IL-15-producing splenic B cells play pathogenic roles in the development of autoimmune hepatitis

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Graphical abstract
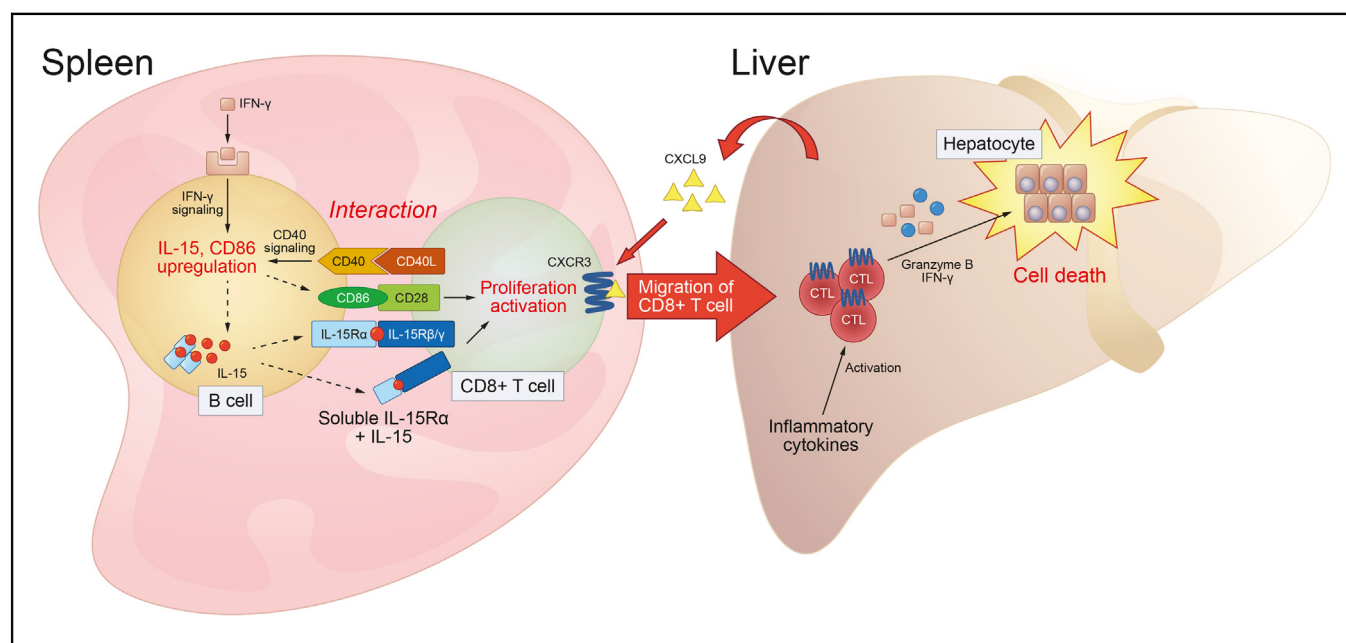
Highlights

- B cells highly expressed IL-15 in an experimental AIH model and patients with AIH.

- B cells exacerbated experimental AIH via the expansion of CD8^{+} T cells.

- IFN-γ and CD40 signalling were required for IL-15 expression in B cells.

- CD40L^{+}CD8^{+} T cells were expanded and in contact with B cells in the spleen.

- CD40L^{+}CD8^{+} T cells promoted IL-15 expression in B cells with the interaction.

Impact and Implications

IL-15-producing B cells were shown to exacerbate experimental AIH via cytotoxic T lymphocyte expansion. CD40L^{+}CD8^{+} T cells promoted IL-15 expression in B cells, indicating the mutual interaction of both cells. High serum IL-15 concentrations, IL-15^{+} B-cell counts, and CD40L^{+}IL-15Rα^{+}CD8^{+} T-cell counts were confirmed in the blood of patients with AIH.

IL-15-producing splenic B cells play pathogenic roles in the development of autoimmune hepatitis

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Background & Aims: B-cell depletion therapy with an anti-CD20 is an effective treatment strategy for patients with refractory autoimmune hepatitis (AIH). However, the mechanisms underlying B-cell action are unclear.

Methods: Herein, we used the adeno-associated virus IL-12 model, in which hepatic IL-12 expression triggers liver injuries characteristic of AIH. We also analysed the clinical samples of patients with AIH.

Results: B-cell depletion using anti-CD20 or splenectomy was found to improve liver functions and decrease the cytotoxic CD8$^+$ T-cell (cytotoxic T lymphocyte [CTL]) count in the liver. This improvement was reversed by the adoptive transfer of splenic B cells derived from AAV IL-12-treated mice to splenectomised mice as it caused the hepatic CTL count to increase. RNA-sequencing analysis identified IL-15 as a key factor in pathogenic B cells, which promotes CTL expansion and subsequent migration to the liver via the CXCL9/CXCR3 axis. Indeed, IL-15 neutralisation ameliorated hepatitis by suppressing splenic and hepatic CTLs *in vivo*. The close distribution of B220$^+$ B cells and CD8$^+$ T cells in the spleen of AIH mice suggested mutual interactions. Mechanistically, IFNγ and CD40L/CD40 signalling were indispensable for the expression of IL-15 in B cells, and *in vitro* co-culture experiments revealed that splenic CD40L$^+$CD8$^+$ T cells promoted IL-15 production in B cells, which led to CTL expansion. In patients with AIH, high serum IL-15 concentration and IL-15$^+$ B-cell counts, positively correlating with serum alanine aminotransferase levels, support translation and potential therapeutic targeting in human AIH.

Conclusions: This investigation elucidated the roles of IL-15-producing splenic B cells that occur in concert with pathogenic CD8$^+$ T cells during the development of AIH.

Impact and Implications: IL-15-producing B cells were shown to exacerbate experimental AIH via cytotoxic T lymphocyte expansion. CD40L$^+$CD8$^+$ T cells promoted IL-15 expression in B cells, indicating the mutual interaction of both cells. High serum IL-15 concentrations, IL-15$^+$ B-cell counts, and CD40L$^+$IL-15Rα^+CD8$^+$ T-cell counts were confirmed in the blood of patients with AIH.

Introduction

Autoimmune hepatitis (AIH) is an immune-mediated chronic liver disease caused by an autoimmune response to hepatocytes. It is characterised by high transaminase serum levels, elevated serum IgG levels, and interface hepatitis. Approximately 80% of patients respond well to a standard therapy of corticosteroids alone or in combination with azathioprine.[1,2] However, 20% of patients show no response, insufficient response, or intolerance (adverse effects) to standard therapy.[3]

Rituximab (anti-CD20) has been found to be effective when used with B-cell depletion therapy in non-responders who did not experience therapeutic effects with the standard therapy and in patients who were intolerant to steroids.[4–6] A similar therapeutic effect has been confirmed in paediatric patients with AIH, and rituximab is also considered a third-line treatment in the clinic.[7,8] Recently, belimumab, an antibody drug targeting the B-cell growth factor, has been reported to be an effective treatment for refractory AIH.[9] Accumulated evidence thus suggests that B-cell-targeting therapy is a promising approach for AIH treatment.

AIH is a T-cell-mediated disease in which antigen-activated CD4$^+$ and CD8$^+$ T cells differentiate and proliferate as effector cells.[2] B cells are also considered to contribute to autoimmunity through autoantibody production, antigen presentation, or cytokine production; however, the precise molecular mechanism has not yet been elucidated, possibly because an appropriate AIH

model had not yet been developed.[10] B-cell depletion exacerbated hepatitis in the S100-induced hepatitis model, which has been widely used in AIH studies; thus, the therapeutic effects of B-cell depletion could not be reproduced.[11] Similar results were obtained in AIH model mice using the NOD/Ltj line.[12] Although Beland *et al.*[13] successfully reproduced the therapeutic effects of B-cell depletion using their mouse model, specific functional molecules remain unidentified. In the current study, we used an adeno-associated virus (AAV) IL-12 model, in which liver-specific IL-12 expression triggers the onset of liver injuries characteristic of AIH.[14] In this experimental AIH (eAIH) model, C57BL/6 mice are i.v. injected with AAV8-expressing single-chain IL-12 under the control of a liver-specific promoter, followed by the development of chronic liver injury with B-cell differentiation.[14,15] The clear rise in the levels of alanine aminotransferase (ALT) and autoantibodies (anti-nuclear antibody and anti-smooth muscle antibody production), as well as interface hepatitis, were confirmed in this model.[14] Consequently, the AAV IL-12 model was selected for this study as it reproduces important characteristics of autoreactive B-cell-related AIH pathology.

IL-12 is an important factor for chronic inflammation and autoimmune diseases affected by Th1 (type 1 helper CD4+ T) and CD8^{+} T cells and has been acknowledged as an exacerbating factor in inflammatory bowel disease and rheumatoid arthritis.[16–18] Neutralising antibodies against IL-12 and IL-23 are used to treat inflammatory bowel disease. IL-12 has been recognised as a key factor contributing to AIH and has been used to construct multiple AIH models.[19,20] Using comprehensive transcriptome analyses, we successfully identified the molecular mechanisms underlying the interaction between B cells and cytotoxic CD8^{+} T cells in the spleen, which lead to the development of AIH.

Materials and methods

Animals
Male C57BL/6 mice (8–10 weeks old) were purchased from CLEA (Tokyo, Japan). Male and female C57BL/6-Ly5.1 mice (8–10 weeks old) were obtained from The Jackson Laboratory (ME, USA). All mice were maintained under specific-pathogen-free conditions with 12-h light/dark cycle in the Animal Care Facility of Keio University School of Medicine. All animal experiments complied with the relevant ethical regulations and were approved by the Animal Ethics Committee of Keio University (No. 14082).

