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Integrative molecular profiling of autoreactive CD4 T cells in autoimmune hepatitis

Amédée Renand¹, Iñaki Cervera-Marzal^{2**}, Laurine Gil^{2**}, Chuang Dong^{2**}, Alexandra Garcia¹, Erwan Kervagoret¹, Hélène Auble³, Sarah Habes⁴, Caroline Chevalier³, Fabienne Vavasseur³, Béatrice Clémenceau⁵, Anaïs Cardon¹, Jean-Paul Judor¹, Jean-François Mosnier^{1,6}, Florence Tanné⁷, David-Axel Laplaud^{1, 9}, Sophie Brouard¹, Jérôme Gournay^{4, 8}, Pierre Milpied^{*2}, Sophie Conchon^{*1}

1 Université de Nantes, CHU Nantes, Inserm, Centre de Recherche en Transplantation et Immunologie, UMR 1064, ITUN, F-44000 Nantes, France

2 Aix Marseille Université, CNRS, INSERM, Centre d'Immunologie de Marseille-Luminy, CIML, Marseille, France

3 Centre d'Investigation Clinique gastro-nutrition, CHU Nantes, Nantes, France

4 Service Hepato-gastro-entérologie et Assistance Nutritionnelle, CHU Nantes, Nantes, France

5 Université de Nantes, CHU Nantes, INSERM, CRCINA, F-44000, NANTES, France

6 Service Anatomie et Cytologie Pathologiques, CHU Nantes, Nantes, France

7 Service d'hépatogastroentérologie, CHU Cavale Blanche, Brest, France

8 Institut des Maladies de l'Appareil Digestif, IMAD, CHU Nantes, Nantes, France

9 Service de Neurologie et CIC 1413, CHU Nantes, Nantes, France

*equal contribution

**equal contribution

Corresponding author : Sophie Conchon, CRTI INSERM UMR1064, 30 Bd Jean Monnet, 44093 Nantes cedex 1, France, tel: +33 240087507; Fax:+33 240087411; mail: sophie.conchon@univ-nantes.fr

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List of abbreviations : Autoimmune hepatitis : AIH; Soluble Liver Antigen : SLA; single-cell RNA-seq : scRNA-seq; T cell receptor : TCR; Non-Alcoholic SteatoHepatitis: NASH; autoimmune liver diseases: AILD; T follicular helper: TFH; T peripheral helper cell: TPH; rheumatoid arthritis: RA; systemic lupus erythematosus: SLE; Candidas Albicans : C.Alb; Immunoglobulin G : IgG; Alanine/Aspartate Aminotransferases: ALT/AST.

Keywords : Autoimmune hepatitis; autoreactive CD4 T cells, single cell RNAseq; Soluble Liver Antigen; Autoreactive TCR clonotypes.

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Abstract (Word Count 275)

Background & Aims: In most autoimmune disorders, crosstalk of B cells and CD4 T cells results in the accumulation of autoantibodies. In autoimmune hepatitis (AIH), the presence of anti-Soluble Liver Antigen (SLA) autoantibodies is associated with reduced overall survival, but the associated autoreactive CD4 T cells have not been characterized yet. Here we isolated and deeply characterized SLA-specific CD4 T cells in AIH patients.

Methods: We used brief ex vivo re-stimulation with overlapping SLA peptides to isolate and phenotype circulating SLA-specific CD4 T cells, and integrative single-cell RNA-seq (scRNA-seq) to characterize their transcriptome and T cell receptor (TCR) repertoire. Autoreactive TCRs were cloned and used to identify dominant SLA-derived epitopes. SLA-specific CD4 T cells were tracked in peripheral blood through TCR sequencing, to identify their phenotypic niche. We further characterized disease-associated peripheral blood T cells by high content flow cytometry in n=42 AIH patients and n=17 non-alcoholic steatohepatitis controls.

Results: Autoreactive SLA-specific CD4 T cells were only detected in patients with anti-SLA autoantibodies and had a memory PD-1⁺CXCR5⁺CCR6⁺CD27⁺ phenotype. ScRNA-seq revealed their pro-inflammatory/B-Helper profile. SLA₈₁₋₁₀₀ and SLA₁₇₇₋₂₀₄ contain dominant Tcell epitopes. Autoreactive TCR clonotypes were predominantly found in the memory PD-1⁺CXCR5⁺CD4 T cells which were significantly increased in the blood of AIH patients and supported B cell differentiation through IL-21. Finally, we identified specific T cell phenotypes linked to disease activity and IgG level during AIH.

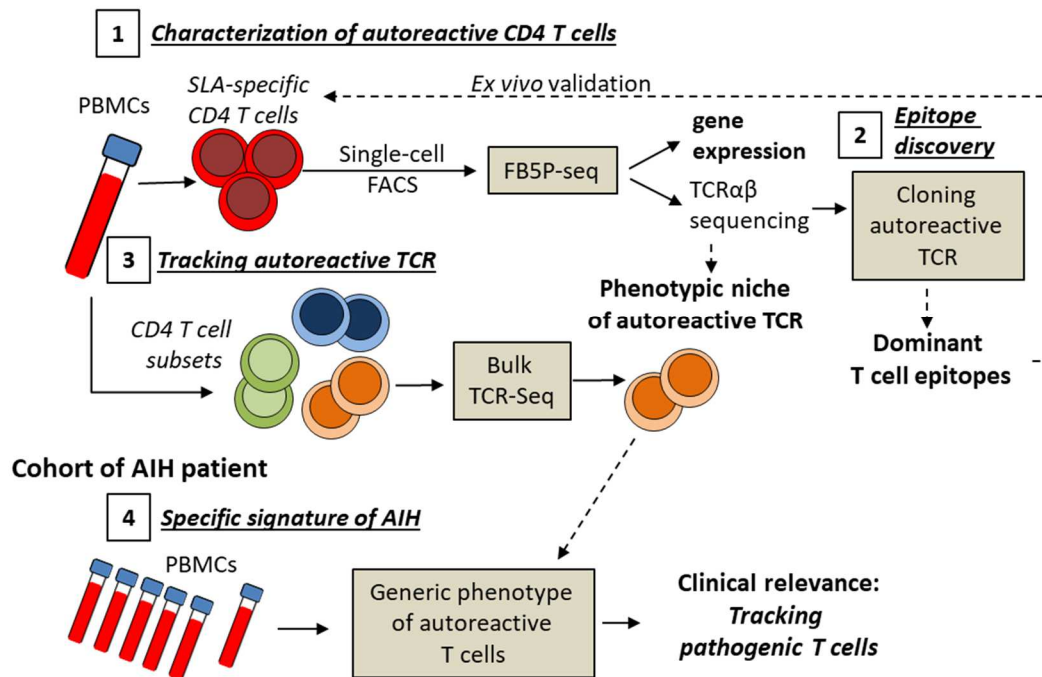
Conclusions: This work provides a deep characterization of rare circulating autoreactive CD4 T cells and the identification of their peripheral reservoir in AIH. We also propose generic phenotype of autoreactive T cells related to AIH disease activity which will be essential to track, delineate and potentially target those pathogenic cells.

Lay summary

One principal characteristic of the autoimmune hepatitis (AIH), like for many other autoimmune diseases, is the accumulation of autoantibodies produced by B lymphocytes following their interaction with autoreactive CD4 T lymphocytes. In this study we identify and characterized with high resolution these CD4 T cells. This will be essential to track, delineate and potentially target them during AIH.

Graphical Abstract

Identify self-reactive CD4 T cells in rare SLA-positive AIH patients



Robust methods to identify the reservoir of autoreactive T cells in AIH: From rare events to clinical relevance

Highlights:

- SLA-specific CD4 T cells have a pro-inflammatory/B-Helper profile (*IL21*, *IFNG* and *TIGIT*).

