPO antibodies at the time of diagnosis.

All the patients were given immunosuppression; n=25 (53.2%) showed complete response, n=16 (34%) showed an incomplete response and n=5 (10.6%) showed relapse after discontinuation of therapy. Thirty seven cases (78.72%) were reactive for autoantibodies, namely ANA and/or SMA and/or SLA. Nine patients (19.15%) were seronegative. Among the nine seronegative patients, 4 were diagnosed as definite AIH and rest of the 5 patients were diagnosed as probable AIH as per Revised IAIHG criteria. Majority of these patients had shown complete response to immunosuppression (n=7, 77.7%) with only two cases with incomplete response and none with relapse. Two out of these nine patients had cirrhosis at the time of presentation in our institute. One patient was a female who presented at the age of 58 years with insidious onset. Her serum IgG levels were raised (1.43 times the upper normal limit), her liver biopsy showed significant interface hepatitis along with F4 fibrosis (Metavir scoring system) and she showed complete response

on immunosuppression. She was heterozygous for HLA-DRB1*03 and had associated Hypothyroidism. The other patient was a 18 years old male who also presented with insidious onset. He had raised serum IgG levels (1.8 times the upper normal limit) and his liver biopsy showed significant interface hepatitis and moderate lymphoplasmacytic inflammation in the portal tracts along with F4 fibrosis (Metavir scoring system). He was also heterozygous for HLA-DRB1*03. However, he showed incomplete response on immunosupression and was advised for liver transplantation. There was no history of alcohol intake or hepatotoxic drug intake in both of these patients and viral markers were negative.

We observed that Anti-SLA ELISA positive cases were poor responders to immunosuppressive therapy, with no case showing complete response compared to 62.5% in cases without Anti-SLA autoantibodies ($\chi 2$ = 8.97, p = 0.004) (Table 2). Strength of association between the two variables (Cramer's V) = 0.442 (Relatively strong Association). Strength of association between the two variables (Bias Corrected Cramer's V) = 0.392 (Moderate Association).

## HLA-DRB1 allele susceptibility

The allele frequency of DRB1*03 was significantly higher in AIH patients as compared to healthy controls (P = 0.0001, OR = 4.83, 95% CI = 2.30 – 10.15, P-value with Bonferroni correction [Pc] = 0.001). The frequencies of the alleles obtained in the patients and controls are shown in Table 3. We also tested by discriminating allele frequencies in different sub-group of patients, categorized on the basis of their clinical features, presence of cirrhosis and response to immunosuppression.

TABLE 1 Baseline characteristics of AIH-1 patients.

| Parameters | |
|---|---|
| Age (Years) | 36.56 ± 13.50 (Mean ± SD) |
| Female: Male | 2.6:1 |
| Time Of Presentation (Age In Years) | 36.34 ± 13.4 (Mean ± SD) |
| Acute presentation of disease | n= 16 (34.04%) |
| Cirrhosis* | n= 20 (42.5%) |
| AST (U/L) | 166 (86-501) Median (IQR) |
| ALT (U/L) | 150.6 (72-342) Median (IQR) |
| ALP (U/L) | 192 (135.3-264) Median (IQR) |
| Total Bilirubin (mg/dL) | 2.8 (1.17-5.50) Median (IQR) |
| Total Protein | 7.60 (6.70-8.8) Median (IQR) |
| Albumin | 3.6 (2.99-4.06) Median (IQR) |
| Serum IgG | |
| WNL | n= 9 (19.14%) |
| Raised | n= 38 (80.85%) |
| Serum IgG (times of Upper normal limit) | 1.43 (1.24-1.87) Median (IQR) |
| Serum profile | |
| ANA | n= 18 (38.3%) |
| SMA | n= 21 (44.7%) |
| ANA & SMA | n= 3 (6.3%) |
| SLA | n= 7 (14.9%) |
| SLA & ANA | n= 1 (2.1%) |
| SLA & SMA | n= 4 (8.5%) |
| Seronegative | n= 9 (19.15%) |

