

Next-Generation Immunosequencing Reveals Pathological T-Cell Architecture in Autoimmune Hepatitis

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BACKGROUND AND AIMS: Autoimmune hepatitis (AIH) is a chronic liver disease that regularly relapses when immunosuppression is tapered. It is thought to be driven by T-cells, whereas the etiologic impact of an apparently deregulated B lineage system, as evidenced by hypergammaglobulinemia and autoantibodies, remains elusive. We set out to investigate T and B cell repertoires supporting chronic inflammation in AIH.

APPROACH AND RESULTS: T and B cell receptor (TCR/BCR) and human leukocyte antigen (HLA) next-generation immunosequencing were used to record immune signatures from a cohort of 60 patients with AIH and disease controls. Blood and liver B lineage immune metrics were not indicative of a dominant directional antigen selection apart from a slight skewing of *IGHV-J* genes. More importantly, we found strong AIH-specific *TRBV-J* skewing not attributable to the *HLA-DRB1* specificities of the cohort. This *TCR* repertoire bias was generated as a result of peripheral T cell (de)selection and persisted in disease remission. Using a clustering algorithm according to antigenic specificity, we identified liver *TCR* clusters that were shared between patients with AIH but were absent or deselected in patients with other liver pathologies.

CONCLUSIONS: Patients with AIH show profound and persisting T-cell architectural changes that may explain high relapse rates after tapering immunosuppression. Liver T-cell clusters shared between patients may mediate liver damage and warrant further study. (HEPATOLOGY 2021;73:1436-1448).

Autoimmune hepatitis (AIH) is a severe chronic inflammatory liver disease characterized by an ongoing autoimmune reaction directed against hepatic autoantigens.^(1,2) Without effective treatment, this disease may evolve toward liver cirrhosis and end-stage liver failure. Although the majority of patients respond to steroids and other immunosuppressive drugs, long-term treatment is usually required, leading to unwanted adverse effects. Moreover, durable remissions are generally not achieved, and patients almost inevitably relapse when immunosuppression is tapered.⁽³⁾

To develop alternative treatment approaches, the complex immunological pathobiology underlying this disease needs to be elucidated. This includes the identification of pathogenic immune cell types that are amenable to direct therapeutic targeting. Liver-infiltrating CD4⁺ T lymphocytes seem to play a major role in the pathogenesis of AIH. This hypothesis is strongly supported by the close human leukocyte antigen (HLA)-DR subtype association of this disease (HLA-DRB1*0301) as well as the prominent liver CD4 T-cell infiltrates.⁽⁴⁾ Yet it is still largely unclear which cell types and processes fuel this T-cell

Abbreviations: AIH, autoimmune hepatitis; ANOVA, analysis of variance; CDR3, complementarity-determining region 3; GLIPH, grouping of lymphocyte interactions by paratope hotspots; HCV, hepatitis C virus; HD, healthy donor; HLA, human leukocyte antigen; Ig, immunoglobulin; IGH, immunoglobulin heavy chain; MAIT, mucosal-associated invariant T cell; NGS, next-generation immunosequencing; PBC, primary biliary cholangitis; PBMC, peripheral blood mononuclear cell; PCA, principal component analysis; pGEN, generation probability; sBCR, stereotyped B cell receptor; SHM, somatic hypermutation; TCR, T cell receptor; TRB, T cell receptor beta.

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reaction. Only a few of the potential antigens recognized by autoreactive T-cells have been identified, such as cytochrom P450 (liver kidney microsome (LKM)), asialoglycoprotein receptor, or O-phosphoserine-tRNA:selenocysteinyl-tRNA synthase (SepSecS; also soluble liver antigen (SLA)/liver pancreas (LP)).^(5,6) These targets may be equally recognized based on common circulating autoantibodies found in patients with AIH, suggesting an interplay of B and T-cells in AIH. Beyond this, there are indications suggesting that B cells play a role in AIH pathogenesis: (1) hypergammaglobulinemia (most often characterized by a selective increase in immunoglobulin G [IgG]) associated with intrahepatic inflammatory activity and promptly resolving on immunosuppressive treatment⁽⁷⁾ and (2) the presence of plasma cells within the inflammatory liver infiltrates as a hallmark of AIH. Because it is easy to identify circulating autoantibodies in the blood, this B-cell function traditionally dominated the study of B-cell involvement in a lot of autoimmune diseases.⁽⁸⁾ However, there are many more B-cell functions in these diseases. They go beyond autoantibody production, including strong antigen presentation by major histocompatibility complex class II molecules, delivery of costimulatory signals to T-cells, and cytokine production, such as interferon gamma and interleukins.^(9,10) In inflamed organs with high B-cell infiltration, these cytokines may substantially contribute to the autoimmune reaction. Yet the efficacy of B-cell depletion as an alternative or additional way of therapeutic targeting in AIH is still controversial.⁽¹¹⁻¹³⁾

Here, we used next-generation immunosequencing (NGS) to determine the characteristics of liver-infiltrating and blood-circulating T and B lineage clones and the profiles of T-cell and B-cell regulating cytokines in a large cohort of 60 patients with AIH.

Our analysis suggests dominant antigen-driven selective forces with disease-specific clusters in the T lineage, which are not evident for the B lineage.

Materials and Methods

PATIENT CHARACTERISTICS

Peripheral blood of 60 patients with AIH was collected in ethylene diamine tetraacetic acid Monovette tubes (Sarstedt, Nümbrecht, Germany) between 2017 and 2019 during routine clinical monitoring at the University Medical Center Hamburg-Eppendorf. As control, the blood of healthy donors (HDs) without any hematological abnormalities was collected for scientific purposes. Blood samples from patients with primary biliary cholangitis (PBC) and hepatitis C virus (HCV) served as disease control. Liver cells of 12 patients with AIH were obtained from the tissue collection of the First Medical Department at the University Medical Center Hamburg-Eppendorf (Liver.net Biobank). Liver biopsies from patients diagnosed with nonalcoholic steatohepatitis (NASH; n = 1), nonalcoholic fatty liver disease (NAFLD; n = 3) and hepatic hemangioma (n = 2) were used as a control group. AIH was diagnosed according to the American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines.^(14,15) Table 1 summarizes demographic, clinical, serological, and histological characteristics of the patients with AIH. Written informed consent was obtained from each patient and healthy individual included for the use of their biological material as approved by the Ethics Commission Hamburg (Ethikkommission der Ärztekammer Hamburg, Germany, Project Number PV4081). The

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study was performed in accordance with the Declaration of Helsinki of 1975.