- SLA₈₁₋₁₀₀ and SLA₁₇₇₋₂₀₄ contain dominant T cell epitopes
- Autoreactive TCR clonotypes are enriched in the memory PD-1⁺CXCR5⁺ CD4 T cells
- PD-1 and CD38 co-expression by T cell reflect disease activity
- This work will be essential to track, delineate and target pathogenic T cells in AIH

Introduction

The main feature of autoimmune disorders is an abnormal reactivity of the adaptive immune system against self-antigens. The detection of the resulting self-reactive autoantibodies is one of the main criteria for the diagnosis of autoimmune diseases. Antibody production results from a crosstalk between cognate antigen-specific B cells and CD4 T cells in response to a specific antigen. The CD4 T follicular helper (TFH) cell population, characterized by the expression of the chemokine receptor CXCR5 and of the activation marker Programmed cell death 1 (PD-1), has been identified as the main subset of CD4 T cells responsible for B cell help in antibody responses through a cognate interaction and the production of the cytokine IL-21[1,2]. However, the implication of TFH cells in human autoimmune activation is not yet well characterized, and whether those cells carry self-antigen-reactive TCR has not been studied in many human autoimmune diseases[3–5].

Autoimmune hepatitis (AIH) is a rare disease with an incidence range from 1 to 2 per 100 000 individuals in Europe. Like other autoimmune liver diseases (AILD), AIH is characterized by an immune attack targeting the liver, an hypergammaglobulinemia and the accumulation of auto-antibodies. The presence of some specific auto-antibodies (e.g. anti-SLA, anti-LKM1, anti-LC1 auto-antibodies), which are relevant for diagnosis, and a genetic predisposition linked to HLA class II alleles[6], suggest a predominant role of CD4 T cell subsets in driving autoantibody accumulation and disease progression. Indeed, in AIH, CD4 T cells infiltrate the liver[7], yet it is still debated whether those autoreactive CD4 T cells are related to TH17 cells[8,9], TFH cells[4] or TNF producing cells[10]. In fact, the molecular signature of self-antigen-specific CD4 T cells in AIH has remained elusive due to the difficulty to track such rare cells[10,11], like in other AILD and many autoimmune disorders.

Recent studies have identified B-helper PD-1⁺CXCR5⁺ CD4 T cell populations, named T peripheral helper cell (TPH) or TH10 cells, which support antibody accumulation in rheumatoid arthritis (RA) through IL-21, or in systemic lupus erythematosus (SLE) through IL-10 production [12–15]. However, although phenotypes are similar, the transcriptomic

signatures of identified pathogenic PD-1⁺CXCR5⁻ CD4 T cells are different in RA[12] and in SLE[13], and not known in AIH. Moreover, it is still unknown whether these pathogenic PD-1⁺CXCR5⁻ CD4 T cells carry self-antigen-specific TCRs, a defining characteristic of autoreactive T cells.

In AIH, immunosuppressive treatments induce complete remission but more than 70% of patients relapse when treatment withdrawal is attempted, suggesting a persistence of pathogenic cells such as autoreactive CD4 T cells[7,16]. We previously observed that AIH patients under immunosuppressive treatments persist to have immune dysregulation in their blood and residual CD4 T cell infiltrate is observed in their liver[17]. Moreover, the presence of anti-soluble liver antigen (SLA or SepSecs) autoantibodies in the blood of AIH patients is associated with significantly reduced overall survival[18]. This suggests a link between autoantigen-specific adaptive immunity (autoreactive CD4 T cell response) and the prognosis of the AIH. Thus, there is an urgent clinical need to identify new therapeutic options targeting both the persistence and function of self-reactive T cells in AIH.

Here, we have used *ex vivo* re-stimulation assays based on the up-regulation of CD154 (CD40 Ligand) and 5'-end single-cell RNA-seq to define the surface phenotype, gene expression profile, and TCR $\alpha\beta$ repertoire of a total of 546 self-reactive CD4 T cells in five distinct AIH patients with anti-SLA autoantibodies. We report that circulating autoantigen-specific CD4 T cells invariably carry a memory PD-1⁺CXCR5⁻ phenotype and a pro-inflammatory B-helper molecular and functional profile with close similarity with the reported TPH CD4 cell subset. By cloning autoreactive TCR we also identify two dominant CD4 T cell epitopes derived from the SLA antigen. Our study further identifies a specific T cell phenotypic signature, CD45RA⁻PD-1⁺CD38⁺CXCR5⁻CD127⁻CD27⁺, which correlates with disease activity in AIH patients, and thus could be used to track and/or target pathogenic T cells in AIH.

Methods are described in the Supplementary file

Results

Detection of SLA-specific CD4 T cells with a central memory CD45RA⁻CD27⁺PD-1⁺CXCR5⁻ phenotype in the blood of seropositive (anti-SLA+) AIH patients.

To directly identify autoreactive CD4 T cells based on their antigen specificity, we performed an *ex vivo* short stimulation assay (3 hours) on PBMC with a pool of antigen-derived overlapping peptides. Rare antigen-specific CD4 T cells are activated by cognate peptide-MHC-II, leading to the rapid and specific upregulation of the surface activation marker CD154. The magnetic enrichment step significantly increased the detection of CD154⁺ antigen-specific CD4 T cells which can be analyzed and sorted by FACS (**Supplementary Fig. 2**) [28–30]. We analyzed CD4 T cells from AIH patients with (SLA-pos, n=8) and without (SLA-neg, n=8) anti-SLA autoantibodies (**Table 1 and Supplementary Table 2**) for their capacity to respond to overlapping SLA peptides (**Supplementary Table 3**). Peptides derived from the *Candida Albicans* mannoprotein MP65 (C.Alb) were used as positive control (**Figure 1a,c**). Compared to the condition without antigen, CD154⁺ SLA-specific CD4 T cells were detected only in SLA-pos patients (**Figure 1a-b**). These antigenic responses (SLA and C.Alb) were stable in a longitudinal analysis, supporting the robustness of our re-stimulation assay (**Supplementary Fig. 3**).

The analysis of surface markers related to memory status revealed that both SLA- and C.Alb-specific CD4 T cells exhibited a memory phenotype characterized by low levels of CD45RA, but high levels of CD27 and of CD45RO (**Figure 1d-e**). Classically, the CD4 T cell compartment can be divided in four subsets, with distinct memory status, based on the expression of CD45RA and CD27[31]. Contrary to the heterogeneous distribution of total CD4 T cells, the vast majority of CD154⁺ SLA-specific and CD154⁺ C.Alb-specific CD4 T cells exhibited a central memory T cell (TCM) profile (**Figure 1f**). The frequency of SLA-specific memory CD45RA⁻ CD4 T cells was significantly higher in the group of AIH patients with anti-SLA autoantibodies compared to seronegative patients, whereas C.Alb-specific memory CD45RA⁻ CD4 T cell frequency was similar in both groups, as expected (**Figure 1g**).

We further analyzed the surface expression of CCR6, a chemokine receptor expressed by TH17 cells[32], CXCR5, a chemokine receptor expressed by TFH cells[1], and PD-1, recently described to be expressed by pathogenic CD4 T cells in auto-immune disorders[12,13,15,33] (**Figure 1h-i**). C.Alb-specific CD4 T cells were PD-1⁻CXCR5⁻CCR6⁺, consistent with their expected TH17 phenotype[30,32,34], whereas autoreactive SLA-specific CD4 T cells were PD-1⁺CXCR5⁻CCR6⁻. Neither SLA- nor C.Alb-specific CD4 T cells showed high CXCR5 expression, suggesting they were distinct from circulating TFH cells.

In conclusion, SLA-specific CD4 T cells were detected in the blood of AIH patients with anti-SLA auto-antibodies, and those cells had a central memory CD45RA⁻CD27⁺PD-1⁺CXCR5⁻ phenotype.

scRNA-seq analysis reveals a T peripheral helper (TPH) profile of autoreactive CD4 T cells.

Next we submitted FACS-sorted *ex vivo* activated CD154⁺ C.Alb-specific and SLA-specific memory CD4 T cells from 5 SLA-pos AIH patients to 5'-end single-cell RNA-seq analysis for parallel analysis of gene expression and TCRαβ sequence with the FB5P-seq method[21] (**Figure 2a; Supplementary Table 2 and 4**). Unsupervised analysis of the single-cell gene expression profiles with principal component analysis (PCA) revealed a clear distinction between the two antigen-specific CD154⁺ memory T cell subsets, with SLA- and C.Alb-specific T cells segregating along the first principal component (**Figure 2b, left**). In two-dimensional embeddings of the first 40 principal components with Uniform Manifold Approximation and Projection (UMAP), the distinction between SLA- and C.Alb-specific T cells was evident despite sample-specific batch effects (**Supplementary Fig. 4a-b**). Removal of sample-specific batch effects through bioinformatic integration confirmed the segregation in UMAP embedding (**Figure 2b, right, Supplementary Figure 4a-b**). A total of 313 genes were differentially expressed between C.Alb- and SLA-specific CD4 T cells (**Supplementary Table 5, Figure 2c-d**). The molecular profiles of C.Alb- and SLA-specific CD4 T cells were robust and stable in a longitudinal analysis of samples from one patient

collected at 3 time points over a 6-month period (samples 018-10, 018-13, and 018-14, **Figure 2c**).