*(Continued)*

TABLE 1 Continued

| Parameters | |
|---|---|
| Other Autoimmune diseases | n= 20 (42.5%) |
| **Liver biopsy histopathological findings** | |
| Significant interface hepatitis | n= 41 (87.23%) |
| Lymphoplasmacytic infiltration | n= 29 (61.7%) |
| Hepatocyte resetting | n= 26 (55.32%) |
| Emperipolesis | n= 3 (6.4%) |
| **Diagnosis (Revised IAIHG)** | |
| Probable AIH | n= 11 (23.4%) |
| Definite AIH | n= 36 (76.59%) |
| **Response To Treatment**** | |
| Complete Response | n= 25 (53.2%) |
| Incomplete Response | n= 16 (34.04%) |
| Relapse | n= 5 (10.6%) |

* In two cases both fibroscan and liver biopsy were not available, **one case was lost to follow-up.
AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; U/L, IQR, Interquartile Range; SD, Standard Deviation; IAIHG, International Autoimmune Hepatitis Group; AIH, Autoimmune Hepatitis; WNL, Within Normal Limit.

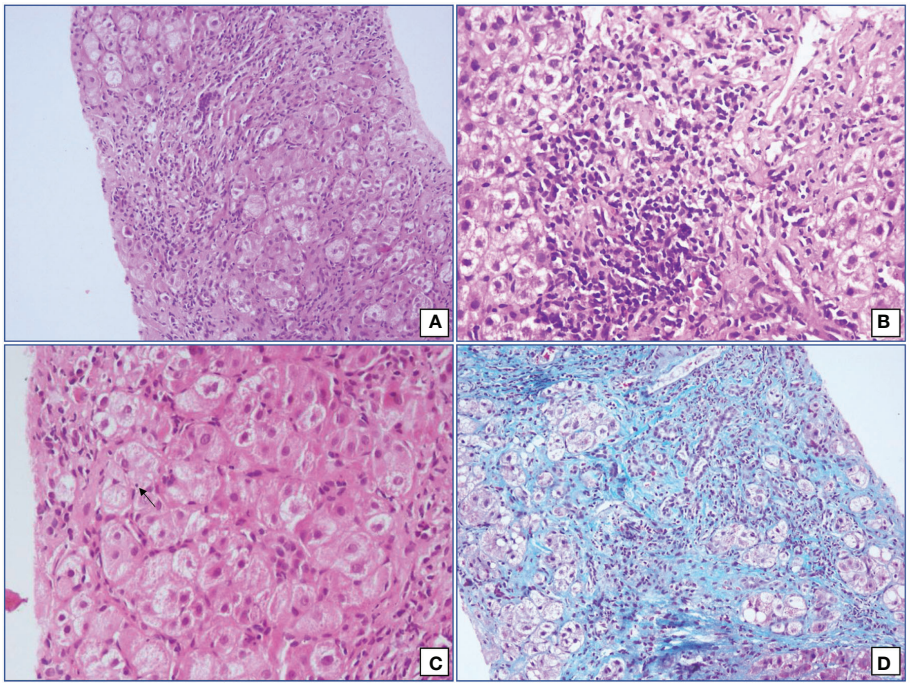


FIGURE 1
Microphotographs from cases of Autoimmune hepatitis showing **(A)** interface hepatitis with expanded portal tracts and significant portal tract inflammation (Hematoxylin and Eosin; 200x), **(B)** moderate lymphoplasmacytic cells infiltrate in an irregular and expanded portal tract (Hematoxylin and Eosin; 400x), **(C)** emperipolesis (Hematoxylin and Eosin; 400x) and **(D)** hepatocyte rosetting (Masson's Trichrome; 200x).

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TABLE 2 Response to treatment in Anti-SLA ELISA positive cases.

| Response to treatment | SLA ELISA | | P value |
|---|---|---|---|
| | Positive (n=7)* | Negative (n=40) | |
| Complete Response | 0 (0.0%) | 25 (62.5%) | 0.004 |
| Incomplete Response | 4 (66.7%) | 12 (30.0%) | |
| Relapse | 2 (33.3%) | 3 (7.5%) | |

*One patient was lost to follow-up.
SLA, Antibodies to Soluble Liver Antigen/liver-pancreas; ELISA, Enzyme-Linked Immunoassay.

However, no significant association was observed between HLA-DRB1 and different categories of the patients.