ISOLATION OF GENOMIC DNA

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density-gradient centrifugation using Biocoll separation solution (Biochrom AG, Berlin, Germany). Genomic DNA of PBMCs and fresh frozen liver tissue was isolated using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Taufkirchen, Germany) in accordance with the manufacturer's instructions.

AMPLIFICATION OF IMMUNOGLOBULIN HEAVY CHAIN AND T CELL RECEPTOR BETA REPERTOIRE FOR NGS

The rearranged *V*, *D*, and *J* gene segments (V(D)J) of the immunoglobulin heavy chain (*IGH*) and T cell receptor beta (*TRB*) loci were amplified together in a multiplex PCR using BIOMED2-FR1 (*IGH*) or *-TRB-A/TRB-B* primer pools and 250 ng of genomic DNA (equating 37,500 genomes). The

primers were purchased from Metabion International AG (Martinsried, Germany). Two consecutive PCR reactions were performed to generate *IGH* and *TRB* fragments tagged with Illumina-compatible adapters for hybridization to the flow cell and seven nucleotide barcodes for sample identification. All PCRs were performed using Phusion HS II (Thermo Fisher Scientific Inc., Darmstadt, Germany). After gelelectrophoretic separation, *IGH* and *TRB* amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), quantified on the Qubit platform (QIAGEN, Hilden, Germany), and pooled to a final concentration of 8 nM. The quality of the *IGH* and *TRB* amplicon pools was controlled on an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) before undergoing NGS.

ILLUMINA NGS AND DATA ANALYSIS

NGS and demultiplexing was performed on an Illumina MiSeq sequencer (600-cycle single indexed, paired-end run, V3 chemistry). Analysis was performed as described.⁽¹⁶⁻¹⁸⁾ Briefly, rearrangements of *IGH* and *TRB* loci was computed with the MiXCR framework.⁽¹⁹⁾

TABLE 1. Cohort and Clinical Characteristics

	AIH (n = 60)	HD (n = 60)	PBC (n = 30)	HCV (n = 30)
Age, years, median (range)	54 (20-79)	50 (23-86)	58 (24-72)	51 (24-71)
	n (AIH)	n (HD)	n (PBC)	n (HCV)
Sex				
Female	50 (83%)	36 (60%)	29 (97%)	26 (87%)
Male	10 (17%)	24 (40%)	1 (3%)	4 (13%)
GOT/GPT > 35 U/L	24 (40%)		11 (37%)	21 (70%)
IgG > 16 g/L	16 (27%)		1 (3%)	
IgA > 4 g/L	2 (3%)		1 (3%)	
IgM > 2.3 g/L	6 (10%)		10 (33%)	
α-LKM-1 > 1:80	5 (8%)		0	
α-AMA > 1:80	3 (5%)		17 (57%)	
α-ANA > 1:80	36 (60%)		19 (63%)	
α-SMA > 1:80	14 (23%)		2 (7%)	
α-SLA > 20 relative U/L	11 (18%)		0	
Cirrhosis	5 (8%)		0	2 (7%)
Treatment	56 (93%)		25 (83%)	0

Abbreviations: α-AMA, α-antimitochondrial antibody; α-ANA, α-antinuclear antibody; α-LKM-1, α-liver-kidney microsomal antibody 1; α-SLA, α-antisoluble liver antigen antibody; α-SMA, α-smooth muscle actin; GOT, glutamic oxaloacetic aminotransferase; GPT, glutamate pyruvate aminotransferase.

As reference for sequence alignment, the default MiXCR library was used for *TRB* sequences and the international ImMunoGeneTics information system® (IMGT) library v3 for *IGH*. Nonproductive reads and sequences with less than two read counts were not considered for further bioinformatic evaluation. Each unique complementarity-determining region 3 (CDR3) nucleotide sequence was defined as one clone. All analyses and data plotting were performed using RStudio (version 1.1.456) and the tcR⁽²⁰⁾, ade4⁽²¹⁾ and tidyverse⁽²²⁾ packages.

IMMUNE REPERTOIRE METRICS

We calculated the clonality of the sequenced *IGH* and *TRB* repertoires using the formula “1- Pielou’s evenness.”⁽²³⁾ In our setting, evenness measures the relative abundance of unique B-cell or T-cell clones in the repertoire and is calculated according to the formula $J = H/\log_2(S)$, with H being the Shannon diversity index and S being the total clone number in a distinct sample (richness).⁽²⁴⁾ A clonality index of 1 indicates that the analyzed sample contains only one clone, whereas 0 indicates complete clonal diversity. Tree analyses were used to visualize T-cell spaces in livers from patients with AIH versus controls. Therefore, clonal spaces were calculated for the entire disease group and plotted using the R package tree map (<https://CRAN.R-project.org/package=treemap>).

IN SILICO GROUPING OF LYMPHOCYTE INTERACTIONS BY PARATOPE HOTSPOTS AND GENERATION PROBABILITY ANALYSIS

We used the grouping of lymphocyte interactions by paratope hotspots (GLIPH) algorithm to cluster TRB sequences that share antigen specificity with a high likelihood.⁽²⁵⁾ Clusters are displayed as consensus sequences derived from the single CDR3 amino acid sequences that constitute the respective cluster. The mean CDR3 amino acid frequency is considered as “cluster size.” The generation probability (pGEN) of the T-cell clusters was calculated using the Optimized Likelihood estimate of immunoglobulin Amino-acid sequences (OLGA) algorithm.⁽²⁶⁾ The pGEN values are \log_2 transformed for plotting purposes.