One advantage of the short re-stimulation assay is to allow transcriptomic characterization of the cytokine expression profile of antigen-specific CD4 T cells[28] (**Figure 2d-e**). C.Alb-specific CD4 T cells showed typical TH17 cytokine and transcription factor profiles[34], with high expression of *IL17A*, *IL23A*, *CCL20*, *CSF2* (GM-CSF), *IL2* and *TNF*, as well as *FOSL2*, *MYC* and *EGR2* among other genes (**Figure 2d-e; Supplementary Table 5**). These cells also expressed genes encoding the surface immune markers *CD200*, *ICOS*, *IL7R* (CD127) and *TNFRSF4* (OX40). By contrast, re-stimulated circulating autoreactive SLA-specific CD4 T cells had a distinct B-helper (*IL21*) and pro-inflammatory (*IFNG* and *TNF*) gene signature (**Figure 2d-e; Supplementary Table 5**). This B-helper profile was also conferred by the expression of the transcription factor *MAF* and by the expression of genes encoding surface immune markers such as *ICOS*, *TIGIT*, *CTLA4* and *SLAMF6*, which were previously found to be elevated on both TFH and TPH cells[12,15,35]. SLA-specific CD4 T cells did not express *CXCR5* (**Figure 1h**) and expressed *IFNG* suggesting their molecular signature was closer to the reported TPH signature (*CD200*, *IL21*, *IFNG*, *ICOS*, *TIGIT*, *CTLA4*, *ITM2A*, *SLAMF6* and *MAF*) than to a TFH signature[12,14]. However, SLA-specific CD4 T cells specifically expressed some additional genes not previously reported in studies of human autoimmune TPH or TFH cells: the transcription factor *NR3C1*, a glucocorticoid receptor involved in immune-regulation processes[36]; *CD109*, involved in the regulation of inflammation[37]; and *CLEC2D* and *KLRB1*, involved in the regulation of the innate immune response[38].

With the 5'-end scRNA-seq method we were able to reconstruct paired variable TCR α /TCR β sequences *in silico* and link them to the transcriptome of antigen-specific CD4 T cells. In all patients, SLA- and C.Alb-specific CD4 T cells were polyclonal, yet we consistently identified some highly frequent TCR $\alpha\beta$ clonotypes indicative of antigen-specific clonal expansions (**Figure 2f; Supplementary Table 6**). We found no evidence for public TCR rearrangements in SLA- and C.Alb-specific CD4 T cells from these five AIH patients (**Supplementary Table**

6). T cells from the same TCR $\alpha\beta$ clonotype were transcriptionally diverse within the overall molecular profile associated with their antigen-specificity (**Figure 2g**).

SLA₈₁₋₁₀₀ and SLA₁₇₇₋₂₀₄ sequences contain dominant T cell epitopes driving the SLA-specific reactivity of CD4 T cells in AIH.

We picked four of the top ten expanded TCR clonotypes of CD154⁺ SLA-specific CD4 T cells from patient #018, which cumulatively represented a quarter of total CD154⁺ SLA-specific CD4 T cells (**Figure 3a; Supplementary Table 6**), and expressed the corresponding recombinant TCR $\alpha\beta$ in the murine T cell hybridoma 58 $\alpha\beta$ ⁻ (**Supplementary Fig. 1**) [25,26]. The tested TCR cell lines reacted strongly to the SLA peptide pool #2, #3 and/or #5 in the presence of antigen presenting cells which were either an autologous immortalized B cell line (1064), or an HLA-DR-match (HLA-DR3^{+/+}) immortalized B cell line (FEB) (**Figure 3b, Supplementary Table 1**). Peptides from pool #2, #3 and #5 were tested individually and peptide p23 (SLA₁₇₇₋₁₉₆), p24 (SLA₁₈₅₋₂₀₄) and p11 (SLA₈₁₋₁₀₀) were identified as reactive (**Figure 3c**). The *ex vivo* PBMC re-stimulation assay in three distinct HLA-DR3⁺ SLA-pos patients with SLA peptides p23 and p24, or with p23, p24 and p11, or with all SLA overlapping peptides as a positive control, resulted in similar frequencies of stimulated CD154⁺ CD4 T cells (**Figure 3d-e**). These results showed that the CD4 T cell reactivity against SLA targeted dominant T cell epitopes, shared by several patients, and recognized by multiple TCR $\alpha\beta$ clonotypes.

The PD-1⁺CXCR5⁻ CD4 T cell population is the main reservoir of auto-reactive CD4 T cells in AIH.

Given the B-helper molecular profile (with TPH similarities) and PD-1⁺CXCR5⁻ surface phenotype of *ex vivo* activated SLA-specific CD4 T cells, we set out to identify the phenotypic niche of untouched autoreactive CD4 T cells in the peripheral blood of AIH patients. We performed bulk TCR β sequencing on DNA from sorted memory CD45RA⁻CXCR5⁺ (CXCR5⁺), CD45RA⁻PD-1⁺CXCR5⁻CCR6⁻ (PD-1⁺), CD45RA⁻PD-1⁻CXCR5⁻CCR6⁺ (CCR6⁺)

and CD45RA⁻PD-1⁻CXCR5⁻CCR6⁻ (PD-1⁻) CD4 T cell subsets from three SLA-pos AIH patients (**Figure 4a; Supplementary Table 2**). SLA-specific TCR β clonotypes identified in the single-cell RNA-seq data were tracked and found highly enriched in the PD-1⁺CD4 T cell subset (**Figure 4b; Supplementary Table 7**). In contrast, C.Alb-specific TCR β clonotypes were mainly found in the CCR6⁺CD4 T cell subset (**Figure 4c; Supplementary Table 7**). These results clearly demonstrated that the CD45RA⁻PD-1⁺CXCR5⁻CD4 T cell population was the major peripheral reservoir of autoreactive CD4 T cells in AIH, distinct from the classical TFH CD45RA⁺CXCR5⁺ CD4 T cell population.

Memory PD-1⁺CXCR5⁻ CD4 T cells with a T peripheral helper (TPH) profile are enriched in the blood of AIH patients.

Given that the PD-1⁺CXCR5⁻ CD4 T cell population was the source of SLA-specific autoreactive CD4 T cells, we tracked this subset in a group of patients with active AIH regardless of anti-SLA antibody serology (n=27; treated or not; elevated level of transaminase and/or elevated level of serum IgG)(**Table 2, Supplementary Table 2**). The proportion of PD-1⁺CXCR5⁻ cells amongst the memory CD3 T cell population was increased in active AIH patients compared to Non-Alcoholic SteatoHepatitis patients (NASH; n=17; control group with hepatic inflammation but with no sign of autoimmunity) (**Figure 5a-b**). The PD-1⁺CXCR5⁺ TFH population was not significantly increased and its frequency was low compared to the PD-1⁺CXCR5⁻ CD4 T cell population in active AIH (**Figure 5c-d**).