## Non-HLA association

SNP arrays were done for 5 SNVs in CTLA-4 and 3 SNVs in PTPN22. The genotype frequencies are given in Table 4. There was a significant difference between the cases and controls in terms of distribution of CTLA-4 CT 60: A/G (p = 0.0003, OR = 5.1, 95% CI = 2.107-12.34). GG genotype of CTLA-4 CT 60 was significantly higher in cases (36.2%) as compared to controls (10.0%). Strength of association between the two variables (Cramer's V) = 0.341 (Moderate Association). Strength of association between the two variables (Bias Corrected Cramer's V) = 0.322 (Moderate Association). On dividing cases based on their gender, GG genotype showed marked susceptibility for AIH while GA genotype was protective, especially in females. There was no significant association of all the 8 SNVs with reference to age at presentation, clinical features at the time of presentation, presence or absence of cirrhosis and response to immunosuppressive therapy and no significant association between HLA-DRB1 typing of all the cases and all the eight SNVs was obtained.

# Discussion

This study is from a tertiary referral centre in North India with an extended panel of screening for autoantibodies, including the AIH specific auto-antibodies against SLA. It is also well characterized with biopsy in 87.2% of patients.

One-third of our patients presented with acute hepatitis; these cases could be acute AIH or the acute exacerbation of AIH. Cirrhosis is more frequently observed in our cohort (43.5%) compared to studies on the Chinese population (17). However, different studies have observed that AIH patients of South Asian origin present late with cirrhosis and have higher mortality rates

TABLE 3 Genotype frequency of HLA DRB1 in cases and controls.

| HLA DRB1 | AIH Patients n=47 | Healthy Controls n= 100 | P value | Pc value | Odds Ratio (95% CI) | Relative Risk (95% CI) |
|---|---|---|---|---|---|---|
| DRB1*01 | 1 (2.1%) | 11 (11%) | 0.131 | 1.703 | 0.18 (0.02-1.40) | 0.24 (0.03-1.62) |
| DRB1*03 | 29 (61.7%) | 25 (255%) | 0.0001 | 0.001 | 4.83 (2.30-10.15) | 2.78 (1.71-4.50) |
| DRB1*04 | 3 (6.4%) | 12 (12%) | 0.449 | 5.837 | 0.50 (0.13-1.87) | 0.60 (0.21-1.70) |
| DRB1*07 | 10 (21.3%) | 22 (22%) | 0.908 | 11.80 | 0.96 (0.41-2.23) | 0.97 (0.55-1.73) |
| DRB1*08 | 0 (0.0%) | 2 (2%) | 0.831 | 10.80 | 0.42 (0.02-8.82) | 0 (Infinity) |
| DRB1*09 | 0 (0.0%) | 0 (0.0%) | - | - | - | - |
| DRB1*10 | 1 (2.1%) | 11 (11%) | 0.131 | 1.703 | 0.18 (0.02-1.41) | 0.25 (0.04-1.62) |
| DRB1*11 | 4 (8.5%) | 19 (19%) | 0.165 | 2.145 | 0.40 (0.13-1.24) | 0.50 (0.20-1.26) |
| DRB1*12 | 0 (0.0%) | 0 (0.0%) | - | – | - | - |
| DRB1*13 | 9 (19.1%) | 18 (18%) | 0.952 | 12.38 | 1.08 (0.44-2.62) | 1.05 (0.58-1.91) |
| DRB1*14 | 7 (14.9%) | 21 (21%) | 0.513 | 6.67 | 0.66 (0.26-1.68) | 0.74 (0.37-1.48) |
| DRB1*15 | 19 (40.4%) | 38 (38%) | 0.920 | 11.96 | 1.11 (0.55-2.25) | 1.07 (0.66-1.73) |
| DRB1*16 | 1 (2.1%) | 2 (2%) | 0.566 | 7.36 | 1.07 (0.09-12.06) | 1.04 (0.21-5.26) |

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TABLE 4 Genotype and allele frequencies of SNVs of CTLA-4 and PTPN22 in cases and controls.