NGS-BASED GENOTYPING OF HLA-DRB1

To genotype samples for the presence of the AIH predisposing *HLA-DRB1* alleles *0301 (NM_001243965.1), *0701 (NM_001359193.1), *0401 (NM_001359194.1), and *1501 (NM_002124.3), a 236 bp fragment of *HLA-DRB1* exon 2 coding for amino acids R52 to V115 was amplified using 250 ng genomic DNA and the primers 5'-ACACTCTTTCCCTACACGACGCTC TTC CGATCTGTCATTTCTTCAATGGGACGGA (forward) and 5'-TGACTGGAGTTCAGAC GTG TGCTCTTCCGATCTCCGCTGCACTGTGAA GCTCT (reverse). Amplification and NGS were performed as described for *IGH* and *TRB*. After aligning the obtained sequence reads to the IMGT/HLA reference database (<http://www.ebi.ac.uk/ipd/imgt/hla/>), all sequences that did not share 100% identity to any known *HLA-DRB1* allele were discarded. Zygosity was determined based on the read count distribution pattern of detected *HLA-DRB1* alleles.

STATISTICAL ANALYSIS

Differences in NGS metrics were studied by ordinary one-way analysis of variance (ANOVA) and student *t* test. Principal component analysis (PCA) differences by Pillai–Bartlett test of multivariate ANOVA. All statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA).

DATA AVAILABILITY

The herein reported sequence data set has been deposited at European Nucleotide Archive under the ID PRJEB37143.

Results

DEEP SEQUENCING OF B AND T-CELL RECEPTOR REPERTOIRES IN A COHORT OF PATIENTS WITH AIH

To gain deeper insights into the adaptive immune architecture of AIH, we performed immuno-NGS on the peripheral B and T-cells of 60 patients with

AIH as well as 60 age-matched and sex-matched control HDs. To ensure that potential immune signatures are disease-specific and not an expression of unspecific liver inflammation or damage also present in other liver diseases, a further 30 patients with PBC and 30 patients with untreated HCV were studied as additional controls. The clinical characteristics of the patients included are summarized in Table 1. The blood samples were sequenced with a sequencing depth of around 30,000 reads per sample for *IGH* and around 50,000 reads per sample for *TRB*. In addition, we studied the *IGH* and *TRB* repertoire from liver tissue of 12 patients with AIH and 6 non-AIH liver control samples (NASH, NAFLD, and hepatic hemangioma). The liver samples were sequenced with a depth of approximately 25,000 reads per sample for *IGH* and 40,000 reads per sample for *TRB*. The latter sequencing depth was chosen to be able to detect every single T-cell and B-cell genome within the 37,500 liver tissue-derived genomes that were used in every sequencing reaction. The depth was calculated based on the maximum number of T and B lineage cells per total liver cells, as estimated by immunohistochemistry of liver sections and exemplarily shown in Supporting Fig. S1.

GLOBAL IMMUNE METRICS FOR B AND T-CELL RECEPTOR REPERTOIRES IN PATIENTS WITH AIH

Global immune repertoire metrics such as richness, diversity, and clonality represent parameters for immunological complexity and for ongoing, successful, or deregulated immune responses.⁽²⁷⁾ To assess these parameters quantitatively in our AIH cohort, we calculated the Shannon diversity index H' and derived the repertoire clonality from Pielou's evenness, both of which are commonly used parameters to describe biodiversity.⁽²³⁾ Although evenness measures relative clonal abundancy in a given repertoire, the Shannon diversity index includes the total read number and clonal size distribution. Figure 1 shows that global immune metrics did not discriminate between AIH and healthy or disease controls either on the level of blood analyzes or in liver tissue studies (Fig. 1). The only exception was seen in the untreated HCV cohort, which showed higher *IGH* diversity and

lower clonality compared with the other subgroups. Moreover, we found a trend toward higher *TRB* clonality in the liver-infiltrating T-cell repertoires of patients with AIH compared with other disease controls (Fig. 1). In addition, we determined the rate of *IGH* somatic hypermutation (SHM) because high levels of SHM are indicative of the number of antigen-experienced memory or plasma cells in the repertoire. Each B-cell clone with <98% identity to the corresponding germline *V* gene was considered hypermutated. Evaluation of our NGS data set showed no differences between *IGH* SHM in AIH versus controls (Fig. 1). Only the HCV subcohort differed significantly from the other subgroups by showing high percentages of sequences with evidence for antigen selection in the peripheral blood.

Besides representing a further marker for immune repertoire diversity, the length (and amino acid composition) of the CDR3 constitutes the major determinant for antigen recognition. CDR3 analysis of our AIH cohort showed an identical Gaussian length distribution pattern for all samples and no differences in the mean CDR3 length of peripheral or liver-infiltrating B and T-cells as compared with their respective controls (data not shown). We also did not detect biases in amino acid composition of the CDR3 sequences (data not shown).

T-CELLS SHOW AN AIH-SPECIFIC BIAS IN *TRBV-J* GENE USAGE

To identify an AIH-associated immune signature, we computed the usage of rearranged productive *IGHV-J* and *TRBV-J* genes in blood-circulating B and T-cells from patients with AIH as well as from PBC and HCV controls and compared each of these groups to HD blood samples. PCA revealed clear biases in *TRBV-J* gene usage in all three disease groups compared with healthy controls, whereas *IGHV-J* rearrangements were markedly less skewed in patients with AIH and patients with PBC (Fig. 2A). The observed T-cell skewing in AIH, PBC, and HCV was disease-specific because comparisons between disease groups resulted in significant differences by PCA. Within the disease groups, AIH and PBC showed more similar T-cell repertoires as compared with AIH versus HCV (Fig. 2B). T-cell repertoires derived from AIH and control liver samples showed skewing in the three-dimensional space ($P = 0.01-0.05$), but no