Viral vectors and induction of eAIH (AAV IL-12 model)
AAV vectors and AAV8 particles were designed and created by VectorBuilder-Cyagen Biosciences Inc, according to previously described methods.[15,21–23] The AAV8 vector for inducing eAIH (AAV IL-12 vector) was constructed with a transgene encoding single-chain IL-12, whereas the control AAV8 vector (AAV Luc vector) was constructed with a transgene encoding luciferase as a control (Fig. S1). Transgene transcription is regulated by the chimeric albumin promoter (Ealb-Pa1AT); therefore, single-chain IL-12 or luciferase is expressed only in the liver. AAV Luc or AAV IL-12 were administered i.v. to each mouse at a dose of 5.0×10^9 viral genomes/kg. eAIH developed in 4–8 weeks.

Patients
Serum samples were obtained from healthy controls (HCs; n = 25), patients with non-alcoholic steatohepatitis (NASH; n = 10), and treatment-naive patients with AIH (n = 9). Peripheral blood mononuclear cells were obtained from blood samples of HCs (n = 9) and treatment-naive patients with AIH (n = 9). All patients were histologically diagnosed. AIH was diagnosed according to the criteria established by the International Autoimmune Hepatitis Group.[24] The clinical information is shown in Table S1A and B. NASH was diagnosed according to previously described criteria.[25] All samples were collected at the Keio University Hospital. Written informed consent was obtained from all the participants. The study protocol was approved by the institutional review board of Keio University Hospital (20120395) and conformed with the ethical guidelines of the 1975 Declaration of Helsinki.

Statistical analysis
Statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad Software Inc., San Diego, CA, USA). Differences between two groups were evaluated using two-sided unpaired Student's *t*-tests. Comparisons of more than two groups were performed using one-way ANOVA, followed by Tukey's multiple-comparisons test. For all analyses, significance was set at *p* <0.05. The sample sizes were determined based on previous studies.[14] Correlations were evaluated using the Spearman rank correlation.

Additional information on materials and methods can be found in the Supplementary methods and CTAT table.