In AIH patients, both PD-1⁺CXCR5⁻ and PD-1⁺CXCR5⁺ TFH CD4 cells were TIGIT^{high}CD127^{low}CD49d^{high} (**Figure 5e**) and expressed ICOS and CTLA4 (**Figure 5f**), similar to SLA-specific CD4 T cells (**Figure 2**). Regarding their capacity to produce IFN γ and IL-21 upon *ex vivo* re-stimulation, PD-1⁺CXCR5⁻ CD4 T cells were mainly IFN γ producing cells, and more than half of IL-21 producing cells also produced IFN γ . In contrast, only 10.8 +/- 5.2% of PD-1⁺CXCR5⁺ TFH produced IFN γ and most IL-21 producing TFH cells did not produce IFN γ (**Figure 5g**). These results are consistent with the TPH profile of PD1⁺CXCR5⁻ CD4 T cells [12] and SLA-specific CD4 T cells. TPH are known to induce B cell

differentiation, thus PD-1⁺CXCR5⁻ memory CD4 T cells from AIH patients were tested for their B helper capacity (**Figure 5h-j**). Memory PD-1⁺CXCR5⁻ CD4 T cells from AIH patients were more potent than PD-1⁻CXCR5⁻ CD4 T cells at inducing the differentiation of B cells into CD27⁺CD38⁺ plasmablasts (**Figure 5h**) and IgG secretion (**Figure 5j**). We could not test the B-helper activity of PD-1⁺CXCR5⁺ CD4 T cells due to insufficient cell number of that subset. Blocking soluble IL-21 with decoy IL-21R drastically reduced the proportion of differentiated plasmablasts (**Figure 5i**), suggesting that the B-helper capacity was mostly IL-21-dependent.

Altogether, these data demonstrated that AIH patients, independently of the presence of anti-SLA auto-antibodies, presented in their blood an increased frequency of a CD4 T cell subset expressing PD-1 but not CXCR5, and that this subset presented a strong similarity with the TPH population, reported to be involved in other auto-immune diseases[12,14,15].

PD-1 and CD38 co-expression by memory CD45RA⁻CXCR5⁻CD127⁻CD27⁺ T cells is an immune correlate of AIH activity.

In a parallel effort to determine the clinical relevance of tracking the autoreactive CD4 T cell population and to delineate its specific phenotype, we performed 11-color flow cytometry on peripheral blood T cells from 11 patients with an active AIH versus 11 patients with NASH (**Figure 6a, Supplementary Table 2**), monitoring markers identified in the scRNA-seq data or in previous phenotypic observations (**Figure 1, 2 and 5**), with a focus on activation and differentiation markers (CD38, CD127 and CD27). Unsupervised analysis of these data with self-organizing maps and hierarchical clustering identified 14 phenotypic T cell metaclusters (**Figure 6a, Supplementary Fig. 5 and 6**). Three metaclusters were significantly increased in AIH patients compared to NASH patients (**Figure 6b**). They were respectively memory CD4⁺ (cluster 5), CD8⁺ (cluster 8) and double negative (cluster 12) T cells, and all shared the CD45RA⁻PD-1⁺CD38⁺CXCR5⁻CCR6⁻CD127⁻CD27⁺ phenotype (**Figure 6c, Supplementary Figure 7**).

Next we set out to analyze the link between these identified T cell subsets (clusters 5, 8 and 12) and the clinical status of AIH patients in a group of 27 active AIH patients, a group of 15 AIH patients under remission (treated) and a group of 17 NASH patients (**Table 2, Supplementary Fig. 8 and Supplementary Tables 2 and 8**). The cluster 5 and cluster 8 T cell subsets were the most significantly enriched subsets in the active AIH group compared to the NASH group (**Figure 5d-e, Supplementary Table 8**).

Interestingly, cluster 5 cells remained significantly enriched in the group of AIH patients under remission compared to the NASH group, whereas cluster 8 cells were significantly decreased during remission compared to the active AIH group (**Figure 5d-e, Supplementary Table 8**).

Only the frequency of cluster 5 and 8 T cell subsets correlated with biological markers of disease (**Figure 5f, Supplementary Table 9**). Cluster 5 CD4 T cell subset frequency correlated with serum IgG levels and cluster 8 CD8 T cell subset frequency correlated with transaminase activity.

Thus, fine immuno-phenotyping based on the characterization of autoreactive CD4 T cells identified T cell subsets significantly associated with AIH disease activity. Our data suggested that the clinical relevance of monitoring autoreactive TPH cells could be enhanced by analyzing additional markers such as CD27, CD127 and CD38. Finally, the co-expression of PD-1 and CD38 by CD45RA⁻CXCR5⁻CCR6⁻CD127⁻CD27⁺ CD4 and/or CD8 T cells may be essential to track, delineate and potentially target pathogenic T cells involved in AIH pathogenesis.

Discussion

The combination of *ex vivo* re-stimulation with 5'-end scRNA-seq, further confirmed by bulk TCR β sequencing, provided strong evidence that the central memory CD45RA $^-$ CD27 $^+$ PD-1 $^+$ CXCR5 $^-$ CCR6 $^-$ CD4 T cell population, with B-helper function and a pro-inflammatory signature, is the major reservoir of autoreactive CD4 T cells in AIH. This subset supports B cell differentiation through the production of IL-21, which constitutes a new possible therapeutic target for AIH treatment. It was previously reported that IL-21 could play an important role in AIH pathogenesis [4,41]. Authors reported the principal source of IL-21 as the classical TFH cell population (PD-1 $^+$ CXCR5 $^+$ CD4 $^+$). Our data strongly suggest that the CD45RA $^-$ CD27 $^+$ PD-1 $^+$ CXCR5 $^-$ CD4 T cell population could be another major source of IL-21 participating to AIH pathogenesis.

Results presented in our study confirm the idea that autoreactive CD4 T cells supporting B cell differentiation in multiple autoimmune disorders share a similar phenotype characterized by the expression of PD-1 and the absence of CXCR5, comparable to the recently described gluten-specific CD4 T cells in celiac disease[33], TPH cells or TH10 cells in rheumatoid arthritis and in lupus[12–14]. By tracking autoreactive TCR β in blood CD4 T cell subsets, we confirmed that autoreactive CD4 T cells are PD-1 $^+$ CXCR5 $^-$, and are distinct from the classical TFH cell population[12–14,33]. The PD-1 $^+$ CXCR5 $^-$ memory CD4 T cell population identified in AIH patients in our study expressed CD27 but did not express CCR6, suggesting that these cells might be phenotypically distinct from pathogenic CD4 T cells identified in previous studies on gluten-specific CD4 T cells (CD27 $^-$)[33], autoreactive CD4 T cell described in infants with autoimmune diabetes (CCR6 $^+$)[42] and expanded effector CD4 T cells in rheumatoid arthritis (CD27 $^-$)[43].

For the first time we propose a complete transcriptomic analysis of autoreactive (SLA-specific) CD4 T cells in AIH, and we reveal their specific molecular signature characterized by an *IL21* and *IFNG* cytokine signature. In autoimmune disorders, distinct autoreactive CD4 T cell molecular signatures have been reported, including a TFH and TPH

signature[3,5,12,14,15,33,44], a TH10 and Tr1/Treg (regulatory T cell) signature[13,45], and a TH17-like signature[32,42,46]. Here, we have shown that SLA-specific CD4 T cells had a transcriptomic signature close to the TPH signature although *CXCL13*, one of the major genes characterizing TPH cells, was only detected in a small fraction of SLA-specific T cells. Overall, SLA-specific CD4 T cells express a specific transcriptomic signature characterized by the transcription factors *NR3C1*, *EGR2*, *STAT4*, *STAT3*, *IRF4* and *MAF*; the cytokines *IL21*, *IFNG* and *TNF*; and the immune-regulatory molecules *CLEC2D*, *CTLA4*, *TIGIT*, *KLRB1* and *CD109*. These observations suggest a disease dependent immune signature of autoreactive CD4 T cells. Thus, the direct characterization of the transcriptomic signature of autoreactive CD4 T cells in diverse autoimmune disorders is primordial to elucidate the physiopathology of these cells.

The expression of PD-1 and the overexpression of genes related to immune regulation (*NR3C1*, *TIGIT* and *CTLA4*) could result from the chronic activation of autoreactive CD4 T cells. However, those autoreactive CD4 T cells persist with a pro-inflammatory profile (*IFNG* and *TNF*), which suggests a form of resistance or adaptation, rather than functional exhaustion. Thus, targeting regulatory pathways, including CD109, CLEC2D or KLRB1, may be interesting new therapeutic options. NK cells are enriched in the liver compared to blood[48] and a recent study has shown the presence of high numbers of KLRB1⁺CD4 T cells with a pro-inflammatory cytokine signature (TNF and IFNG) in the inflamed liver[49]. This suggests that the NK-like signature we observed for autoreactive CD4 T cells in AIH may be imprinted by liver-specific factors.