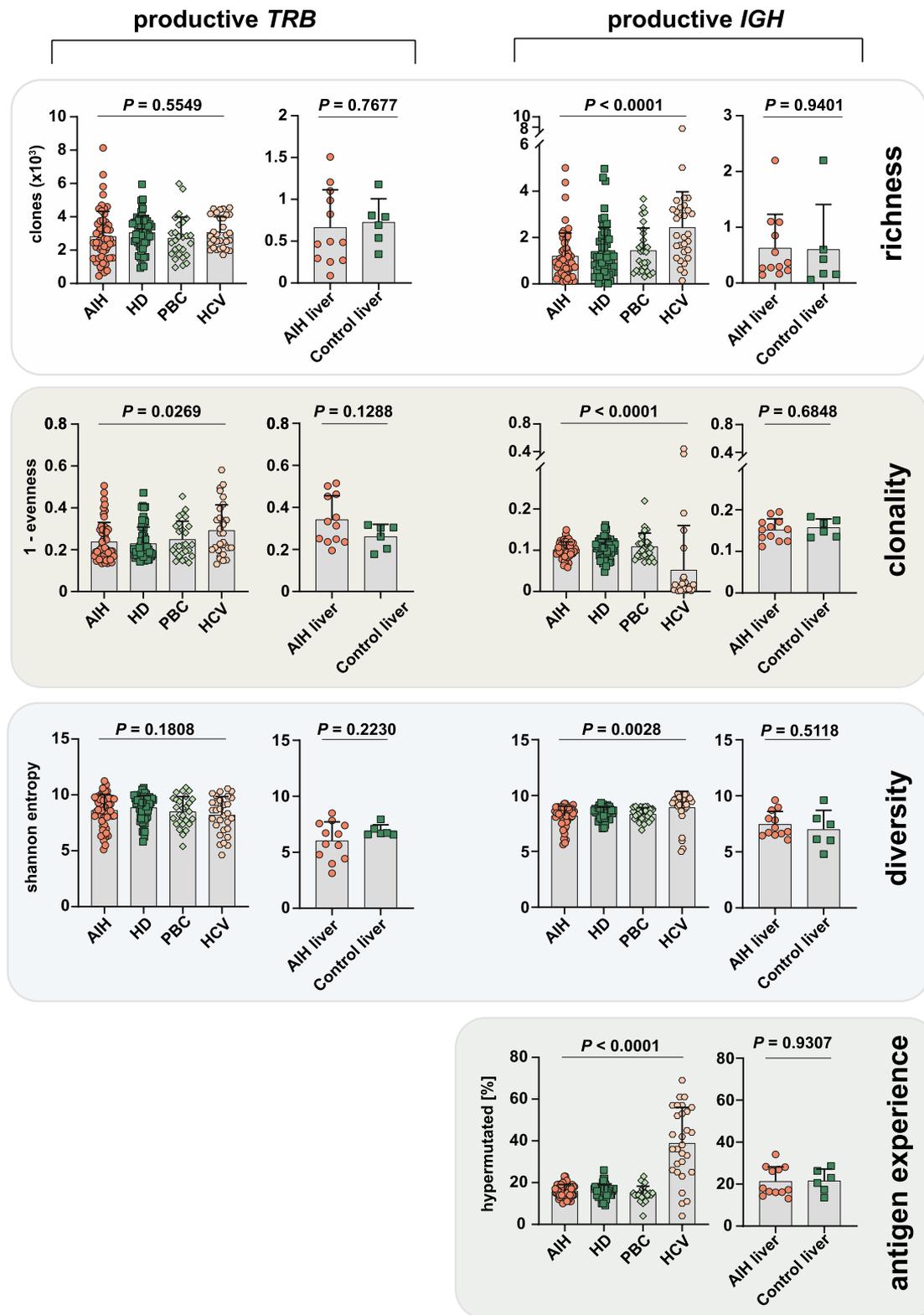


FIG. 1. Mean richness, clonality, Shannon diversity, and hypermutation rate for the productive *IGH* and *TRB* repertoires derived from the peripheral blood ($n = 60$) and livers ($n = 12$) of patients with AIH. Metrics were compared with patients with PBC ($n = 30$), HCV infection ($n = 30$) and HDs ($n = 60$) on the blood level or to non-AIH liver biopsies ($n = 6$). Statistical analysis: ordinary one-way ANOVA for blood samples, unpaired two-tailed student t test for liver samples.

