INTRODUCTION

Autoimmune hepatitis (AIH) is a type of chronic autoimmune inflammation directed against liver tissue. Most patients are identified late in the disease process because the development of AIH is associated with a long delay between the initiation of autoimmunity and the diagnosis of symptomatic disease. Approximately 40% of patients have liver cirrhosis at the time of diagnosis. Because of the delayed onset of overt disease, the identification of the key determinants and the priming process remains challenging. Additionally, little is known about the aetiology of AIH because there are few reliable animal models that reflect the disease.1 There is controversy over the induction of chronic AIH and the priming of the autoimmune T cell response. Specifically, over whether it originates in the liver or spleen.

Some studies have shown that T cell priming occurs in the liver or in the draining liver lymph nodes.2,3 Other studies have demonstrated that the spleen is essential for initiating an immune response to hepatocytes.4,5 Moreover, AIH is generally thought to result from the T lymphocyte-mediated destruction of hepatocytes. In this context, we have already shown that experimental murine AIH (emAIH) can be induced by CD4+ T cells alone.6 Despite the early onset of autoantibodies, the role of B cells in the maintenance of autoimmune responses remains controversial. Thus, it is important to characterize the role of the spleen and B cells in the initiation of chronic emAIH.

Abbreviations: Ad, adenovirus; AIH, autoimmune hepatitis; ALT, alanine transaminase; AST, aspartate aminotransferase; emAIH, experimental murine autoimmune hepatitis; FTCD, formiminotransferase cyclodeaminase; HAV, hepatitis A virus; HE, haematoxylin and eosin; IHLs, intrahepatic lymphocytes; mHAI, modified hepatitis activity index; Tregs, regulatory T cells.

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2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animal care and experiments were performed in accordance with the institutional and national guidelines. All animal experiments were performed according to protocols approved by the animal welfare commission of Hannover Medical School and the local ethics animal review board (Lower Saxony State Office for Consumer Protection and Food Safety).

2.2 | Mice

Animals were maintained under specific pathogen-free conditions at the central animal facility of Hannover Medical School (Hannover, Germany). Female and male NOD/Ltj mice were bred in the same animal facility. Some mice were splenectomized 2 weeks before the administration of adenovirus expressing formiminotransferase cyclodeaminase (Ad-FTCD). Mice were injected intravenously with a total of $4 \times 10^7$ infectious particles containing Ad-FTCD in PBS. All mice were sacrificed 12 weeks post-infection.

2.3 | Adenovirus construction

The generation of Ad-FTCD was described before. Briefly, FTCD was amplified by PCR from cDNA generated from human liver cells; its sequence was verified by sequencing both DNA strands. The constructs were cloned into the Ad transfer vector pShuttle-CMV (Stratagene). By homologous recombination, this shuttle vector was recombined with pAdEasy-1, which carried deletions in the E1 and E3 regions (Stratagene). The genome of the generated adenovirus could be amplified only within the HEK 293 packaging cell line, which complements the essential regions. The purification of recombinant adenovirus was performed using a cesium chloride gradient, and the adenoviral stocks were quantified using an Adeno-X™ Rapid Titer Kit (Clontech).

2.4 | Flow cytometry

Organs were minced, and intrahepatic lymphocytes (IHLs) were separated using a 40%/70% Percoll (GE Healthcare) gradient. Red blood cells in the spleen were lysed, and lymphocytes were subsequently stained with appropriate combinations of anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-Ki-67, anti-B220, anti-Foxp3, anti-CD62L and anti-CD44. All data were acquired with an LSRII SORP interfaced with FACSDiva software (BD Biosciences).

2.5 | Serum analysis

Blood samples were collected via the retro-orbital route. Aspartate aminotransferase (AST) and alanine transaminase (ALT) were determined by photometric enzyme activity assays with an Olympus AU400 Chemistry Analyzer using serum, as described before.