| Genotype | AIH Patients (n=47) | Healthy Controls (n= 100) | p value OR (95% CI) |
|---|---|---|---|
| CTLA-4 CT60 (rs 3087243) | | | |
| AA | 16 (34.0%) | 32 (32.0%) | p=0.954 OR=1.097 (0.525-2.288) |
| GA | 14 (29.8%) | 58 (58.0%) | p=0.002 OR=0.307 (0.146-0.644) |
| GG | 17 (36.2%) | 10 (10.0%) | p=.0003 OR=5.100 (2.107-12.34) |
| A | 46 (48.9%) | 122 (61.0%) | p=0.068 OR=0.612 (0.373-1.005) |
| G | 48 (51.1%) | 78 (39.0%) | p=0.068 OR=1.632 (0.995-2.676) |
| CTLA-4 49 (rs 231775) | | | |
| AA | 22 (46.8%) | 47 (47.0%) | p=0.876 OR=0.994 (0.620-1.596) |
| AG | 18 (38.3%) | 45 (45.0%) | p=0.557 OR=0.758 (0.373-1.540) |
| GG | 7 (14.9%) | 8 (8.0%) | p=0.319 OR=2.013 (0.683-5.929) |
| A | 62 (66.0%) | 139 (69.5%) | p=0.635 OR=0.850 (0.504-1.433) |
| G | 32 (34.0%) | 61 (30.5%) | p=0.635 OR=1.176 (0.697-1.983) |
| CTLA-4 318 (rs 5742909) | | | |
| CC | 41 (87.2%) | 92 (92.0%) | p=0.537 OR=0.594 (0.193-1.823) |
| CT | 6 (12.8%) | 8 (8.0%) | p=0.537 OR=1.683 (0.548-5.163) |
| C | 88 (93.6%) | 192 (96.0%) | p=0.547 OR=0.611 (0.205-1.815) |
| T | 6 (6.4%) | 8 (4.0%) | p=0.547 OR=1.636 (0.551-4.859) |
| CTLA-4 1661 (rs4553808) | | | |
| AA | 39 (83.0%) | 77 (77.0%) | p=0.540 OR=1.456 (0.646-3.554) |
| AG | 8 (17.0%) | 14 (14.0%) | p=0.817 OR=1.260 (0.488-3.251) |
| GG | 0 (0.0%) | 9 (9.0%) | p=0.033 OR=0.101 (0.005-1.781) |
| A | 86 (91.5%) | 168 (84.0) | p=0.117 OR=2.048 (0.904-4.637) |
| G | 8 (8.5%) | 32 (16.0%) | p=0.117 OR=0.488 (0.215-1.106) |

*(Continued)*

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TABLE 4 Continued

| Genotype | AIH Patients (n=47) | Healthy Controls (n= 100) | p value OR (95% CI) |
|---|---|---|---|
| CTLA-4 1722 (rs733618) | | | |
| AA | 33 (70.2%) | 79 (79.0%) | p=0.337 OR=0.626 (0.284-1.379) |
| AG | 11 (23.4%) | 20 (20.0%) | p=0.798 OR=1.222 (0.530-2.816) |
| GG | 3 (6.4%) | 1 (1.0%) | p=0.184 OR=6.750 (0.682-66.75) |
| A | 77 (81.9%) | 178 (89.0%) | p=0.137 OR=0.559 (0.281-1.113) |
| G | 17 (18.1%) | 22 (11.0%) | p=0.137 OR=1.786 (0.898-3.552) |
| PTPN22 1858 (rs 2476601) | | | |
| AA | 4 (8.5%) | 5 (5.0%) | p=0.646 OR=1.767 (0.452-6.911) |
| GA | 1 (2.1%) | 0 (0.0%) | p=0.319 OR=6.484 (2.590-162.3) |
| GG | 42 (89.4%) | 95 (95.0%) | p=0.360 OR=0.442 (0.121-1.609) |
| A | 9 (9.6%) | 10 (5.0%) | p=0.217 OR=2.012 (0.788-5.132) |
| G | 85 (90.4%) | 190 (95.0%) | p=0.217 OR=0.497 (0.194-1.268) |
| PTPN22 2740 (rs 1217412) | | | |
| AA | 28 (59.6%) | 53 (53.0%) | p=0.568 OR=1.307 (0.647-2.639) |
| AG | 16 (34.0%) | 45 (45.0%) | p=0.281 OR=0.630 (0.306-1.297) |
| GG | 3 (6.4%) | 2 (2.0%) | p=0.379 OR=3.341 (0.538-20.72) |
| A | 72 (76.6%) | 151 (75.5%) | p=0.953 OR=1.062 (0.596-1.890) |
| G | 22 (23.4%) | 49 (24.5%) | p=0.953 OR=0.941 (0.529-1.675) |
| PTPN22 1123 (rs 2488457) | | | |
| CC | 2 (4.3%) | 8 (8.0%) | p=0.624 OR=0.511 (0.104-2.507) |
| GC | 14 (29.8%) | 34 (34.0%) | p=0.749 OR=0.823 (0.389-1.743) |
| GG | 31 (65.9%) | 58 (58.0%) | p=0.459 OR=1.403 (0.681-2.890) |
| C | 18 (19.1%) | 50 (25.0%) | p=0.301 OR=0.710 (0.387-1.302) |
| G | 76 (80.9%) | 150 (75.0%) | p=0.301 OR=1.407 (0.768-2.579) |