After reconstitution of autoreactive TCR $\alpha\beta$ in T cell hybridoma, we were able to perform T cell epitope discovery and to validate the data *ex vivo* with our re-stimulation assay. The T cell epitopes for the SLA antigen we identified here had been previously described in a transgenic mouse model[11], and our study highlights their relevance for auto-immune activation in AIH patients. This may drive new clinical therapeutic strategies by offering the possibility to target autoreactive CD4 T cells.

Finally, based on the data generated from the scRNA-seq and from the phenotypic analysis of circulating PD-1⁺CXCR5⁻CD4 T cells in AIH patients, we have identified a more specific phenotype of memory CD4 and CD8 T cells (CD45RA⁺PD-1⁺CD38⁺CXCR5⁻CD127⁻CD27⁺) associated with an active AIH disease. We showed a direct association between the frequency of CD45RA⁺PD-1⁺CD38⁺CXCR5⁻CD127⁻CD27⁺CD8 T cells and transaminases levels suggesting its potential involvement in the destruction of the liver parenchyma. This subset was downregulated during remission suggesting a sensibility to immunosuppressive treatment. Because the level of AST/ALT in the group of NASH patients was low we cannot exclude that this T cell subset was linked to the degree of liver inflammation and additional control disease groups will be needed to conclude on the AIH specificity. We also observed that the frequency of CD45RA⁺PD-1⁺CD38⁺CXCR5⁻CD127⁻CD27⁺CD4 T cells correlated with serum IgG levels during active AIH and remained high during remission. In agreement with our previous study[17], the persistence of this subset during remission suggests residual auto-immune activity under treatment. The high rate of relapse is a major challenge when wishing to limit long term immunosuppression and associated side effects in AIH patients. It will therefore be most interesting to investigate whether a higher frequency of this subset in the blood of patients in remission can be predictive of relapse after treatment withdrawal. Thus, the tracking of autoreactive T cell activity in patients' blood will be of interest for better treatment management in the clinic.

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TABLES:

Table 1 . Clinical characteristics of patients for SLA-specific CD4 T cell reactivity tracking (Fig.1a-c), expressed as mean (range or percentage)

	SLA-neg AIH	SLA-pos AIH	<i>adjusted p-value</i>
Number (n)	8	8	
Age (years)	60 (39-74)	37 (19-72)	<i>ns</i>
Female (n)	7 (87%)	7 (87%)	<i>ns</i>
Disease duration (years)	4 (0-10)	11 (0-23)	<i>ns</i>
IgG (g/L)	14 (9.07-22.01)	17 (8.27-32.69)	<i>ns</i>
AST (IU/L)	343 (23-2527)	98 (18.5-518.2)	<i>ns</i>
ALT (IU/L)	347 (14-2889)	140 (10-809)	<i>ns</i>
Active AIH	3/8	4/8	

IgG: immunoglobulin G; ALT/AST: Alanine/Aspartate Aminotransferases

Table 2 . Clinical patterns of AIH and NASH patients (Fig.6), expressed as mean (\pm SD or percentage)

	Active AIH	Remission AIH	NASH
Number (n)	27	15	17
Age (years)	58 (\pm 19)	54 (\pm 18)	57 (\pm 15)
Female (n)	20 (74%)	9 (60%)	6 (35%)
Disease duration (years)	1 (\pm 3)	7 (\pm 6)	5 (\pm 4)
IgG (g/L)	28 (\pm 15)	11 (\pm 2)	NA
AST (IU/L)	583 (\pm 629)	27 (\pm 10)	44 (\pm 20)
ALT (IU/L)	573 (\pm 602)	25 (\pm 12)	60 (\pm 40)
Untreated (n)	19 (70%)	1 (7%)	9 (53%)
Under treatment (IS) (n)	8 (30%)	14 (93%)	0
At diagnosis (n):			
ANA \geq 1/80	25/26 (96%)	10/14 (71%)	4/9 (44%)
SMA \geq 1/80	18/27 (67%)	10/15 (67%)	0/9 (0%)
SLA+	1/27 (4%)	4/15 (27%)	0/7 (0%)
LKM1+	0/27 (0%)	1/15 (7%)	0/4 (0%)
LC1+	0/17 (0%)	1/10 (10%)	NA
Seronegatif	0/27 (0%)	0/15 (0%)	5/9 (55%)

IgG: immunoglobulin G; ALT/AST: Alanine/Aspartate Aminotransferases; ANA : anti-nuclear-antibodies; SMA: Smooth-muscle antibodies; SLA: Soluble Liver Antigen antibody, LKM1: Liver kidney microsomal type 1 antibody, LC1: Liver cytosol antibody.

FIGURES AND LEGENDS

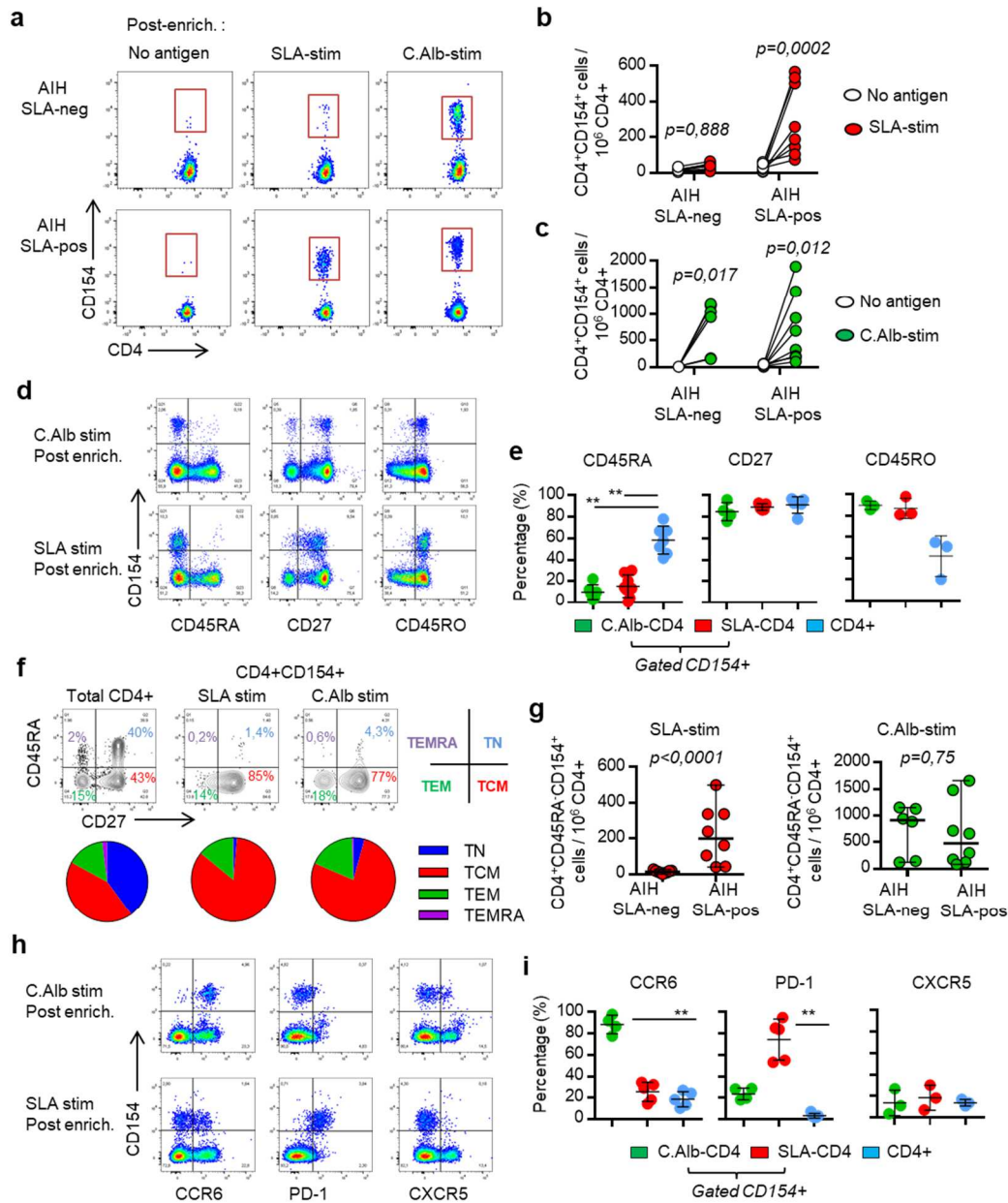


Figure 1. Detection of SLA-specific CD4 T cells with a central memory CD45RA⁺ CD27⁺PD-1⁺CXCR5⁻ phenotype in the blood of seropositive (anti-SLA⁺) AIH patients. a-c) Dot plots (a) and frequencies (b, c) of CD154⁺CD4⁺ T cells, after short stimulation with SLA (SLA-stim) or MP65 (C.Alb-stim) peptides, or without antigen (No antigen), from AIH SLA-neg (b: n=8; c: n=6) and AIH SLA-pos (b, c: n=8) patients. d-e) Dot plot (d) and frequency (e) of CD45RA⁺, CD27⁺ and CD45RO⁺ CD154⁺CD4⁺ T cells after peptide stimulation (C.Alb-CD4: green;SLA-CD4: red). f) Dot plot and graphic repartition of TN