Results

Numbers of functional T and B cells increase in the liver and spleen of the AAV IL-12 model
First, we created a similar AAV vector and constructed an AAV IL-12 model using previous studies as a reference (Fig. 1A and Fig. S1).[15,21–23] We analysed the pathology 1–8 weeks after AAV IL-12 administration to determine the appropriate endpoint for the eAIH model (Fig. 1A). Serum ALT and aspartate aminotransferase (AST) levels peaked at 4 weeks (Fig. 1B). Increased serum levels of ALT, IgG, and anti-nuclear antibody and interface hepatitis were also observed within 4 weeks (Fig. 1B–D). Additionally, liver mononuclear cell (LMNC) and splenocyte counts increased over time, suggesting lymphocyte proliferation, maturation, and subsequent migration into the liver (Fig. S3A). Among the immune cell subsets, the numbers of CD4^{+} T, CD8^{+} T, and B cells increased over time in both the liver and the spleen (Fig. 1E and F). Similar to other AIH models, monocytes/macrophages (MO/Mφ) and dendritic cell (DC) counts rose in the liver (Fig. S3A). Based on these results, we determined the appropriate endpoint of this model, and consequently, pathology was analysed at Week 4. At this point, the number of interferon-gamma (IFN-γ)[+]CD4^{+} T cells and IFN-γ^{+}/granzyme B^{+}CD8^{+} T cells had increased in both the liver and the spleen (Fig. 1G and H). In addition, plasma cell differentiation was confirmed in the AAV IL-12-administered mice (Fig. 1I), and CD95^{+}CD86^{+} B-cell counts were increased in the liver and spleen (Fig. 1J), as reported in patients with AIH.[26] We also detected AAV in the liver, and the expression of vector sequence mRNA peaked at Week 1 (Fig. S3B), whereas the expression of IL-12 gradually increased only in the liver and peaked at Week 4 (Fig. S3C). The results suggest that this model is suitable for exploring the mechanisms related to T and B cells in AIH pathology.
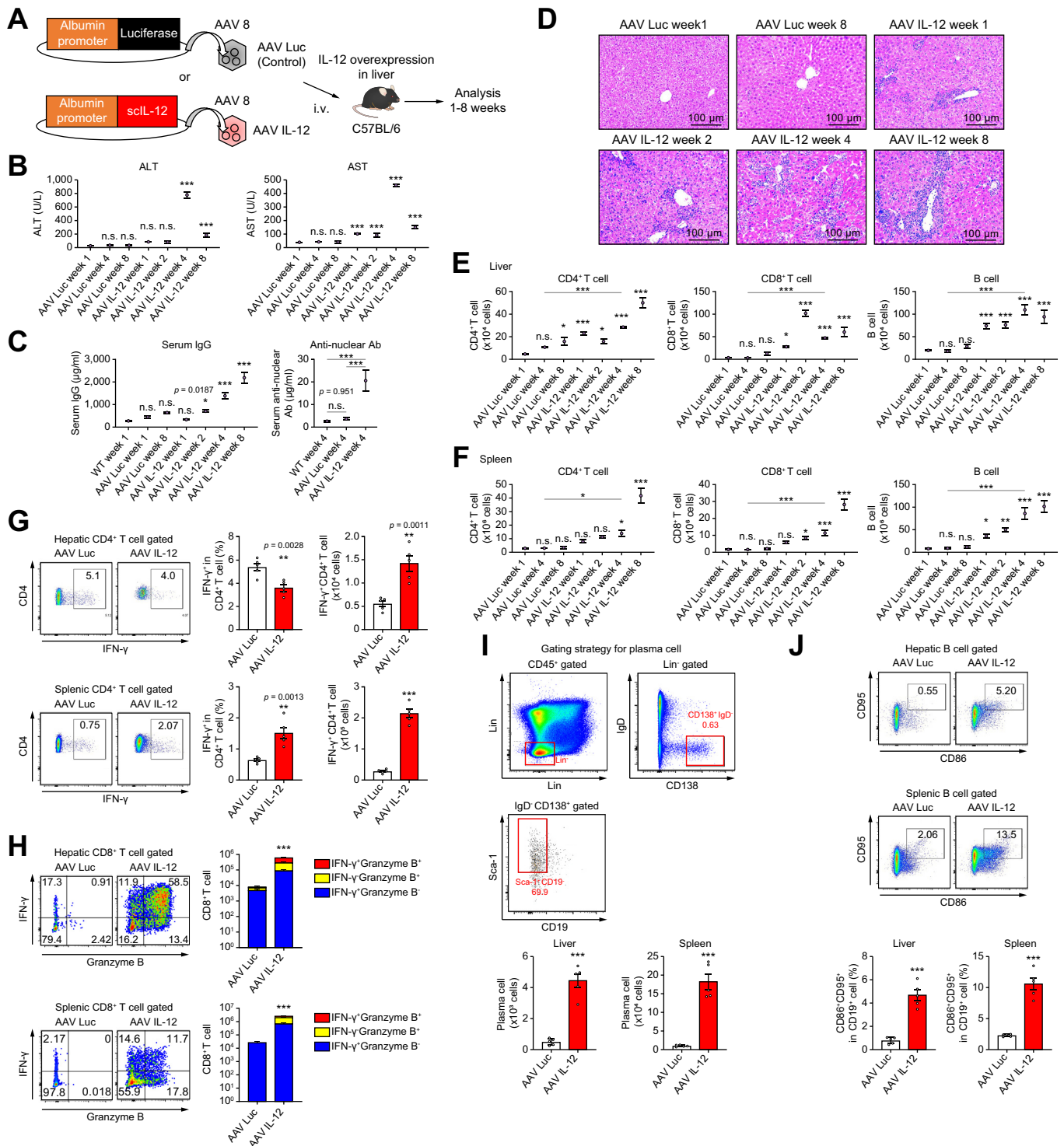
Fig. 1. Numbers of functional T cells and B cells increased in both the liver and spleen of AAV IL-12 model mice. (A–F) AAV Luc (control AAV) or AAV IL-12 was administered i.v. into C57BL/6 mice. A series of evaluations were conducted from 1 to 8 weeks (n = 4–5). (G–J) Evaluations were conducted at Week 4 (n = 5). (A) Schematic representation of the AAV IL-12 model. (B) Serial analysis of serum ALT and AST levels after administration of AAV Luc or AAV IL-12 at the indicated time points. (C) Serial analysis of serum IgG and anti-nuclear Ab levels at Week 4. (D) Representative H&E staining of liver sections. (E and F) Number of hepatic/splenic CD4$^+$ T, CD8$^+$ T, and B cells. (G) Representative flow-cytometry staining and the proportion and the number of hepatic/splenic IFN-γ^+CD4$^+$ T cells. (H) Representative flow-cytometry staining and the number of IFN-γ^+/granzyme B$^+$ among hepatic/splenic CD8$^+$ T cells. (I) Gating strategy for plasma cell and plasma cell counts in the liver and spleen. Plasma cells were identified as part of the CD45$^+$leneage$^-$ (CD4$^-$CD8$^-$Gr-1$^-$F4/80$^-$TER119$^-$) IgD$^-$CD138$^+$ Sca-1$^+$CD19$^-$ cells. (J) Representative flow-cytometry staining and the proportion of CD86$^+$CD95$^+$ among B cells in the liver and spleen. Data are presented as the mean ± SEM. *p <0.05, **p <0.01, and ***p <0.001. One-way ANOVA with Tukey's multiple-comparisons *post hoc* test (B, C, E, and F) or unpaired two-tailed Student's *t* tests (G–J) was applied. AAV, adeno-associated virus; Ab, antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IFN-γ, interferon-gamma; scIL-12, single-chain IL-12.
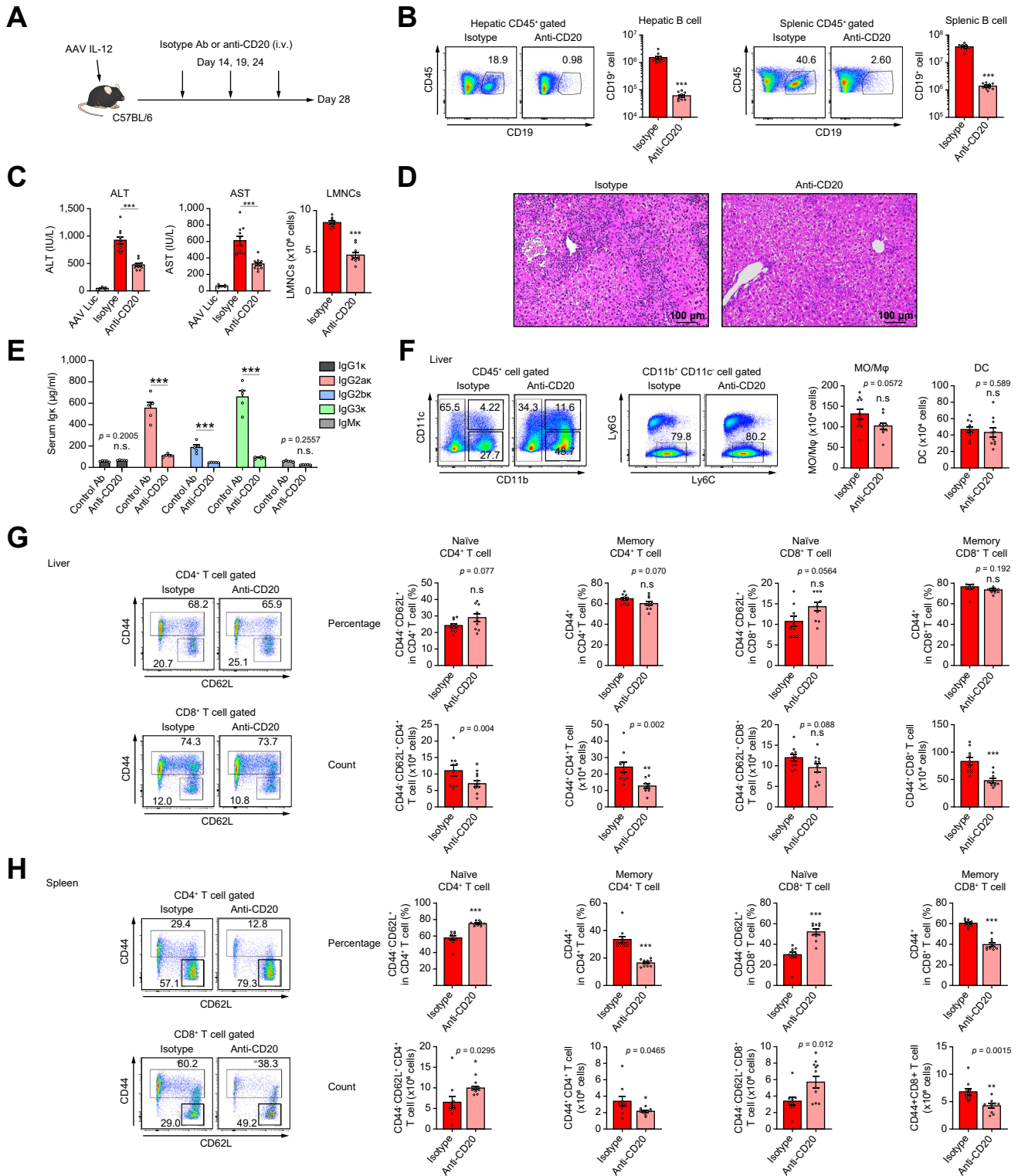
Fig. 2. Therapeutic depletion of B cells reduced the number of hepatic T cells and ameliorated AIH pathology. (A) Study design: anti-CD20 or isotype Ab was i.v. administered to mice 14, 19, and 24 days after AAV IL-12 administration (n = 5 for the AAV Luc group; n = 10 for the isotype group or anti-CD20 group). (B) Representative CD45/CD19 staining of CD45$^+$ gated LMNCs and splenocytes, and hepatic/splenic B-cell count. (C) Serum ALT levels, AST levels, and LMNC counts. AAV Luc means control AAV-treated mice. (D) Representative H&E staining of the liver sections. (E) Concentrations of each serum Igκ (n = 5). (F) MO/Mφ and DC counts in the liver. (G and H) Representative CD62L and CD44 staining in hepatic (G) and splenic (H) CD4$^+$ and CD8$^+$ T cells. Proportion and number of CD44$^-$CD62L$^+$ (naive) or CD44$^+$ (memory) cells among the splenic CD4$^+$ T and CD8$^+$ T cells are also shown. Data are presented as the mean ± SEM. $^*p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001. Unpaired two-tailed Student's t tests (B, C, and F–H) were applied. Data are combined from two independent experiments. AAV, adeno-associated virus; Ab, antibody; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DC, dendritic cell; LMNC, liver mononuclear cell; MO/Mφ, monocytes/macrophages.
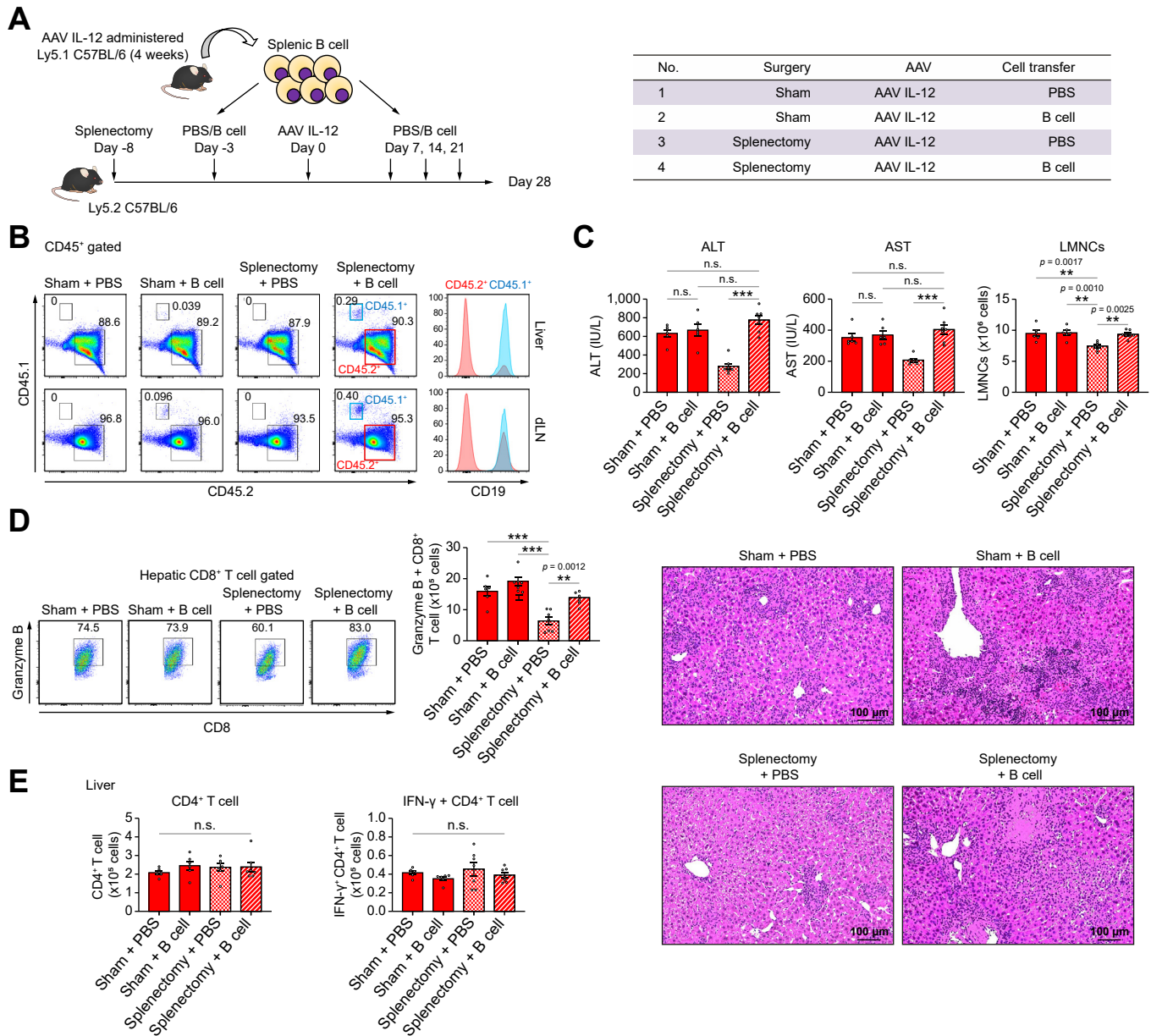
Fig. 3. Adoptive transfer of pathogenic B cells increased hepatic CTL and exacerbated hepatitis in splenectomised mice. (A) Study design: pathogenic B cells were isolated from the spleens of eAIH Ly5.1 mice (donor). Splenectomy was performed on Day -8, and pathogenic B cells or vehicle (PBS) was injected i.v. to Ly5.2 mice (host) on Days -3, 7, 14, and 21. AAV IL-12 was administered on Day 0, followed by evaluation on Day 28 (n = 6 for the sham group; n = 7 for the splenectomy group). (B) Detection of transferred CD45.1⁺ B cells. Representative flow-cytometry staining and the histogram of CD19 staining of CD45.1⁺ or CD45.2⁺ gated LMNCs or liver dLN cells. (C) Serum ALT levels, LMNC counts, and representative H&E staining of liver sections. (D) Representative flow-cytometry staining and the number of granzyme B⁺CD8⁺ T cells in the liver. (E) CD4⁺ T-cell and IFN-γ⁺CD4⁺ T-cell counts. Data are presented as the mean ± SEM. **p <0.01 and ***p <0.001. One-way ANOVA with Tukey's multiple-comparisons *post hoc* test (C–E) was applied. Data are combined from two independent experiments. AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CTL, cytotoxic T lymphocyte; dLN, draining lymph node; eAIH, experimental autoimmune hepatitis; IFN-γ, interferon-gamma; LMNC, liver mononuclear cell.