CTLA-4, Cytotoxic T-Lymphocyte Antigen-4; PTP, Protein Tyrosine Phosphatases; SNV, Single Nucleotide Variant.

than patients of East Asian origin (6, 18). An extended serological workup was included, involving Anti-SLA ELISA and IIF for ANA, AMA, LKM and SMA autoantibodies.

Out of all cases, 19.15% (n=9) were seronegative, which is as described in the literature (19). We divided the cases into Anti-SLA positive and Anti-SLA negative groups and analyzed various parameters to see if they presented any different from each other. Anti-SLA positivity was seen in 14.9% (n=7) of patients. Autoantibodies against SLA have a high specificity (99%) for the diagnosis of AIH (20). Among 4.2% (n=2) of all the patients, Anti-SLA was the only antibody detected, which is much less as compared to previous reports (21). In 10.6% (n=5) of our patients, Anti-SLA positivity was present along with ANA/SMA positivity. After comparing Anti-SLA positive and Anti-SLA negative groups on various parameters with each other, we found that Anti-SLA positive cases showed significantly poor response to immunosuppression compared to Anti-SLA negative cases. Similarly, Ma Y et al. in a study had found that Anti-SLA positive autoimmune liver disease patients have a more severe disease clinical course, take longer time to respond to immunosuppression and have more incidences of relapse (22). On the other hand, Zhi-Xian Chen et al. demonstrated that treatment response was comparable between the groups, 72% and 71.3% in Anti-SLA positive and negative groups, respectively (23). Zhi-Xian Chen et al. conducted a meta-analysis and they also described a significant risk of relapse in the Anti-SLA positive groups (23). Zachou et al. also showed treatment relapse after corticosteroid withdrawal and thus proposed lifelong immunosuppression in these patients (24). Therefore, including SLA antibodies in screening panel can increase AIH detection in the population as well as help in their plan of treatment. In North India, the prevalence spectrum of autoimmune liver diseases is different from that described in Caucasian populations. Here, AIH is the most common autoimmune liver disease, followed by AIH/PBC overlap (25).

The distribution of HLA-DRB1 showed a positive association of DRB1*03 allele with AIH patients from India. This is in conjunction with the studies in Caucasian European and North American populations, where along with HLA-DRB1*03:01, HLA-DRB1*04:01 and HLA-DRB1*01:01 were also significantly associated. Even in the Brazilian population, HLA-DRB1*03:01 has a secondary association with the disease (26). Similarly, DRB1*03 is also a susceptible allele for various other autoimmune disorders such as autoimmune thyroiditis (27), PR3-ANCA-associated vasculitis (28), systemic lupus erythematosus, multiple sclerosis, and myasthenia gravis (29).

Previously Kaur et al., 2014 described HLA-DRB1*04 and HLA-DRB1*08 as susceptible alleles for AIH-1 in the North Indian population (11). It was also observed that HLA-DRB1*04 was significantly associated with pediatric AIH-1 patients, and DRB1*08 was associated with adult AIH-1 patients. The discordance of these results with our study can be explained by the inclusion of only adult patients in the present study, of which 87.2% were biopsy-proven. Additionally, the presence of SLA positivity is also demonstrated to be associated with HLA-DRB1*03 (30). Further salient reports on HLA association with AIH in different ethnic groups are illustrated in Supplementary Table 1.