(CD45RA⁺CD27⁺; blue), TCM (CD45RA⁻CD27⁺; red), TEM (CD45RA⁻CD27⁻; green) and TEMRA (CD45RA⁺CD27⁻; purple) on total CD4 T cells, and CD154⁺CD4⁺ T cells. g) Normalized frequency of memory CD45RA⁻CD154⁺CD4⁺ cells in AIH SLA-neg and AIH SLA-pos patients after peptide stimulation. h-i) Dot plot (h) and frequency (i) of CCR6⁺, PD-1⁺ and CXCR5⁺ CD154⁺CD4⁺ T cells after peptide stimulation (C.Alb-CD4: green; SLA-CD4: red). The Mann-Whitney U test (g), the Kruskal-Wallis test and Dunn's Multiple Comparison test (e, i), the Sidak's multiple comparisons test (b, c) were used. **:p<0.01.

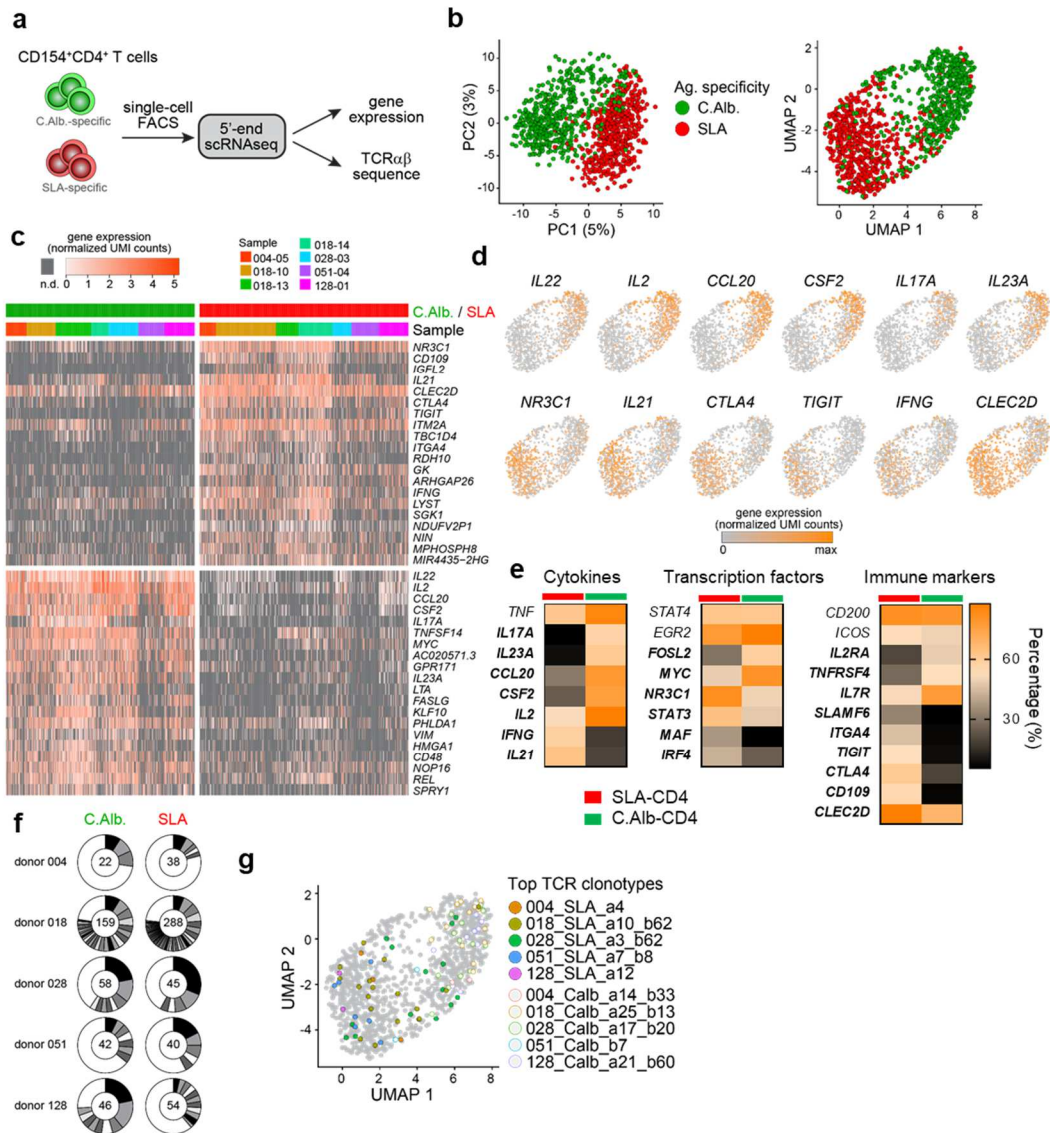


Figure 2. scRNA-seq analysis reveals a TPH profile of autoreactive CD4 T cells. a)

Experimental design. b) PCA projection on PC1 and PC2 (percent of total variability, along axis) before batch effect removal (left), and UMAP projection after batch effect removal

(right) of single-cell transcriptomes of n=546 SLA-specific (red) and n=493 C.Alb.-specific

(green) CD4 T cells isolated from 5 distinct AIH patients. c) heat map representation of the

top 20 genes differentially expressed. d) Single-cell gene expression UMAP plots showing

expression levels of specific marker genes. e) heat map representation of gene expression

related to cytokines, transcription factors or immune markers from SLA-specific and C.Alb.-

specific CD4 T cells. Bold genes are significantly different between groups. f) Distribution of

TCRαβ clones among each patient's antigen-specific T cells. Numbers indicate the number

of single cells analyzed per patient. Black and grey sectors indicate the proportion of TCR $\alpha\beta$ clones expressed by ≥ 2 cells, white sector: unique clonotypes. g) Same UMAP plot as in b) with the top TCR $\alpha\beta$ clones identified per patient overlaid (full circle, SLA-specific; empty circle, C.Alb.-specific).