Therapeutic depletion of B cells reduced hepatic T-cell numbers and ameliorated AIH pathology

Previous reports have shown the therapeutic effects of B-cell depletion using rituximab (anti-CD20 antibody) in patients with refractory AIH.[4–7] Although the efficacy of B-cell depletion has also been demonstrated in an eAIH model, the precise underlying mechanisms are unknown.[13] We also examined the efficacy of anti-CD20 with respect to hepatitis in the AAV IL-12 model

(Fig. 2A). Treatment with anti-CD20 was sufficient to ablate B cells in the liver and spleen (Fig. 2B). A partial amelioration of hepatitis was confirmed based on the results of serological and histological analyses, with a decreased number of LMNCs (Fig. 2C and D). Notably, decreased serum IgG levels were observed in multiple subtypes (Fig. 2E). Anti-CD20 treatment also decreased memory T-cell counts without affecting hepatic myeloid cells counts (Fig. 2F–H). The partial therapeutic effect may be
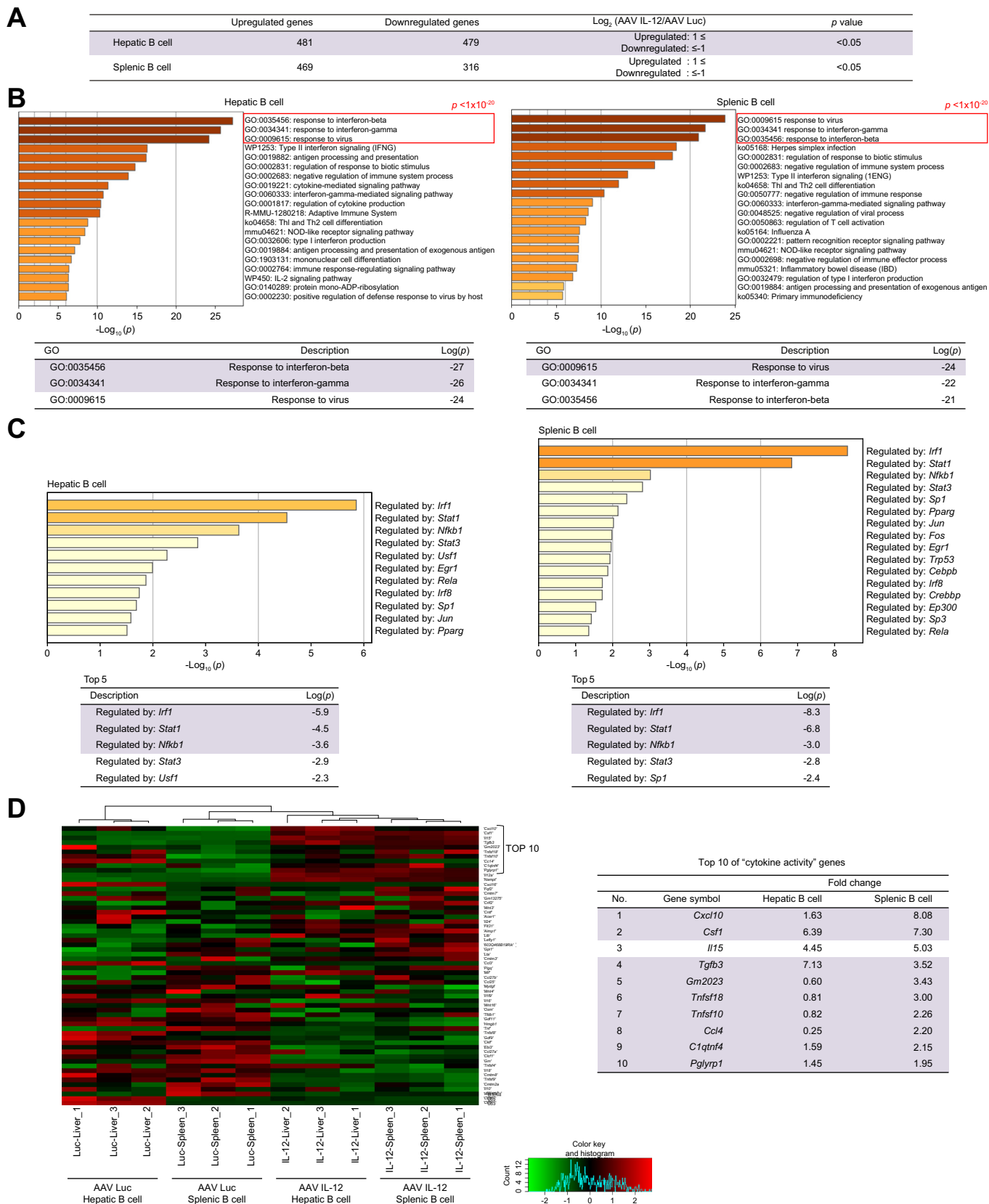
A

	Upregulated genes	Downregulated genes	Log$_2$ (AAV IL-12/AAV Luc)	p value
Hepatic B cell	481	479	Upregulated: 1 ≤ Downregulated: ≤-1	<0.05
Splenic B cell	469	316	Upregulated: 1 ≤ Downregulated : ≤-1	<0.05

B

Hepatic B cell — $p < 1 \times 10^{-20}$

GO:0035456: response to interferon-beta
GO:0034341: response to interferon-gamma
GO:0009615: response to virus
WP1253: Type II interferon signaling (IFNG)
GO:0019882: antigen processing and presentation
GO:0002831: regulation of response to biotic stimulus
GO:0002683: negative regulation of immune system process
GO:0019221: cytokine-mediated signaling pathway
GO:0060333: interferon-gamma-mediated signaling pathway
GO:0001817: regulation of cytokine production
R-MMU-1280218: Adaptive Immune System
ko04658: Th1 and Th2 cell differentiation
mmu04621: NOD-like receptor signaling pathway
GO:0032606: type I interferon production
GO:0019884: antigen processing and presentation of exogenous antigen
GO:1903131: mononuclear cell differentiation
GO:0002764: immune response-regulating signaling pathway
WP450: IL-2 signaling pathway
GO:0140289: protein mono-ADP-ribosylation
GO:0002230: positive regulation of defense response to virus by host

-Log$_{10}$(p)

GO	Description	Log(p)
GO:0035456	Response to interferon-beta	-27
GO:0034341	Response to interferon-gamma	-26
GO:0009615	Response to virus	-24

Splenic B cell — $p < 1 \times 10^{-20}$

GO:0009615 response to virus
GO:0034341 response to interferon-gamma
GO:0035456: response to interferon-beta
ko05168: Herpes simplex infection
GO:0002831: regulation of response to biotic stimulus
GO:0002683: negative regulation of immune system process
WP1253: Type II interferon signaling (1ENG)
ko04658: Th1 and Th2 cell differentiation
GO:0050777: negative regulation of immune response
GO:0060333: interferon-gamma-mediated signaling pathway
GO:0048525: negative regulation of viral process
GO:0050863: regulation of T cell activation
ko05164: Influenza A
GO:0002221: pattern recognition receptor signaling pathway
mmu04621: NOD-like receptor signaling pathway
GO:0002698: negative regulation of immune effector process
mmu05321: Inflammatory bowel disease (IBD)
GO:0032479: regulation of type I interferon production
GO:0019884: antigen processing and presentation of exogenous antigen
ko05340: Primary immunodeficiency

-Log$_{10}$(p)

GO	Description	Log(p)
GO:0009615	Response to virus	-24
GO:0034341	Response to interferon-gamma	-22
GO:0035456	Response to interferon-beta	-21

C

Hepatic B cell

Regulated by: *Irf1*
Regulated by: *Stat1*
Regulated by: *Nfkb1*
Regulated by: *Stat3*
Regulated by: *Usf1*
Regulated by: *Egr1*
Regulated by: *Rela*
Regulated by: *Irf8*
Regulated by: *Sp1*
Regulated by: *Jun*
Regulated by: *Pparg*

-Log$_{10}$(p)

Top 5

Description	Log(p)
Regulated by: *Irf1*	-5.9
Regulated by: *Stat1*	-4.5
Regulated by: *Nfkb1*	-3.6
Regulated by: *Stat3*	-2.9
Regulated by: *Usf1*	-2.3

Splenic B cell

Regulated by: *Irf1*
Regulated by: *Stat1*
Regulated by: *Nfkb1*
Regulated by: *Stat3*
Regulated by: *Sp1*
Regulated by: *Pparg*
Regulated by: *Jun*
Regulated by: *Fos*
Regulated by: *Egr1*
Regulated by: *Trp53*
Regulated by: *Cebpb*
Regulated by: *Irf8*
Regulated by: *Crebbp*
Regulated by: *Ep300*
Regulated by: *Sp3*
Regulated by: *Rela*

-Log$_{10}$(p)

Top 5

Description	Log(p)
Regulated by: *Irf1*	-8.3
Regulated by: *Stat1*	-6.8
Regulated by: *Nfkb1*	-3.0
Regulated by: *Stat3*	-2.8
Regulated by: *Sp1*	-2.4

D

Top 10 of "cytokine activity" genes

No.	Gene symbol	Fold change	
		Hepatic B cell	Splenic B cell
1	*Cxcl10*	1.63	8.08
2	*Csf1*	6.39	7.30
3	*Il15*	4.45	5.03
4	*Tgfb3*	7.13	3.52
5	*Gm2023*	0.60	3.43
6	*Tnfsf18*	0.81	3.00
7	*Tnfsf10*	0.82	2.26
8	*Ccl4*	0.25	2.20
9	*C1qtnf4*	1.59	2.15
10	*Pglyrp1*	1.45	1.95

Color key and histogram
Count

Fig. 4. Gene expression characteristics of pathogenic B cell in the AAV IL-12 model. (A) Number of upregulated and downregulated genes identified in pathogenic hepatic/splenic B cells compared with the control B cells. (B) Pathway and process enrichment analysis of upregulated genes in pathogenic hepatic and splenic B cells. (C) TRRUST analysis of upregulated genes in pathogenic hepatic and splenic B cells. (D) Heat map of 'Cytokine activity' gene expression and the top 10 upregulated 'Cytokine activity' genes. AAV, adeno-associated virus; GO, Gene Ontology; TRRUST, Transcriptional regulatory relationships unravelled by sentence-based text-mining.

attributable to residual inflammatory myeloid cells. Memory T-cell counts decreased in the liver and spleen, whereas the proportion of memory T cells decreased only in the liver (Fig. 2G and H). These results suggest that B cells play a pathogenic role in the development of hepatitis by affecting T cells.