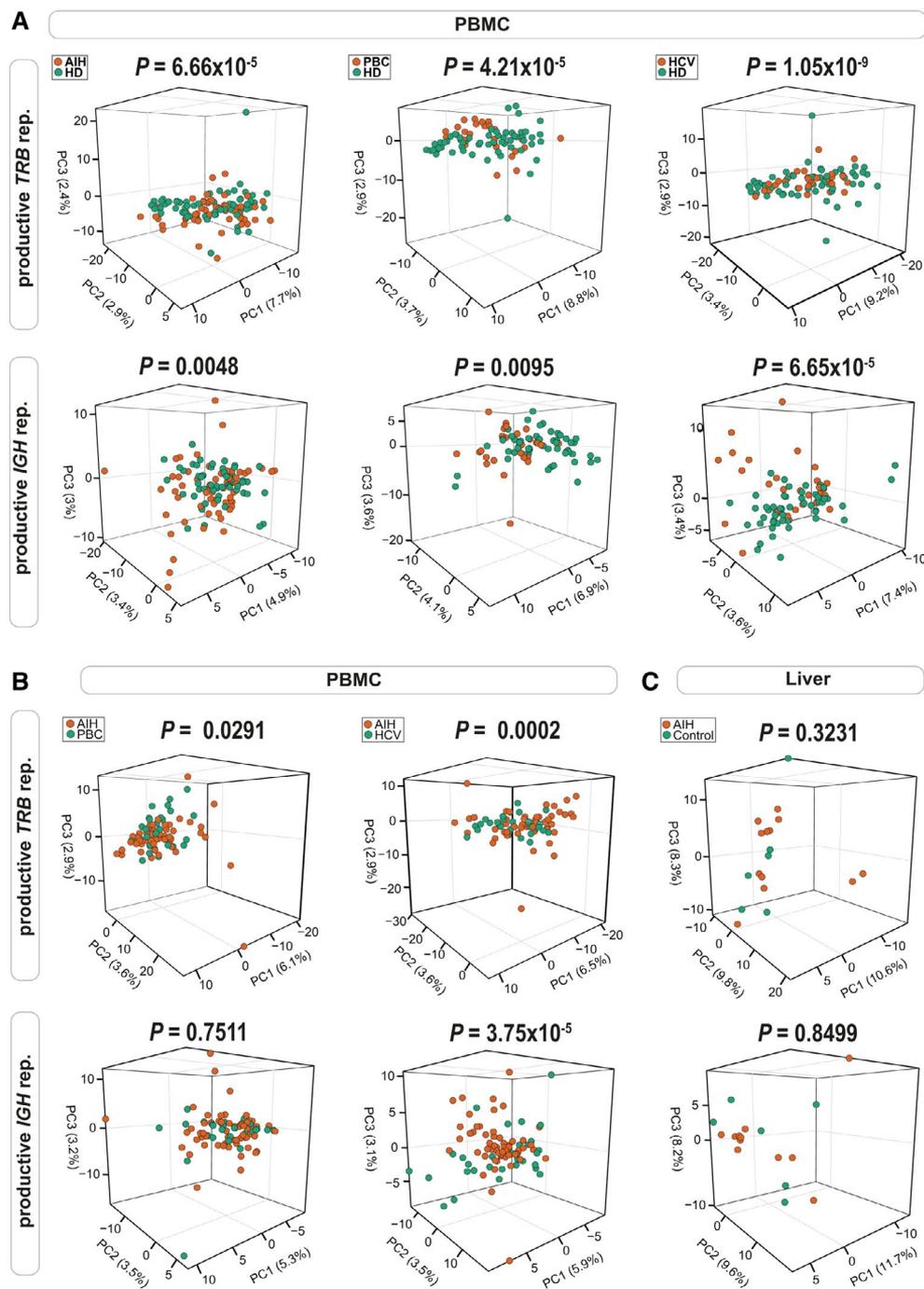


FIG. 2. PCA of productive *TRB* and *IGH V-J* gene usage in the peripheral blood and livers of patients with AIH. (A) The repertoires of AIH ($n = 60$), PBC ($n = 30$), and HCV ($n = 30$) PBMCs were compared with PBMCs from HDs ($n = 60$). (B) Comparison of AIH repertoires with PBC and HCV. (C) Comparison of *TRB* and *IGH* repertoires in AIH liver samples ($n = 12$) with non-AIH liver biopsies ($n = 6$). The contribution of the first three principal components (PC1 to PC3) is shown in percent. Statistical analysis: Pillai–Bartlett test of multivariate ANOVA of all principal components.

significant skewing was observed if all n dimensions of the PCA were considered ($P = 0.3231$), potentially because of the relative paucity of samples (Fig. 2C).

Interestingly, not just a single rearrangement but a broad variety of overrepresented or underrepresented rearrangements proved to underlie the AIH *IGH* and

TRB signatures, as shown in Fig. 3. Among others, we found differential usage of *TRBV6* and *TRBV20* family gene variants representing a key feature of mucosal-associated invariant T (MAIT) cells.⁽²⁸⁾ Calculation of the *V* gene contributions to the (weaker) *IGHV* bias revealed that especially *IGHV5-10-1*, *IGHV5-51*, *IGHV1-69*, *IGHV1-46*, and *IGHV3-23* genes were differentially used in AIH and control samples.

Complete biochemical remission of disease on immunosuppressive treatment interestingly had no impact on the T-cell architectural bias in our patients (Fig. 4A). Similar results were obtained for the weaker *IGHV-J* skewing in the B-cell repertoire (not shown).

Genetic studies have long established that susceptibility to AIH is conferred by distinct polymorphisms in the *HLA-DRB1* gene.^(2,4) These risk alleles code for lysine (K) or arginine (R) at position 71 of *HLA-DRB1* and are found in more than 90% of patients.^(1,2) To rule out the *TRB* repertoire shift in AIH being merely caused by overrepresentation of a specific *HLA-DRB1* subtype, we performed *HLA-DRB1* deep sequencing

of the critical region in exon 2 in the AIH samples and 45 randomly chosen HDs and PCA-plotted the *TRBV-J* gene usage according to risk allele status. The PCA analysis showed no bias in *TRBV-J* gene usage associated with *HLA* risk allele status, suggesting that the AIH-specific *TRBV-J* skewing was independent of the *HLA-DRB1* status (Fig. 4B).

GLIPH ANALYSIS REVEALS LIVER T-CELL CLUSTERS ASSOCIATED WITH AIH

Although the AIH T-cell repertoire bias was found in both blood and liver, we assumed that the liver repertoires may be more informative because they should be enriched for the functionally most relevant clones. Therefore, we studied these repertoires in more detail. As suggested by the liver metrics data (Fig. 1), the clonal distributions between AIH livers and control livers were similar, as shown in a summary tree diagram of six AIH versus six control livers (Fig. 5A).