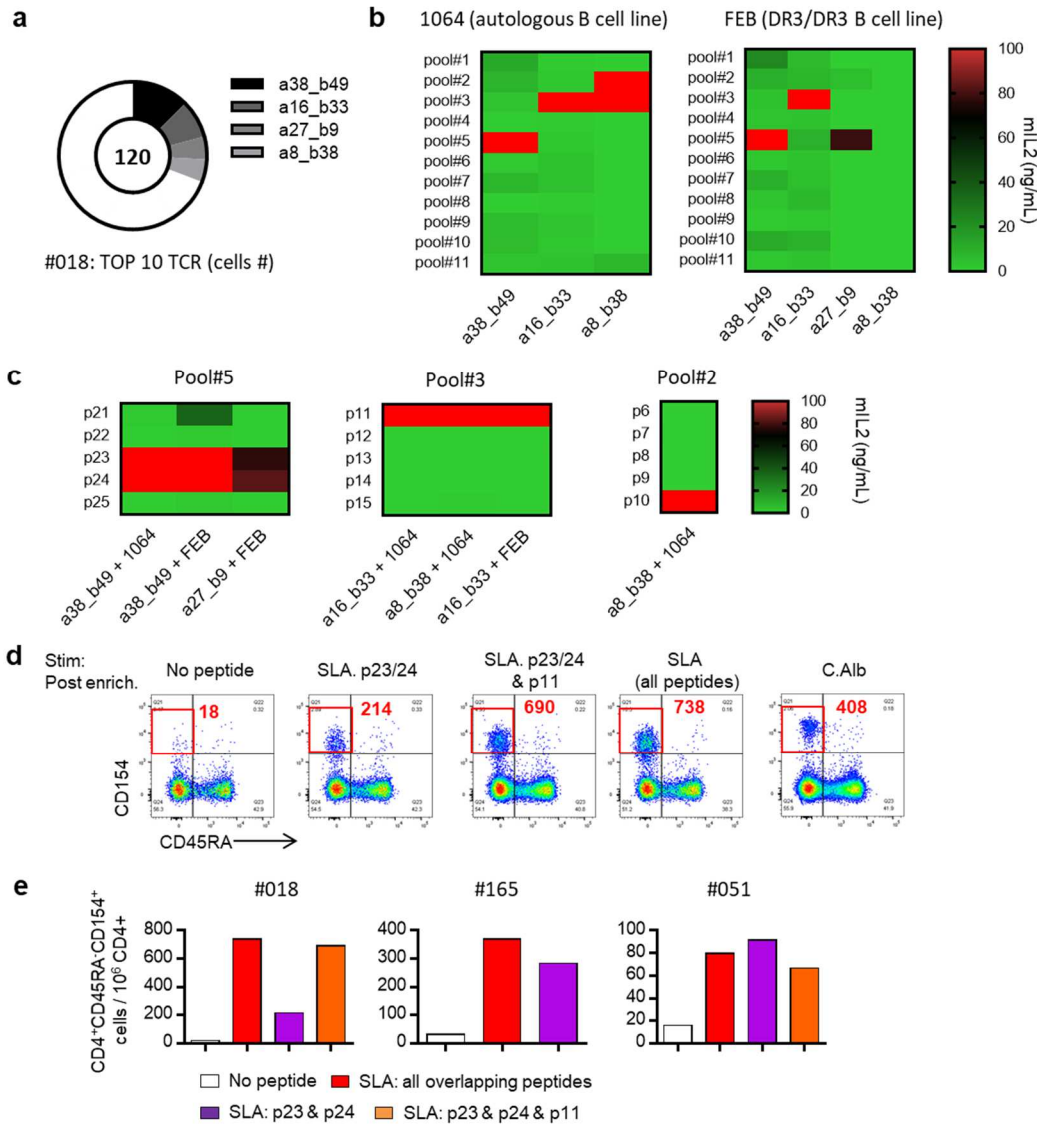


Figure 3. SLA₈₁₋₁₀₀ and SLA₁₇₇₋₂₀₄ sequences contain dominant T cell epitopes driving the SLA-specific reactivity of CD4 T cells in AIH. a) Distribution of the four tested TCRαβ clonotypes among the 120 SLA-specific T cells expressing the top 10 most frequent clonotypes from patient #018. Sectors indicate the proportion of cells with tested TCRαβ clonotypes. b-c) heatmap representation of murine IL2 production after stimulation of the selected TCRαβ clone lines (columns) with SLA-peptide pools (b) or individual peptides (derived from the indicated pool) (c) (rows) presented by autologous (1064) or DR3 homozygous (FEB) immortalized B cell lines. d) Dot plot of the CD154 expression on CD4 T cells, after stimulation with identified peptides and controls. The number of CD154⁺CD45RA⁻

CD4⁺ cells per million total CD4 T cells is indicated in red in the dot plots for patient #018, and in the bar graphs (e) for three distinct HLA-DR3⁺ AIH patients (#018, #165, #051).

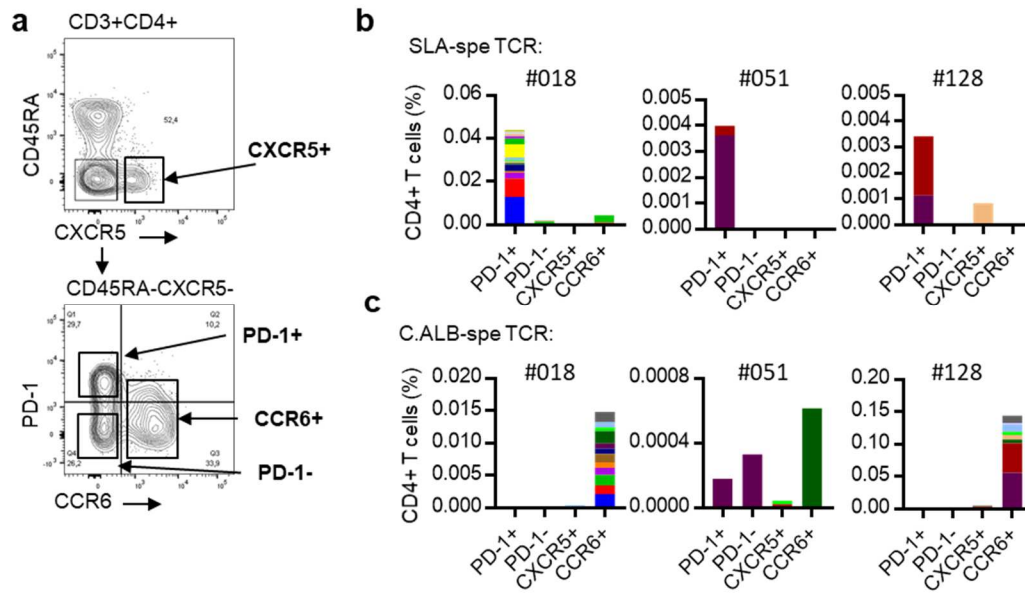


Figure 4. The PD-1⁺CXCR5⁻ CD4 T cell population is the reservoir of autoreactive CD4 T cells in AIH. a) Gating strategy to isolate **CXCR5⁺**, PD-1⁺CXCR5⁻CCR6⁻ (**PD-1⁺**), PD-1⁻CCR6⁺CXCR5⁻ (**CCR6⁺**) and PD-1⁻CXCR5⁻CCR6⁻ (**PD-1⁻**) CD4 memory T cells in the blood of AIH patients. b, c) Frequency, among bulk sequencing TCR β clonotypes from indicated subsets defined in (a), of the major TCR β clonotypes of SLA-CD4 T cells (b) and of C.Alb-CD4 T cells (c) identified at the single-cell level (Fig.2) in three AIH patients. Each color in the bars represents a distinct TCR β clonotype.