Adoptive transfer of pathogenic B cells increased hepatic CD8+ T-cell count and exacerbated hepatitis in splenectomised mice

We hypothesised that B cells play a pathogenic role especially in the spleen owing to their high abundance in this organ. Thus, we adopted a splenectomy model to exclude splenic B cells. To clarify whether splenic B cells contribute to pathogenesis, we examined the effects of the adoptive transfer of splenic B cells to splenectomised mice with AAV IL-12 administration. For this experiment, B cells were isolated from the spleens of Ly5.1+ mice administered with AAV IL-12, and Ly5.2+ mice were used as hosts (Fig. 3A). Transferred Ly5.1+ B cells were detected in the liver and liver-draining lymph nodes of the host mice (Fig. 3B). Although ALT levels were decreased by splenectomy, the adoptive transfer of pathogenic B cells ablated this improvement to the same level as that in sham-treated mice (Fig. 3C). Liver histology and hepatic granzyme B+CD8+ T-cell counts revealed a similar trend (Fig. 3C and D), whereas the hepatic CD4+ T-cell counts did not change (Fig. 3E). These results indicated that splenic B cells play a substantial role in the development of hepatitis via the expansion of CD8+ T cells (CTLs). We also confirmed that CD8+ T-cell depletion ameliorated eAIH (Fig. S4A–C). Furthermore, we evaluated B-cell function using the concanavalin A-induced acute hepatitis model as hosts (Fig. S5A and B). Pathogenic B cells were isolated from the spleens of Ly5.1 mice administered AAV IL-12. Consistent with the result of the AAV IL-12 model, the transfer of splenic B cells ablated the reduction in the severity of liver injuries by splenectomy with an increase in hepatic CTLs (Fig. S5C–E).

IL-15 expression increased in the pathogenic B cells of the AAV IL-12 model mice

Next, we performed RNA-sequencing (RNA-seq) analysis to identify the essential molecules expressed in B cells that contribute to the pathology. B cells were sorted from the liver and spleen of control or eAIH mice and used for comparative analysis (Fig. 4A). Enrichment analysis was performed to identify the key bioprocesses related to the upregulated genes. Process enrichment analysis showed that 'response to interferon-beta' and 'response to interferon-gamma' were accelerated in both pathogenic hepatic and splenic B cells when compared with the control B cells (Fig. 4B). Transcriptional factor analysis indicated that Ifr1 and Stat1, which are regulated by IFN signalling, might be active (Fig. 4C). To identify the functional molecules, we selected a series of genes under the Gene Ontology term 'Cytokine activity' and found that the expression of IL-15 was significantly upregulated in pathogenic B cells (Fig. 4D and Table S2). IL-15 is a well-known cytokine that contributes to the maintenance and proliferation of memory CD8+ T cells and CTLs.[27] Thus, we hypothesised that B-cell-derived IL-15 plays a key role in the induction of CTL and the subsequent development of eAIH.

The increased protein expression of IL-15 in hepatic/splenic B cells was confirmed by flow cytometry (Fig. 5A). B cells accounted for approximately 55% of the IL-15-expressing immune cells in the spleen of eAIH mice (Fig. 5B). In the liver, by contrast, macrophages and neutrophils are the main IL-15-

producing cells, whereas B cells accounted for less than 1% (Fig. S6A). Among splenic B cells, CD86+CD95+ B cells showed higher IL-15 expression (Fig. 5C). Furthermore, the expression of the IL-15 receptor (IL-15Rα and IL-15Rβ) was elevated in both hepatic/splenic CD4+ and CD8+ T cells of eAIH mice, with the highest expression in splenic CD8+ T cells, suggesting higher sensitivity to IL-15 signalling (Fig. 5D, and Fig. S6A and B). Among splenic and hepatic CD8+ T cells, the proportion and number of IL-15Rα+IFN-γ+/granzyme B+CD8+ T cells were markedly increased in eAIH mice (Fig. 5E). Next, we examined the localisation of CD8+ T cells and B cells using immunostaining. Lymphocytes formed a systemic structure in the spleen of control mice (Fig. 5F). By contrast, the structure of the B220+ B-cell region was replaced by the global distribution of expanded B cells with the scattered localisation of mixed B cells and T cells in eAIH mice (Fig. 5F). Enlarged images confirmed that B cells and CD8+ T cells were closely localised (Fig. 5F). Lymphocytes appear to infiltrate around the portal vein of the liver in eAIH mice, but the proximity of the B cells and CD8+ T cells was less evident when compared with that in the spleen (Fig. 5G). The close distribution of B cells and CD8+ T cells in the spleen suggests a direct interaction and contribution to the development of hepatitis.

IL-15 neutralisation ablated AIH by decreasing hepatic CTLs

To verify whether CTL proliferation and the subsequent development of hepatitis depend on IL-15, we examined the effects of IL-15 neutralisation in vivo (Fig. 6A). As expected, serum ALT levels were significantly low in mice administered with anti-IL-15 (Fig. 6B). Splenocyte count decreased through IL-15 neutralisation, whereas the LMNCs were unaffected, suggesting that anti-IL-15 has a more potent effect on the spleen (Fig. 6B). Consistently, total CD8+ T-cell, CD44+IL-15Rα+CD8+ T-cell, and granzyme B+ CD8+ T-cell counts were decreased in the spleen and liver (Fig. 6C and E). There was no significant change in the number of IL-15+ B cells, which is the producer of IL-15 (Fig. 6D). Unlike CD8+ T-cell counts, the hepatic CD4+ T-cell counts did not change, indicating that IL-15 neutralisation was more effective for the accumulation of CD8+ T cells in the liver (Fig. 6E).

Splenic B cells promoted the proliferation and maintenance of memory CD8+ T cells via IL-15

To verify the interaction between B and CD8+ T cells, splenic CD8+ T cells derived from eAIH mice were co-cultured with splenic B cells isolated from eAIH or control mice (Fig. 7A). In this experiment, highly activated splenic CD8+ T cells cannot survive because of the weak CD3/CD28 stimulation in vitro (Fig. 7B). The proliferation (carboxyfluorescein diacetate succinimidyl ester (CFSE) low) and maintenance of CD44+CD8+ T cells were induced by co-culture with pathogenic B cells, but not with control B cells (Fig. 7B). Notably, the neutralisation of IL-15 suppressed the proliferation of CD44+CD8+ T cells (Fig. 7B). Furthermore, we confirmed that hepatic IL-15Rα+CD8+ T-cell count had decreased by splenectomy and was recovered by the adoptive transfer of splenic B cells in our in vivo experiment (Fig. 7C). These results suggest that IL-15 derived from splenic B cells contributes to the proliferation and maintenance of memory CD8+ T cells.