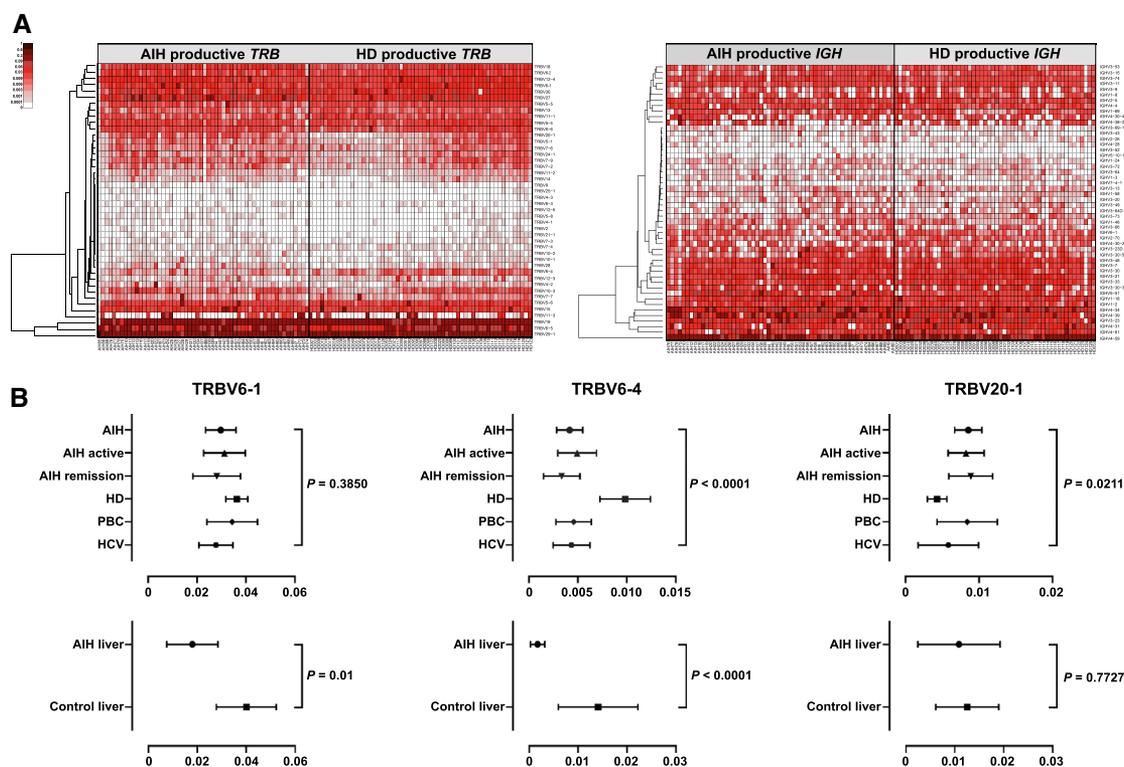


FIG. 3. Differential *V* gene usage in the productive *TRB* and *IGH* repertoires in the peripheral blood and livers of patients with AIH. (A) Heatmap of differentially used *V* genes in the productive *TRB* and *IGH* repertoires of AIH ($n = 60$) as compared with HDs ($n = 60$). (B) Frequency of differentially used *TRBV* genes characteristic for MAIT cells in AIH, PBC, HCV, and HD samples. Statistical analysis: ordinary one-way ANOVA.

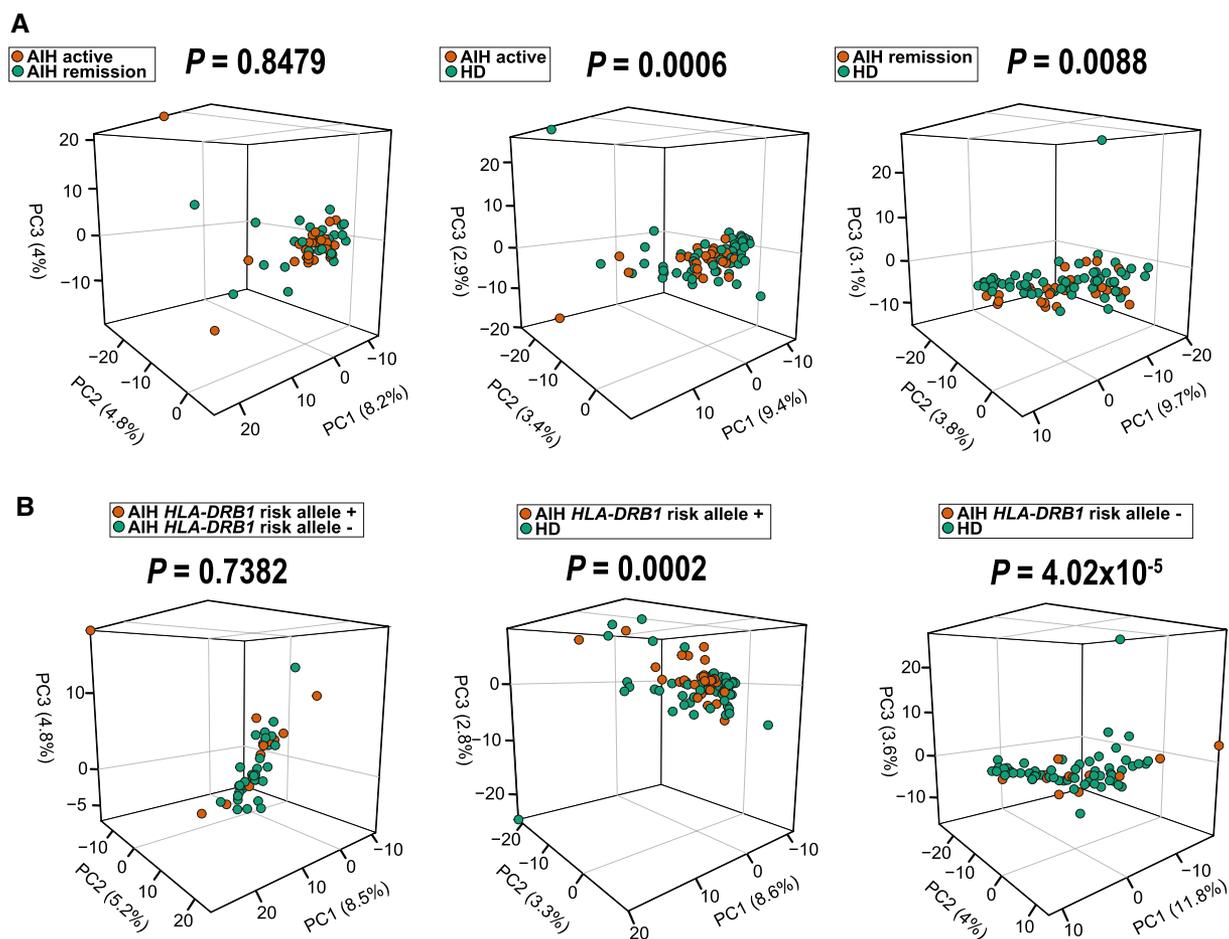


FIG. 4. PCA of *TRB* and *IGH V-J* gene usage in AIH in relation to disease status and the presence of *HLA* risk variants. (A) PCA of productive *TRBV-J* gene rearrangements in patients with active AIH ($n = 32$) versus AIH in remission ($n = 28$). (B) PCA of *TRBV-J* gene usage in AIH subcohorts with ($n = 32$) or without ($n = 12$) predisposing *HLA-DRB1* risk variants. AIH subcohorts were also compared with HDs ($n = 22$). The contribution of the first three principal components (PC1 to PC3) is shown in percent. Statistical analysis: Pillai-Bartlett test of multivariate ANOVA of all principal components.

This suggested that the AIH liver T-cell architecture is characterized by a quite diverse range of antigen-reactive T-cells rather than by expansions of single reactive T cell clones.