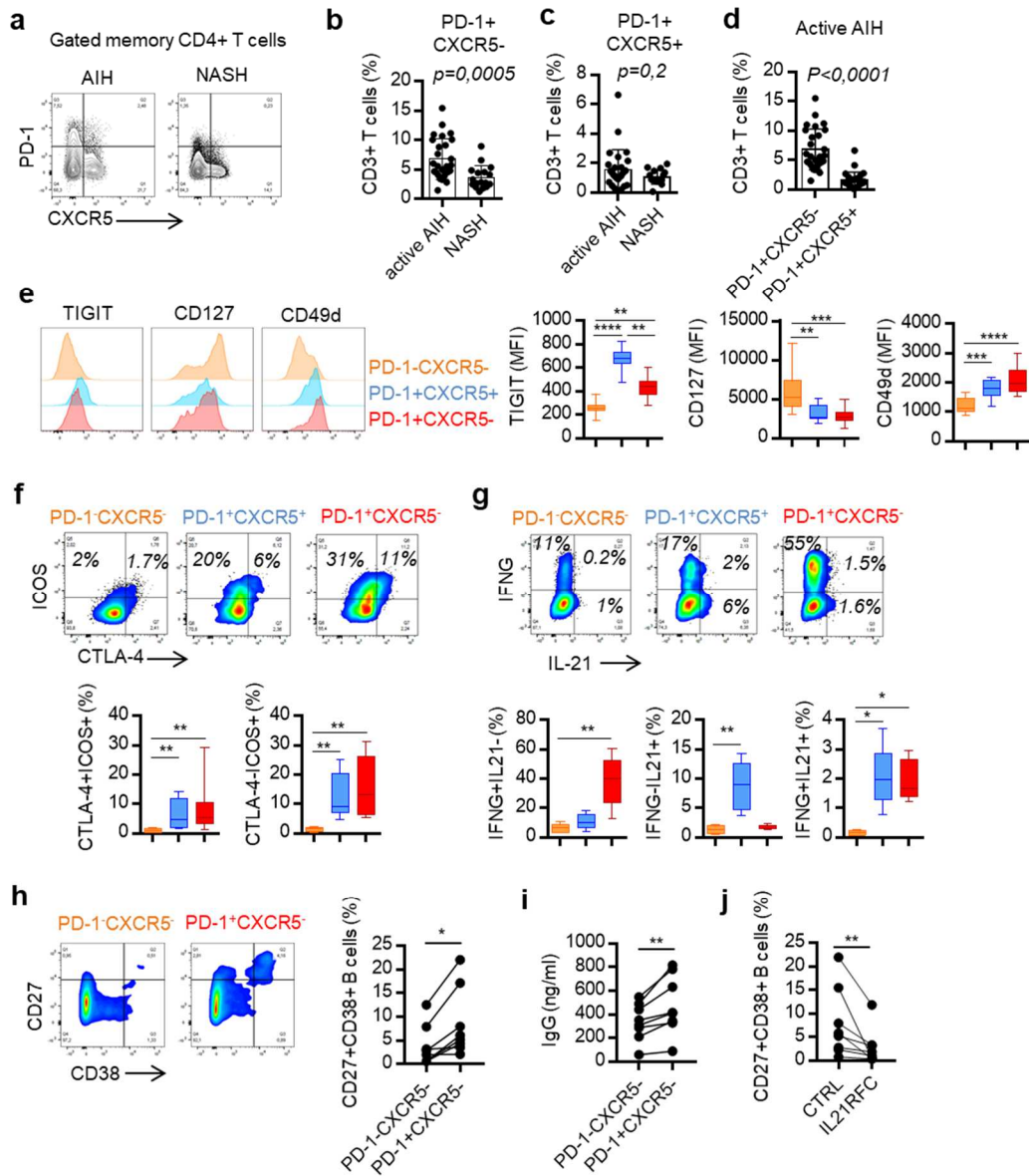


Figure 5. Memory PD-1⁺CXCR5⁻ CD4 T cells with a T peripheral helper (TPH) profile are enriched in the blood of AIH patients. a-d) Representative dot plots and graph of the CD45RA⁻PD-1⁺CXCR5⁻ (b and d) and the CD45RA⁻PD-1⁺CXCR5⁺ (c and d) CD4⁺ T cell populations in active AIH (n=27) and NASH (n=17) patients. e) Median of fluorescence intensity for indicated markers in indicated CD4 T cell subsets in AIH patients (n=12). f and g) Percentage of cells expressing ICOS and/or CTLA4 (f; n=8), and IFNG and/or IL21 (g; n=5) in indicated CD4 T cell subsets in AIH patients. h and j) Percentage of plasma cells (h, j; CD27⁺CD38⁺) and concentration of IgG (i) after a 7 day co-culture with autologous B cells and indicated memory CD4 T cell subsets with IL21RFC or without (CTRL) (j). Each point

represents a duplicate experiment per patient (n=8). The Mann-Whitney U test (b-d), the Kruskal-Wallis test and Dunn's Multiple Comparison Test (e-g) or the Wilcoxon matched-pairs signed rank test (h-j) were used. *:p<0.05; **:p<0.01; ***:p<0.001; ****:p<0.0001.

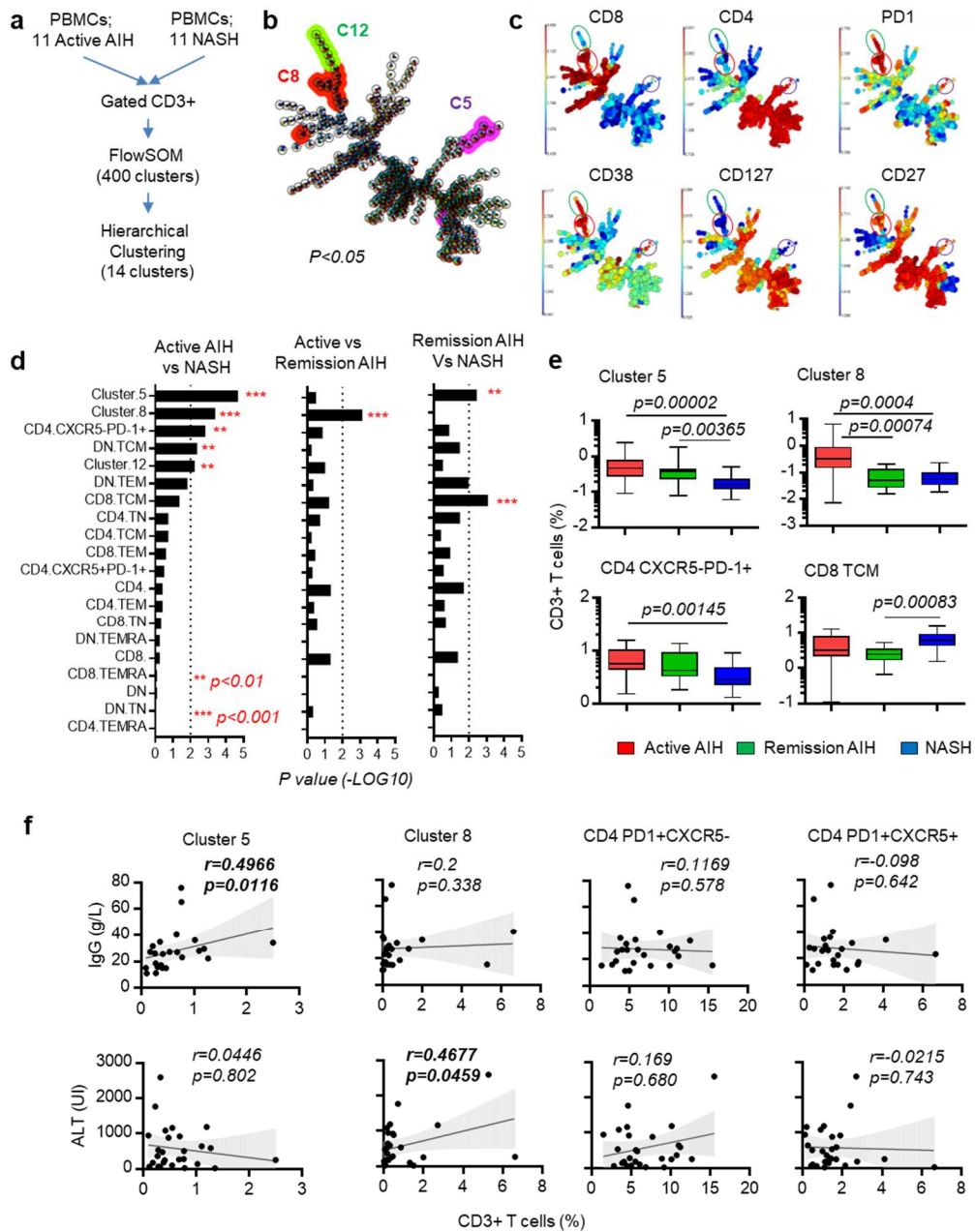


Figure 6. PD-1 and CD38 co-expression by memory CD45RA-CXCR5- CD127-CD27+ T cells is an immune correlate of AIH activity. a) Strategy of the flow cytometry analysis of the combined T cell population from n=11 active AIH patients and n=11 NASH patients. b) Self-organizing map visualization of the phenotypic analysis, highlighting the three metaclusters (C5, C8, C12) expanded in AIH patients ($p < 0.05$) after hierarchical clustering with FlowSOM. c) Expression of indicated markers visualized within the self-organizing map. d, e) Graph representation of the adjusted p-value (d) and of the frequency amongst CD3+ T

cells (e) for T cells subsets indicated after comparisons between Active AIH (n=27), Remission AIH (n=15) and NASH (n=17) patients, as indicated. f) Spearman correlation of the frequency of indicated T cell subsets with the serum IgG and ALT levels in active AIH patients (n=27). The Shapiro-Wilk normality test and the Bonferroni multiple test correction were used. **:p<0.01; ***:p<0.001.