However, it is still unclear how the production of IL-15 in B cells was regulated. As demonstrated, RNA-seq analysis showed that IFN and NF-κB signalling were enhanced in B cells of eAIH mice (Fig. 4B and C). Therefore, B cells were stimulated with IFN-γ, which was markedly upregulated among IFN-related genes in the liver and spleen at the onset of hepatitis (Week 4) (Fig. S7A),
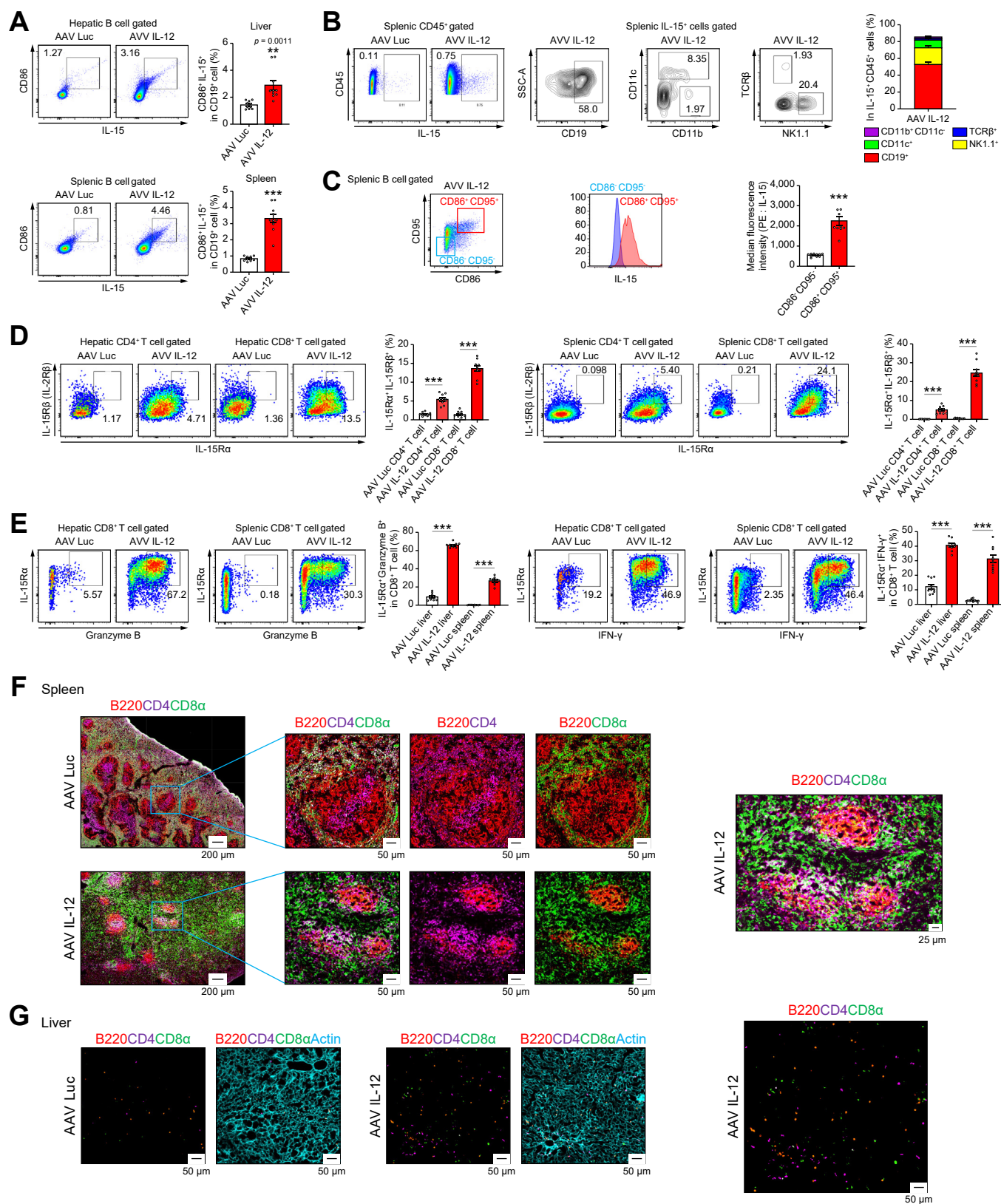
Fig. 5. Pathogenic B cells express IL-15 at the protein level, and splenic CD8⁺ T cells highly express its receptor. (A) Representative flow-cytometry staining and the proportion of CD86⁺IL-15⁺ among B cells in the liver and spleen (n = 10). (B) Representative flow-cytometry staining and the proportion of CD19⁺, CD11b⁺, CD11c⁺, TCRβ⁺, and NK1.1⁺ among splenic IL-15⁺CD45⁺ cells (n = 10). (C) Median fluorescence intensity of IL-15 in splenic CD86⁻CD95⁻ or CD86⁺CD95⁺ B cells (n = 10). (D) Representative flow-cytometry staining and the proportion of IL-15Rα⁺IL-15Rβ⁺ among hepatic/splenic CD4⁺ T cells and CD8⁺ T cells (n = 10). (E) Representative flow-cytometry staining and the proportion of granzyme B⁺IL-15Rα⁺ and IFN-γ⁺IL-15Rα⁺ among hepatic/splenic CD8⁺ T cells (n = 10). (F and G)
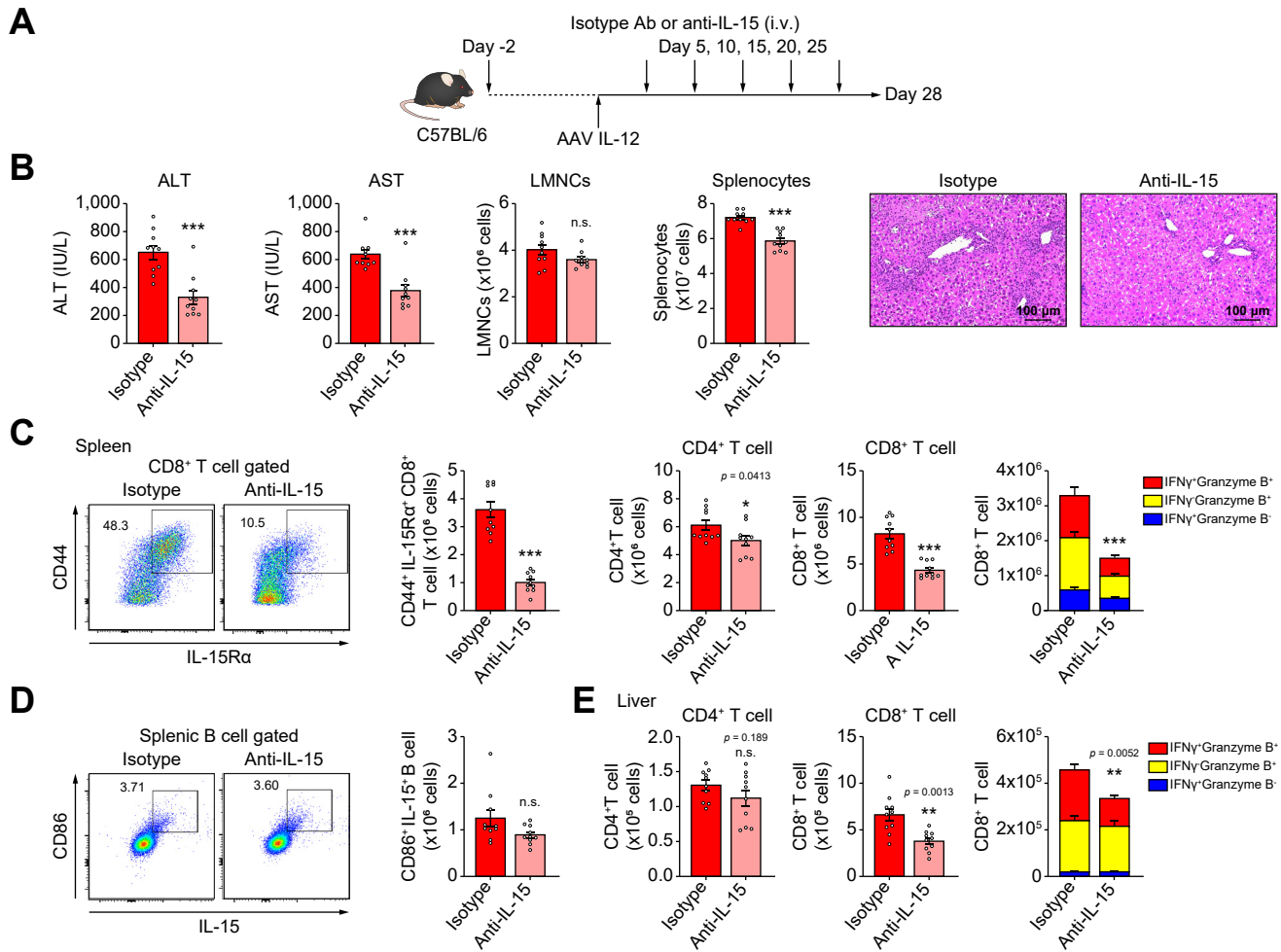
Fig. 6. IL-15 neutralisation ablated AIH by decreasing hepatic CTL numbers. (A) Study design: AAV IL-12 was administered to mice 2 days after i.v. administration of anti-IL-15 or isotype Ab. Each Ab was then administered i.v. every 5 days for 4 weeks (n = 10). (B) Serum ALT levels, LMNC counts, splenocyte counts, and H&E staining of the liver sections. (C) Representative flow-cytometry staining and the number of CD4$^+$ T cells, CD8$^+$ T cells, CD44$^+$IL-15Rα$^+$CD8$^+$ T cells, and IFN-γ$^+$/granzyme B$^+$ CD8$^+$ T cells in the spleen. (D) Representative flow-cytometry staining and number of CD86$^+$IL-15$^+$ B cells in the spleen. (E) The number of CD4$^+$ T cells, CD8$^+$ T cells, and IFN-γ+/granzyme B$^+$CD8$^+$ T cells. Data are presented as the mean ± SEM. *p <0.05, **p <0.01, and ***p <0.001. Unpaired two-tailed Student's t tests (B–E) were applied. Data are combined from two independent experiments. AAV, adeno-associated virus; Ab, antibody; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-gamma; IL-15R, IL-15 receptor; LMNC, liver mononuclear cell.

and anti-CD40 (agonist antibody) as B-cell-related inducers of NF-κB signalling.[28] The combined stimulation with IFN-γ and anti-CD40 upregulated the gene expression of *Il15, Cxcl10*, and *Csf1* in the splenic B cells isolated from wild-type mice (Fig. 7D), which were identified as the top three 'cytokine activity' genes in the RNA-seq analysis. These results suggested that CD40L$^+$ cells play an important role in the induction of IL-15$^+$ B cells. Given that CD86 expression was also upregulated by the same stimuli, this subset of CD86$^+$ B cells might produce IL-15, which aligns with our *in vivo* results (Fig. 5C). Flow-cytometry analysis showed that splenic CD8$^+$ T cells exhibited upregulated expression of CD40L in eAIH (Fig. 7E). Most splenic CD40L$^+$CD8$^+$ T cells

were IL-15Rα$^+$, suggesting that they were responsive to IL-15 (Fig. 7F). Of the splenic CD8$^+$ T cells, the proportion of CD40L$^+$IL-15Rα$^+$ cells were higher in the central memory T-cell and effector memory T-cell subsets (Fig. S7B). Additionally, the co-expression of CD8α and CD40L and their close distribution to B220$^+$ B cells were observed in the spleen of eAIH mice by immunohistochemistry (Fig. S7C). Co-culture of CD8$^+$ T cells and B cells isolated from the spleen of eAIH mice in the presence of IFN-γ revealed that CD8$^+$ T cells increased the proportion of CD86CD95$^+$ and IL-15$^+$ B cells, whereas CD40L neutralisation significantly decreased each proportion, supporting our hypothesis (Fig. 7G and H). These results collectively suggest that