The genetic complexity of autoimmune diseases has led to many research studies regarding the association of non-HLA genes. In AIH, associations of CARD10, SH2B3, and ICOS genes were suggested in GWAS by de Boer et al. in 2014 (2). Higuchi et al. in 2021 have enlisted the association of other genes, namely KIR, PTPN22, SH2B3, TNFAIP3, STAT4, TNIP1, CTLA-4, FAS and TNF (31) (Supplementary Table 2). We studied SNVs of CTLA-4 and PTPN22 genes. We had selected a larger number of polymorphisms in these two genes (a total number of 8 SNPs) to be able to report ethnic genetic variations in our cohort of AIH. The other reason for choosing these SNPs was that we had already studied these SNPs in ANCA associated vasculitis patients and thus had planned to look for the same SNPs in Autoimmune Hepatitis (28). From India, there has not been any study regarding non-HLA association gene polymorphisms with AIH. In this study, we found GG genotype of CTLA-4 CT 60 to be significantly associated with AIH patients (p=0.0003). In contrast, no significant difference in the genotype frequencies was observed in analyzing +49 A/G (rs 231775), -318 C/T (rs 5742909), -1722 C/T (rs 733618) and -1661 A/G (rs 4553808) polymorphisms of CTLA-4 gene. Agarwal et al. analyzed +49 A/G (rs 231775) polymorphism of the CTLA-4 gene and detected a high G allele frequency in patients compared to controls (32). They have described the association of GG genotype with greater mean AST levels and higher frequency of detection of Anti-TMA (Anti-Thyroid Microsomal Antigen) and HLA-DRB1*03:01. However, we could not find any relation between these polymorphisms and biochemical parameters, clinical signs and symptoms, treatment outcome and HLA-DRB1 typing.

Mutation in PTNP22 gene may increase susceptibility to various autoimmune diseases such as Type 1 Diabetes Mellitus, Rheumatoid Arthritis etc. A study in the Japanese population found a significant difference in minor allele frequencies of rs 1217412 in patients compared to controls (33). Li et al. analysed -1123 C/G/T (rs 2488457) and +1858 C/T (rs 2476601) polymorphisms in Chinese Han population and found that risk of AIH-1 was lower in carriers of T allele than CC genotype (CT + TT versus CC) in +1858 C/T (rs2476601), [OR (95% CI) = 0.65 (0.44–0.93)] (34). Similar to our results, Li et al. also didn't find any association between -1123 C/G/T (rs 2488457) and AIH-1 risk. Moreover, earlier studies also have demonstrated the absence of +1858 C/T association with SLE (35), vitiligo (36) and ANCA-associated vasculitis (28) except for rheumatoid arthritis (37) and type 1 diabetes (38).

Analysis based on clinical categorization such as the presence of cirrhosis, response to treatment, increased serum IgG levels and concurrent other autoimmune disorders indicates

no significant correlation with non-HLA genes. This was similar to results demonstrated in North European Caucasoid population (32). On the contrary, Fortes et al. confirmed the association of PTPN22 1858 C/T with cirrhosis, increased serum IgG levels and treatment response in Mestizo Venezuelan population (39). In the North European Caucasoid population, there was a synergy between CTLA-4 +49A/G and DRB1*03:01 in susceptibility to AIH-1 (32). However, we could not demonstrate any such association between them. The small sample size and low-resolution HLA typing are the major limitations of this study. This is a pilot study, and it needs to be validated in a larger cohort.

## Conclusion

In a rare disease like AIH, we were able to describe HLA and non-HLA polymorphisms in a fairly large single-centre study. HLA-DRB1*03 is the susceptibility allele, and CTLA-4 CT60 is an independent predictor of disease. AIH remains underdiagnosed in India and picked up in the late stage. Screening should be enhanced in the country with an extended AIH panel to diagnose SLA-positive cases as they portend resistance to therapy. In future, registries and multicentric collaboration throughout the country should aim at a GWAS from India. It could unravel ethnic variations in genetics as well as the phenotype of the disease.

## Data availability statement

Datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics statement

The study involving human participants was reviewed and approved by IEC, Postgraduate Institute of Medical Education and Research, Chandigarh. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

NA: executed the study, analyzed the data, and wrote the manuscript. JS: contributed in execution of study, analyzed the data and helped in manuscript writing. RM: designed the study, analyzed the data, edited and corrected the manuscript. SA: contributed in execution of Real-Time PCR for SNV polymorphism. AD: reported and evaluated the histopathology of liver. ST: helped in recruitment of patients, treated the patients, and provided the clinical details of the patients. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.984083/full#supplementary-material

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