To reduce the complexity of the liver data sets with many individual T cell receptor (TCR) sequences, we used the GLIPH algorithm to cluster TCRs that shared antigen specificity.^(25,29) To identify T-cell clusters of pathophysiological relevance for AIH, we combined the GLIPH analysis with an estimate of the pGEN of each individual *V-J* recombination using the OLGA algorithm.⁽²⁶⁾ We reasoned that shared T-cell clusters associated with AIH and rather low generation probabilities most likely encompass T-cells with

pathophysiological relevance. This analysis revealed a set of 595 T-cell clusters that were exclusively found in livers from patients with AIH, whereas only 203 exclusive clusters were detected in the control livers (Fig. 5B). Between samples, 724 clusters were shared. The most enriched AIH-exclusive clusters exhibited the consensus CDR3 motifs “IDSD,” variants of the motif “S...SYE” as well as “R.GNQP.” In 3 out of 4 cases with matched blood samples, we also found blood T-cells exhibiting these CDR3 motifs at the time point of active disease, suggesting that these may also be peripherally tracked. The CDR3 motifs found as part of the clusters do not appear in the VDJdb database⁽³⁰⁾ as public sequences.

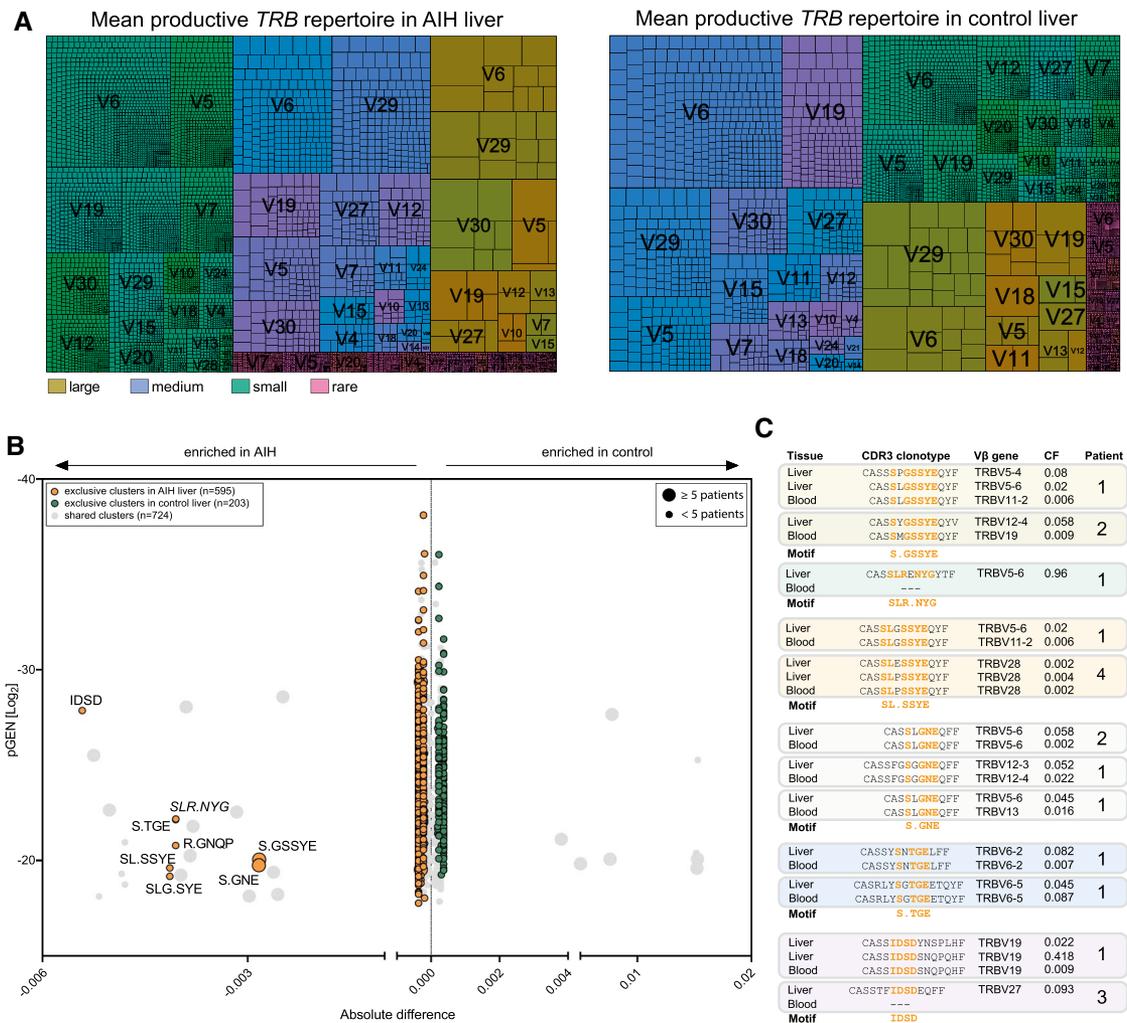


FIG. 5. Clonal distribution and GLIPH clustered T-cells in the livers of patients with AIH. (A) Comparative tree diagram of the clonal T-cell architecture in AIH and control livers (six each). (B) GLIPH-calculated T-cell clusters from AIH versus control livers. Enrichment of clusters in AIH versus control is plotted to the left and right. Dot sizes reflect the number of individuals sharing a distinct cluster. Clusters exclusively found in AIH are colored in orange, those exclusively found in control livers are colored in green, and those found in both groups are colored in gray. Amino acid motifs are shown for the AIH-exclusive clusters only. (C) CDR3 clonotype sequences from liver clusters in cases with available matched blood samples (patients 1-4). In 3 out of 4 patients, AIH liver clusters were also found in peripheral blood. Abbreviation: CF, clone frequency.

Discussion

The current understanding of the principal pathobiological mechanism underlying AIH is that it is a T-cell-mediated autoimmune response directed against liver autoantigens.⁽²⁾ Although hypergammaglobulinemia and autoantibodies are important diagnostic features of AIH, the role of B cells in the etiology of this disease remains to be clarified. Moreover, a better understanding of T-cell and B-cell interplay with

potential immune repertoire or cytokine-related biomarkers could open up avenues for therapeutic targeting or targeted de-escalation of treatment.