Representative fluorescent photomicrographs of the spleen (F) and liver (G) from control mice and eAIH mice, stained with anti-CD8α (green), anti-B220 (B-cell marker; red), anti-CD4 (magenta), and phalloidin (staining actin filaments; light blue). Data are presented as the mean ± SEM. **p <0.01 and ***p <0.001. Unpaired two-tailed Student's t tests (A and C–E) were applied. Data are combined from two independent experiments. AAV, adeno-associated virus; eAIH, experimental autoimmune hepatitis; IFN-γ, interferon-gamma: IL-15R, IL-15 receptor.
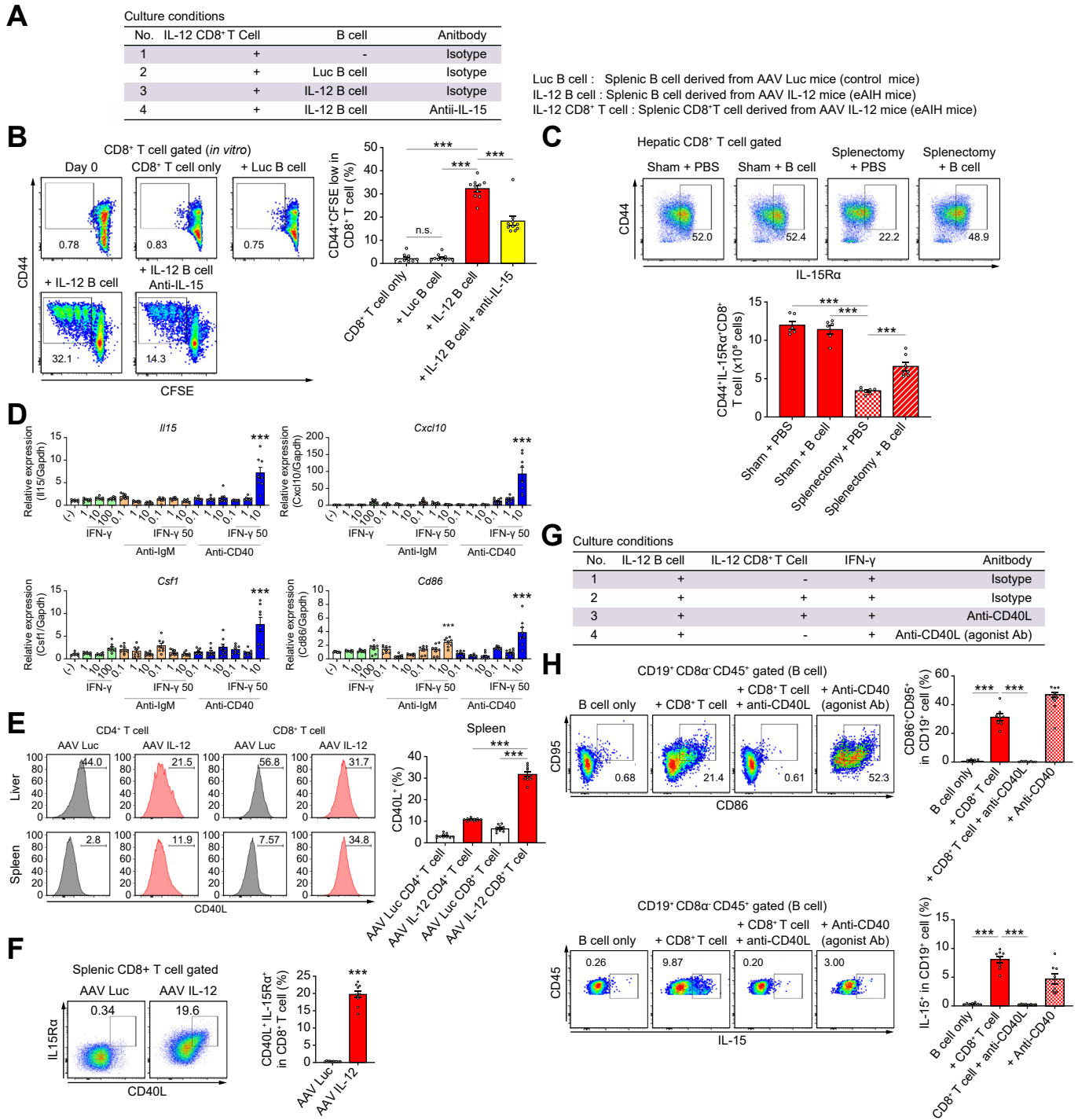
Fig. 7. IL-15⁺ B cells interacted with CD40⁺CD8⁺ T cells. (A) Condition of B cells and CD8⁺ T cells co-cultured for IL-15 neutralisation. CD8⁺ T cells were isolated from the spleens of eAIH mice and co-cultured with splenic B cells of control mice or eAIH mice (n = 10). (B) Representative flow-cytometry staining and the proportion of CD44⁺CFSE^{low} among cultured CD8⁺ T cells in each condition. (C) Hepatic CD44⁺IL-15Rα⁺CD8⁺ T-cell counts in sham/splenectomised mice with injection of PBS/pathogenic B cells (n = 6 for the sham group; n = 7 for the splenectomy group). (D) B cells were isolated from the spleens of wild-type mice and stimulated with 1–100 ng/ml IFN-γ, 0.1–10 μg/ml anti-IgM, or 0.1–10 μg/mL anti-CD40 agonist antibody (n = 10). For combined stimulation, B cells were exposed to 50 ng/ml IFN-γ. mRNA expression of *Il15*, *Cxcl10*, *Csf1*, and *Cd86* in cultured B cells was quantified. (E) The proportion of CD40L⁺ among CD4⁺ T or CD8⁺ T cells in the liver and spleen following AAV Luc or AAV IL-12 administration (n = 10). (F) Representative flow-cytometry staining and proportion of CD40L⁺IL-15Rα⁺ among the CD8⁺ T cells in the spleen (n = 10). (G) The condition of the B cells and CD8⁺ T cells co-cultured for CD40L neutralisation. B cells and CD8⁺ T cells were isolated from the spleens of eAIH mice (n = 8). (H) Representative flow-cytometry staining and the proportion of CD86⁺CD95⁺ and IL-15⁺ among cultured B cells. Data are presented as the mean ± SEM. ***p <0.001. One-way ANOVA with Tukey's multiple-comparisons *post hoc* test (B–E and H) or unpaired two-tailed Student's *t* tests (F) were applied. Data are combined from two independent experiments. AAV, adeno-associated virus; CFSE, carboxyfluorescein diacetate succinimidyl ester; eAIH, experimental autoimmune hepatitis; IFN-γ, interferon-gamma; IL-15R, IL-15 receptor.

splenic IL-15+ B cells and CD40L+ CD8+ T cells mutually interact to acquire pathological functions.

CD8+ T cells expanded by IL-15 in the spleen migrated into the liver in a CXCL9/CXCR3-dependent manner

We hypothesise that memory CD8+ T cells and CTLs proliferate by induction from splenic B cells and subsequently migrate to the liver to damage hepatocytes. We tested this hypothesis by investigating the underlying mechanisms regulating migration into the liver. C-X-C motif chemokine receptor 3 (CXCR3) expression was characteristically increased in the CD8+ T cells of the inflamed liver (Fig. S8A and B). In thymic CD8+ T cells, this tendency was not observed, suggesting that the thymus was not the direct source of CXCR3+ CD8+ T cells (Fig. S8C). The gene expression of CXCR3 ligands also increased in the liver, and *Cxcl9* was upregulated most prominently (Fig. S8D). Although there were other upregulated chemokines, elevation of the corresponding receptors in hepatic CD8 T cells could not be confirmed (Fig. S8A). Therefore, we evaluated the effect of C-X-C motif ligand 9 (CXCL9) neutralisation *in vivo* (Fig. S9A). Anti-CXCL9 administration ameliorated eAIH (Fig. S9B). Although the LMNC count was decreased by anti-CXCL9, the splenocyte count did not change significantly (Fig. S9C). Anti-CXCL9 treatment significantly decreased the numbers of hepatic granzyme B+CD8+ T cells and IL-15Rα+CXCR3+CD8+ T cells, which received IL-15 and had the potential to migrate into the liver and function as CTLs (Fig. S9D and F). Interestingly, the number of each type of CD8+ T cells in the spleen remained unchanged after the

administration of the anti-CXCL9 (Fig. S9E and G). The results in this model indicate that the CXCL9/CXCR3 axis plays a key role in the development of hepatitis through the migration of CD8+ T cells into the inflamed liver. Notably, IL-15 neutralisation maintained the high expression level of Cxcl9 in the liver (Fig. S10A), whereas CXCR3+CD62L+CD8+ T cells, which probably migrated from the spleen, decreased in number (Fig. S10B). These findings suggest that CD8+ T cells proliferate in the spleen in an IL-15-dependent manner and are an important source of liver CTLs.

Numbers of IL-15+ B cells and CD40L+IL-15Rα+CD8+ T cells increased in the blood of patients with AIH

To explore the translational potential of these results in humans, we analysed the clinical samples of patients with AIH. Serum concentrations of IL-12 and IFN-γ in these patients, which are the triggers of AAV IL-12 model, were higher than those in HCs and patients with NASH (Fig. 8A). Notably, a high concentration of IL-15 and IL-15 expression in B cells were confirmed in the serum of patients with AIH (Fig. 8A and B). We noticed a significantly positive correlation between ALT levels and the expression level of IL-15 in B cells (Fig. 8B). In addition, the induction of CD40L+IL-15Rα+ memory CD8+ T cells was also observed in patients with AIH, suggesting the interaction with IL-15+ B cells in patients with AIH (Fig. 8C). Unlike that in the mouse model, the proportion of CXCR3+ in peripheral CD8+ T cells decreased (Fig. 8D). Combined with high serum concentration of CXCR3 ligands (CXCL9 and CXCL10) and the expansion of memory CD8+ T cells,
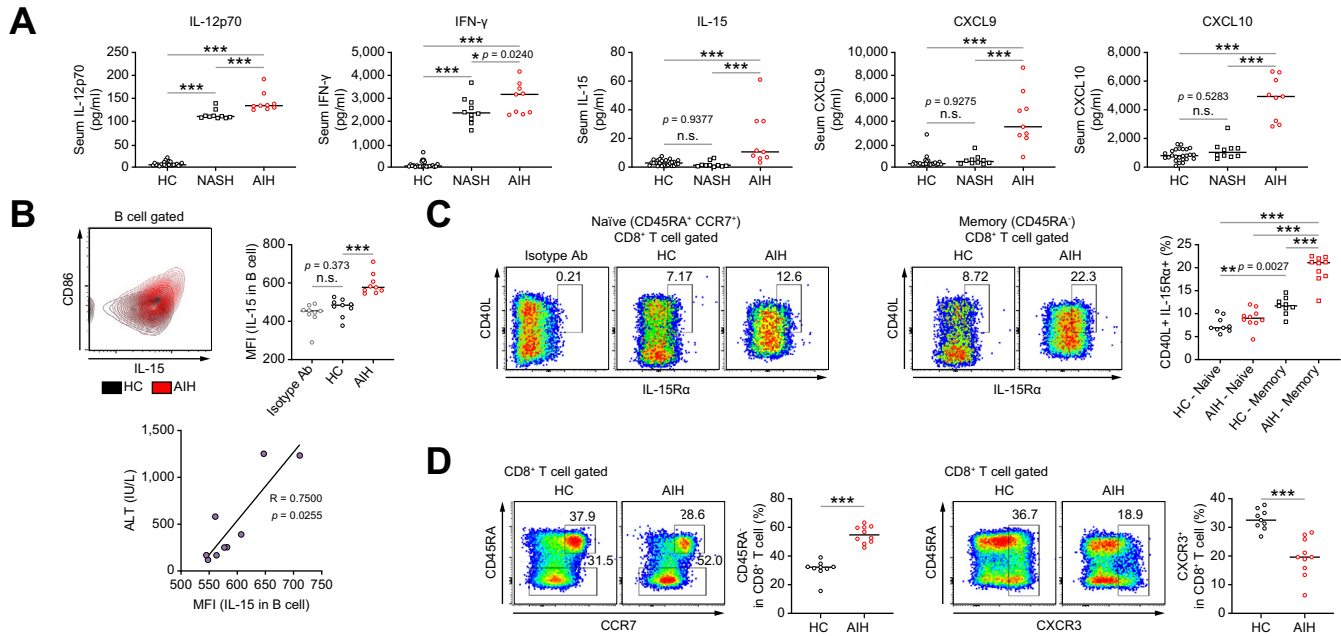