2.6 | Histology

Murine livers were fixed in formalin and embedded in paraffin or were embedded in Tissue-Tek® OCT™ Compound (Sakura) without fixation for cryosectioning (8 µm). Paraffin-embedded sections (5 µm) were prepared for haematoxylin and eosin (HE) staining. After staining, the sections were examined in a blinded manner by a pathologist using the approved modified hepatitis activity index (mHAI) for AIH.

2.7 | Immunofluorescence

Immunofluorescence microscopy was performed as described before. Briefly, cryosections were rehydrated, blocked, stained with anti-CD4, anti-CD8, anti-Foxp3 and DAPI, and were analysed with an AxioImagerM1 using AxioVision 4.8 software (Zeiss).

2.8 | Real-time PCR using Fluidigm technology

2.8.1 | Nucleic acid isolation and cDNA synthesis

Total RNA was isolated from frozen liver samples with an RNeasy Mini Kit (Qiagen) and was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher). Then, 2 µg of RNA was reverse transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

2.8.2 | Pre-amplification and quantitative RT-PCR (Fluidigm)

The pre-amplification of cDNA was performed using Fluidigm® PreAmp Master Mix and TaqMan® Assays according to the manufacturers’ guidelines. Quantitative RT-PCR was performed in a 48.48 Dynamic Array IFC using the pre-amplified samples. IFC priming and
loading were performed using a Juno instrument using the prime script Prime 48.48 GE and Load Mix 48.48 GE. RT-PCR data were generated using a BioMark™ HD instrument. The gene TaqMan® Assays that were utilized can be found in the corresponding figure.

2.8.3 | Bioinformatics analysis

The normalization of the Ct values was performed by subtracting the mean values of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta actin (Actb) from the mean values of the genes of interest. Heat map and PCA analysis of the delta Ct values were plotted via Qlucore software (P < .05 and q < 0.2).

2.9 | Statistics

An unpaired, two-tailed Student’s t test was performed using GraphPad Prism version 7.00 for Mac (GraphPad Software, www.graphpad.com). Significant differences with P ≤ .05 are indicated by *, and very significant differences (P ≤ .01) are indicated by **. P > .05 was considered to be not significant (ns).

3 | RESULTS

By performing splenectomy before the induction of the disease, the site of the onset of AIH should be revealed. Therefore, we removed the spleen 2 weeks before the induction of AIH by Ad-FTCD. Splenectomy did not affect the hepatic histology, transaminases or lymphocyte compartments (data not shown). The analysis of the animals 12 weeks after adenoviral AIH induction showed inflammation in both the splenectomized or non-splenectomized groups before the adenoviral induction of emAIH (Figure 1A). Surprisingly, the splenectomized animals had a markedly mHAI compared to animals with a spleen (Figure 1B). However, the lymphatic infiltrate size was not significantly larger (Figure 1C). The liver weight was unchanged in both groups (Figure 1D), and the removal of spleen, which represents the loss of a lymphocyte compartment, was not compensated for by an increase in lymphocytes in the liver (Figure 1E). Additionally, no change was observed in the level of aminotransferase (Figure 1F,G).

Because T cells were identified as the definitive drivers of emAIH, it is noteworthy that there was a reduced number of