To gain deeper insights into the adaptive immune architecture in AIH and identify disease-specific immune signatures, we performed immuno-NGS on blood and liver tissue in a large cohort of patients with AIH. Our key finding was a clearly biased signature of *TRBV-J* gene usage in peripheral and liver-infiltrating T-cells of patients with AIH, independent

of AIH predisposing *HLA-DRB1* alleles. This signature was unaffected by immunosuppressive treatment and not related to complete biochemical disease remission. This suggests that treatment acted on T-cell functionality rather than on the underlying pathological T-cell architecture in this disease that has a high relapse rate.

Of note, one of the main drivers of the signature that was found was a lack of rearranged *TRBV6-1* and *TRBV6-4* genes, whereas rearranged *TRBV20* was enriched. Preferential usage of *TRBV6* and *TRBV20* family gene variants represents a key feature of different subsets of MAIT cells, highly conserved innate-like lymphocytes characterized by expression of a semi-invariant *TCR-alpha* chain.^(31,32) MAIT cells are highly abundant in mucosal tissues and constitute 5% of circulating and up to 40% of liver-residing T-cells.⁽²⁸⁾ They secrete proinflammatory cytokines and seem to contribute to immune responses against invading pathogens.^(33,34) The loss of MAIT cell subsets in the peripheral blood has been reported for a diverse range of autoimmune diseases, including systemic lupus erythematosus, asthma, rheumatoid arthritis, Sjögren syndrome, and multiple sclerosis⁽²⁸⁾ as well as common variable immunodeficiency.⁽³⁵⁾ In addition, a recent report showed a reduced frequency of circulating MAIT cells in patients infected with HCV and an inverse correlation between intrahepatic MAIT cell depletion and the grade of liver inflammation and fibrosis.⁽³⁶⁾ Although their observed reduction in many autoimmune conditions might to some degree be due to their sensitivity to corticosteroids,⁽²⁸⁾ MAIT cells are promising candidates to further expand functional concepts of autoimmune diseases, including AIH.

The factors triggering AIH and the target specificity of autoreactive lymphocytes remain to be identified in AIH etiology. To approach this, we performed a GLIPH analysis to identify intrahepatic T-cells with the same antigenic specificity shared by different patients with AIH. Indeed, we were able to identify individual T-cell clusters that were exclusive for AIH livers, indicating that the T-cell clones within the clusters could be involved in mediating liver damage. Notably, in 3 of 4 cases with corresponding blood samples, single-cluster clones were also found to be circulating in the periphery. Future studies may find these TCRs to be blood-trackable disease markers.

Unexpectedly, we did not find strong evidence for antigen-driven repertoire shifts in B cells of patients with AIH, unlike the clear picture observed for the T

lineage. With only a slight skewing of *IGHV-J* genes in AIH B lineage repertoires, there were no other features pointing at a directional force in the form of some specific antigen targeted by a relevant proportion of B lineage cells in our AIH cohort. This is in line with the finding of polyclonal hyper-IgG in these patients, whereas autoantibodies only make up a small proportion of the repertoire. Also, the absence of a strong B-cell signature in the liver itself may prompt us to speculate that the autoantibody-producing B cells may not reside in the liver but reside in other lymphoid organs that, unfortunately, were not accessible for us in the context of this research project.

We noticed that the slight skewing of the B-cell repertoire was mostly driven by the underrepresentation of certain *IGHV* rearrangements. Of particular interest are the rearrangements of the *IGHV1-69*, *IGHV1-46*, *IGH5-51*, and *IGHV3-23* genes commonly used in stereotyped B cell receptors (sBCRs) during restricted (or stereotyped) B-cell responses.⁽³⁷⁾ Stereotyped B cells express B cell receptors (BCRs) that share characteristic *V(D)J* sequence elements but originate from various individuals with different genetic backgrounds and immunological histories.⁽³⁷⁾ Originally discovered in B-cell neoplasia (especially *IGHV1-69* in chronic lymphocytic leukemia⁽³⁸⁾), restricted B-cell responses have also been described in autoimmune disorders like pemphigus,⁽³⁷⁾ Sjögren syndrome⁽³⁹⁾ or celiac disease.^(39,40) Besides mirroring the presence of autoreactive BCRs or autoantibodies in AIH, the observed skewing of sBCR-related *IGHV* genes might also provide a pathomechanistic hint because sBCRs are of exceptional importance as the source for broadly neutralizing antibodies (BNAs). BNAs can recognize conserved epitopes even in pathogens with high genetic diversity like influenza,⁽⁴¹⁾ human immunodeficiency virus,⁽⁴²⁾ and HCV.⁽⁴³⁾ For the latter, *IGHV1-69*-restricted B cells are currently exploited for vaccine development because they produce antibodies that efficiently target and neutralize the HCV E2 envelope glycoprotein needed for cell entry through CD81.⁽⁴⁴⁾ Taking into account that AIH might be triggered by molecular mimicry after exposure to viral antigens and environmental agents, underrepresentation of stereotyped B cells targeting these pathogens might be a contributing factor to the loss of tolerance in AIH development.

Collectively, the findings from this study underpin a profound and persisting T-cell bias in AIH, most

likely permitting relapsing episodes of acute liver damage when immunosuppression is tapered. Our findings also raise the question as to whether treatment directed at the B-cell lineage (e.g., rituximab or belimumab) can result in meaningful clinical benefit. Given the high number of investigated individuals—considering the rareness of the disease—and given the depth and breadth of state-of-the-art immune-NGS performed, we believe our data to be solid and thus contribute significantly to the understanding of adaptive immunity in AIH. One means of clinical exploitation resulting from this study could involve defining the T-cell clusters associated with AIH and using them as part of a diagnostic tool. The sequencing data deposited with this work have the potential to serve as a valuable resource in achieving this. Furthermore, clonal tracking should be prospectively investigated within future biomarker trials.

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Author Contributions: M.B., C.W.-N. and A.W.L. conceived the study design. C.S. performed most of the experiments. A.O. performed DNA extraction and PCR for liver specimens. E.W. and D.S. wrote R scripts for data analysis. L.F. and N.B. performed the GLIPH analysis. N.W. and J.S.-Z.-W. provided samples. M.B., C.S., D.S. and A.W.L. analyzed and interpreted the data. C.S. and M.B. wrote the manuscript. All authors reviewed the manuscript.

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