Fig. 8. Serum IL-15 concentration and IL-15-related lymphocyte numbers were increased in the blood of patients with AIH. (A) Serum levels of IL-12p70, IFN-γ, IL-15, CXCL9, and CXCL10 in patients with AIH (n = 9), patients with NASH (n = 10), and HCs (n = 25). (B) Representative flow-cytometry staining and the MFI of PE (IL-15) in B cells in the blood of HCs or patients with AIH. Correlation of ALT levels and the MFI of PE (IL-15) in B cells. (C) Representative flow-cytometry staining and the proportion of CD40L+IL-15Rα+ cells among CD8+T cells. (D) Representative flow-cytometry staining and proportions of CD45RA− (memory) and CXCR3+ among CD8+ T cells. (B–D) Patients with AIH (n = 9) and HCs (n = 9). Data are presented as the mean ± SEM. *p <0.05, **p <0.01, and ***p <0.001. One-way ANOVA with Tukey's multiple-comparisons *post hoc* test (A–C) or unpaired two-tailed Student's *t* tests (D) was applied. Correlations were evaluated using the Spearman rank correlation (B). Ab, antibody; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; CXCL9/10, C-X-C motif ligand 9/10; CXCR3, C-X-C motif chemokine receptor 3; HC, healthy control; IFN-γ, interferon-gamma; IL-15R, IL-15 receptor; MFI, median fluorescence intensity; NASH, non-alcoholic steatohepatitis; PE, phycoerythrin.

it is assumed that this decrease was the result of migration to the liver.

Discussion

The current study corroborated the clinical observation of the efficacy of anti-CD20 treatment in patients with refractory AIH using the experimental type 1 AIH model, in which liver-specific IL-12 expression triggers the expansion of hepatic CTLs with a recapitulation of the clinical characteristics of human AIH. In this model, we found that IL-15 and CD40L are indispensable for the interactions between B cells and CD8^{+} T cells in the spleen, leading to the subsequent migration of CD8+ T cells to the liver via the CXCL9/CXCR3 axis. The pathogenic role of IL-15-producing B cells in AIH was verified by both *in vitro* and *in vivo* neutralisation assays. IL-15-producing B cells and CD40L^{+}CD8^{+} T cells were also detected in the peripheral blood of patients with AIH, and IL-15 expression in B cells was associated with the disease activity. The key findings of this study are summarised in the graphical abstract.

It remains controversial whether the priming of the autoimmune T-cell response occurs in the liver or spleen during the initiation of AIH. In addition to the previous reports showing that splenectomy improved the survival rate of the murine fatal AIH model,[29] we also confirmed a significant improvement in hepatitis with splenectomy in the AAV IL-12 model. Spleen hypertrophy is a known characteristic of patients with AIH, and spleen stiffness is associated with the progression of liver fibrosis.[1,30] Furthermore, it has been reported that splenectomies in patients with HBV, HCV, alcoholic hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, and AIH significantly suppressed progression to liver fibrosis,[31] suggesting a potential contribution of splenocytes to pathogenesis. In splenectomised mice, the number of CD8^{+} T cells in the liver was significantly decreased, supporting the notion that the migration of CD8^{+} T cells from the spleen plays a key role in this model. As a key cell subset in the spleen, we have demonstrated that the adoptive transfer of splenic B cells cancelled the suppressive effect of the splenectomy with an increased number of hepatic CTLs. Along with the findings that B-cell depletion by anti-CD20 decreased the memory CD8^{+} T-cell counts in both the spleen and the liver, splenic B cells were assumed to play a substantial role in the development of hepatitis in this model.

B cells have been reported to act as antigen-presenting cells (APCs) to induce the activation and proliferation of T cells and exacerbate eAIH.[13] Previous reports have shown that an APC-like CD86^{+} B-cell count was increased in the blood of patients with AIH.[26] Regarding the molecular mechanism of action of APCs, the available information is limited for the costimulatory molecules CD80 and CD86, as no suitable autoantigen has been identified for the study of AIH. In the current study, we successfully identified IL-15 as the functional molecule of pathogenic B cells using RNA-seq analysis. IL-15 is a cytokine that contributes to the proliferation of CD8^{+} T cells.[27] We demonstrated that splenic B cells serve as a source of IL-15, which promotes the proliferation of CD8^{+} T cells. Immunostaining of the spleen revealed an increase in the proportion of B cells in close contact with the CD8^{+} T cells. Given that IL-15 expression was also upregulated in hepatic B cells, it is likewise possible that IL-15-mediated interaction between B cells and T cells could occur in the liver. Accordingly, we confirmed that macrophages and neutrophils are the main IL-15-producing cell populations in the liver of AAV

IL-12 treated mice, whereas B cells accounted for less than 1% of IL-15-producing cells, suggesting that IL-15-mediated B-cell activity plays a smaller role in the liver than in the spleen. In addition to the secretory type, there is also a membrane-bound type of IL-15; therefore, neighbouring B cells could perform more efficient functions. Previous reports have shown that not only lymphoid tissue-derived CD8^{+} T cells but also granzyme B^{+}CD8^{+} mucosal-associated invariant T (MAIT) cells and hepatic resident memory CD8^{+} T-cell (Trm) counts are increased in patients with AIH.[32,33] As IL-15 also contributes to the proliferation of granzyme B^{+} MAIT cells and Trms,[33,34] there may be another target of action other than CD8^{+} T cells in the lymphoid tissues, as we demonstrated.

How IL-15 production is regulated in B cells remains unknown. *In vitro* experiments suggested that IFN-γ signalling is not sufficient to induce IL-15 production in splenic B cells, and the results of this study showed that CD40 signalling was required. AAV IL-12 administration enhanced CD40L expression in splenic IL-15Rα^{+}CD8^{+} T cells (Fig. 7F). Notably, these CD40L-expressing CD8 T cells are distributed close to B220^{+} B cells in the spleen, suggesting that mutual interaction is required for CTL function in the spleen. The interaction of B cells with CD4^{+} T cells has been widely reported, and previous studies have shown the interaction between follicular helper T and B cells in a murine AIH model.[29] In general, CD40L on the cell membranes of CD4^{+} T cells binds to CD40 expressed on DCs and transduces a signal to promote IL-12 expression, known as DC licensing.[35] Furthermore, it has been reported that CD40L is expressed on human and mouse CD8^{+} T cells, especially on memory CD8^{+} T cells, which are abundant in secondary lymphoid tissues.[36–38] These CD8^{+} T cells are called helper CD8^{+} T cells, and it has been suggested that helper CD8^{+} T cells can perform DC licensing as well.[36–38] From our analysis, the increase in the expression of CD40L was remarkable in memory CD8^{+} T cells in the spleen. The close distribution of CD40L^{+}CD8^{+} T cells with B cells and the results from *in vitro* experiments suggest that CD8^{+} T cells promote IL-15 production in B cells via CD40L/CD40 interaction in a manner similar to DC licensing. This mutual interaction may drive a sophisticated mechanism of adaptive immunity and contribute to liver pathology with chemokine-specific migration of the CTLs to the liver. Further characterisation of pathogenic B cells may lead to therapeutic advances targeting B cells beyond anti-CD20 treatment in the future.

Although the AAV IL-12 model is superior for the evaluation of B-cell function in AIH pathology, it does have some limitations. First, the expression of IL-12 induces hepatic CD8^{+} T-cell expansion in the early phase of hepatitis, and B cells contribute to the expansion in the later phase. Thus, the CD4/CD8 ratio for the hepatic T cells is lower than that in actual patients with AIH. Although CD8^{+} T cells contribute to hepatocyte injury in AIH, the interaction between CD4^{+} T cells (*e.g.* Tfh) and B cells may be underestimated in this model. Second, immune cells can react to AAV as hepatitis triggers. As only a single AAV shot is required in the AAV IL-12 model and AAV cannot proliferate without coinfection of a helper virus, it is unlikely that antiviral adaptive immune responses are major contributors in this model. Liver-specific AAV infection also supports the unlikelihood of B cells directly responding to AAV in the spleen (Fig. S3B and C).

Regarding the applicability of the experimental findings in humans, serum levels of IL-12, IFN-γ, IL-15, CXCL9, and CXCL10 were found to be significantly higher in patients with AIH when compared with those in HCs and patients with NASH, suggesting

that they might contribute to the pathogenesis of human AIH. Notably, in patients with AIH, increased IL-15 expression in B cells from the blood of patients with AIH was positively correlated with serum ALT levels, supporting the idea that IL-15-producing B cells may serve as potential therapeutic targets.

Although further studies, especially for validation in human samples, are required, this study clearly demonstrates the indispensable role of splenic IL-15$^+$ B cells in the local expansion of CTLs via mutual interaction and subsequent migration to the liver in the development of AIH.

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Author names in bold designate shared co-first authorship.

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