**FIGURE 1**  Splenectomy does not impact the development of emAIH. A, Splenectomized (black dots) or non-splenectomized NOD/Ltj mice (black squares) received 4 × 10⁹ Ad-FTCD particles. Haematoxylin and eosin staining was performed on liver sections harvested 12 weeks after adenoviral emAIH induction. B, These liver sections were analysed using the approved, blinded mHAI score. C, The infiltrate size, (D) the liver weight and (E) the number of IHLs were measured at the same time-point. F, The AST and (G) ALT levels were measured in the blood serum of mice. Significant differences with P ≤ .05 are indicated by *, and very significant differences (P ≤ .01) are indicated by **
intrahepatic T cells (Figure 2A). The relative and absolute number of B cells increased after splenectomy (Figure 2B), but the IgG serum level did not change (data not shown). Within the T cell compartment, however, there were no further changes in CD4⁺ cells (Figure 2C), CD8⁺ T cells (Figure 2D) and regulatory T cells (Tregs) (Figure 2E), and there were no alterations in the proliferative behaviour of these cells (data not shown). Additionally, the conditions within the CD4⁺ or CD8⁺ naive, activated and memory T cells were unremarkable (data not shown). Finally, we analysed the inflammatory cytokines and TH1-, TH2-, Th17-, Treg- and fibrosis markers by quantitative PCR, but we did not find any differentially regulated genes in a set of 21 genes (Figure 1F). We have already demonstrated through flow cytometry and liver histology that the outcomes of the IHLs differed, because most IHLs were not involved in inflammation and were also present in healthy individuals.⁶,⁹ Therefore, we analysed CD4⁺, CD8⁺ T cells and Tregs in cryosections from these mice (Figure 2G). We found a higher variability in the CD4⁺/CD8⁺ T cell ratio in histological sections (Figure 2H), and most strikingly, the Treg numbers in the CD4⁺ T cell subpopulation were strikingly reduced (Figure 2I) in emAIH.

4 | DISCUSSION
The results show that priming can occur in the liver in the absence of the spleen. While we showed that the spleen is not required for the formation of AIH, its role seems to be protective. However, the exact cause or the underlying mechanism of the formation of AIH remains unclear.

As shown, the mHAI of AIH was more severe in splenectomized mice than in non-splenectomized mice. The number of intrahepatic T cells was reduced, as shown by flow cytometry data, and within
this reduced T cell population, the number of Tregs was disproportionately reduced in the inflamed tissue. Although these results are interesting and provide some hints regarding the underlying mechanisms, the key finding is that long-lasting, intrahepatic inflammation occurred without the spleen. No cellular or molecular inflammatory markers had a pronounced effect after splenectomy. The aminotransferase levels were unchanged. This phenomenon differs from what is observed in patients, but it has already been described in murine chronic hepatitis models.6,10,11

All models that were used to draw conclusions about the induction site of AIH do not reflect human disease. Many animal models indicated that the liver was the site of AIH induction.2,3,12 However, priming in these models tends to result in a self-limited hepatitis flare or hepatic immune tolerance instead of chronic hepatitis. Transgenic T cell models, in which some proportion of the T cells recognize a defined or transgenic target structure, also demonstrated this phenomenon. This does not correspond to actual autoimmune diseases, in which antigen-specific T cells are rare and can only be identified in an ELISPOT at a very low frequency.

In another study that showed a clear role of the spleen for the induction of a hepatic immune response, the time of analysis may have been too soon after induction for a hepatic immune response to be observed.4 In our emAIH model, a hepatic immune response could not be observed before week 12 after induction and was even more pronounced after 30 weeks.6 Therefore, this model described by Backer et al shows acute inflammation but not chronic AIH.

Even though we cannot rule out the effect of lymph nodes, our data are in line with the literature about other diseases, in which the impact of splenectomy on the immune system and immune competence was smaller than expected.13,14

Knowledge about the aetiology of AIH is still very limited. Because of the time lag between the onset and diagnosis of AIH, it is difficult to conclude that that were infections preceding the diagnosis of AIH that caused the initiation of liver-specific autoimmunity. Thus far, in the majority of patients, the induction of AIH cannot be linked to a single environmental agent. Nevertheless, it is well accepted that viral hepatitis can trigger AIH. Therefore, hepatitis A virus (HAV), HCV and HEV have a higher seroprevalence in patients with AIH15,16 than in controls. One could argue that hepatotropic viruses induce disease in the liver and not in the spleen or lymph nodes.

In summary, we demonstrated that the priming of AIH can take place in the liver. We also showed that there is an increase in intrahepatic B cells and a disruption in immunoregulation by Tregs after splenectomy. These results have important implications for understanding the aetiology of AIH.

CONFLICT OF INTEREST
All authors claim that they have no conflict of interest based on a desire for financial gain, prominence, professional advancement or a successful outcome.

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