

Cutting Edge Issues in Autoimmune Hepatitis

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Abstract Autoimmune hepatitis is an inflammatory liver disease affecting mainly females and characterised histologically by interface hepatitis, biochemically by elevated transaminase levels and serologically by circulating autoantibodies and increased levels of immunoglobulin G. Autoimmune hepatitis responds to immunosuppressive treatment, which should be instituted as soon as diagnosis is made. Seropositivity for smooth muscle and/or antinuclear antibody defines type 1 autoimmune hepatitis, while positivity for liver kidney microsomal type 1 antibody defines type 2 autoimmune hepatitis. The aetiology of autoimmune hepatitis is unknown, though both genetic and environmental factors are involved in its expression. The major mechanism of liver damage involves immune reactions against host liver antigens that are not adequately controlled by defective regulatory T cells. Current research aiming at potentiating regulatory T cell function in vitro to reconstitute tolerance in vivo has given promising results.

Keywords Autoimmune hepatitis · Liver · Autoantibody · Humoral immunity · Cellular immunity · Immune regulation

Introduction

Autoimmune hepatitis (AIH) is an inflammatory liver disease, affecting mainly females, characterised histologically by interface hepatitis (Fig. 1), biochemically by

elevated transaminase levels and serologically by the presence of autoantibodies and increased levels of immunoglobulin G (IgG). Clinical presentation is variable [1–3]: AIH can present insidiously, or with symptoms and signs of an acute hepatitis with a fluctuating course, or with complications of portal hypertension, or, more rarely, with fulminant hepatic failure. AIH responds to immunosuppressive treatment, which should be instituted as soon as diagnosis is made, as, if left untreated, the disease is likely to progress to liver failure requiring transplantation. The development of a panel of marker autoantibodies has allowed the subdivision of AIH in distinct types that have clinical and prognostic peculiarities.

Clinical Features

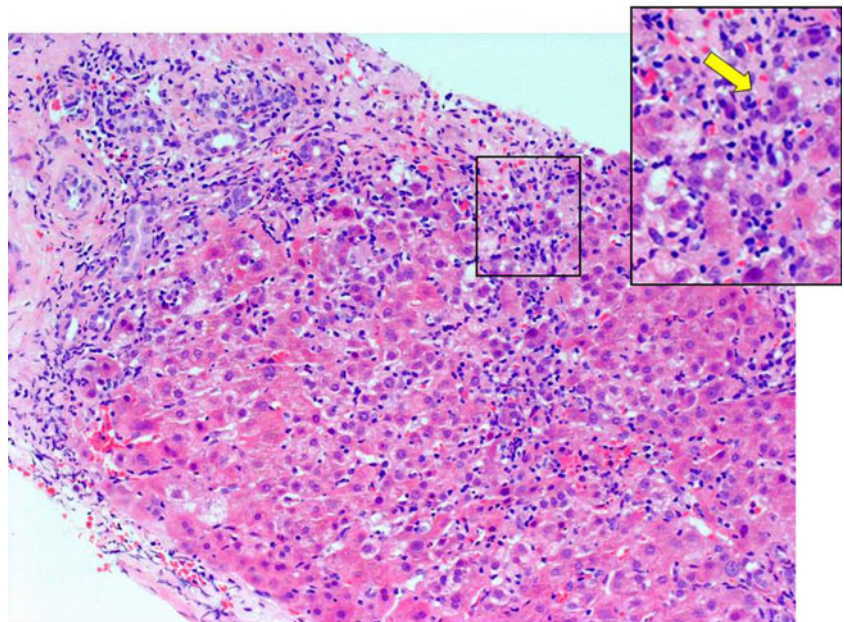
The prevalence of AIH is unknown. It occurs worldwide in children and adults [2]. Reported prevalences range from 1.9 cases per 100,000 in Norway [4] and 1 per 200,000 in the US general population [5], to 20 per 100,000 in females over 14 years of age in Spain [6]. It is likely that all these figures are underestimates.

There are two well-defined types of AIH: seropositivity for smooth muscle (SMA) and/or anti nuclear antibody (ANA) defines type 1 AIH (AIH type 1), while positivity for liver kidney microsomal type 1 (anti-LKM1) or for liver cytosol type 1 (anti-LC1) antibodies defines type 2 AIH (AIH type 2) [1, 2]. A third type of AIH has been suggested, being either negative for conventional autoantibodies or being positive only for anti soluble liver antigen (SLA), an antibody that can also be present in type 1 or 2 AIH and that is associated with a particular severe disease course [7, 8].

AIH type 1 affects all age groups, with two peaks, one in childhood/adolescence, and the other in adulthood around

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Fig. 1 Interface hepatitis: portal and periportal lymphocyte and plasma cell infiltrate (see inset, arrow), extending to and disrupting the parenchymal limiting plate. Swollen hepatocytes, pyknotic necroses and acinar inflammation are present. Haematoxylin and eosin staining (picture kindly provided by Dr. Alberto Quaglia)



the age of 40. About 20% of patients present after the age of 60. AIH type 2 affects mainly children and young adults and has a more aggressive course, leading to fulminant hepatic failure more frequently than type 1 AIH.

The majority of adult patients, usually affected by AIH type 1, have an indolent disease course and come to clinical attention because of non-specific symptoms such as generalised fatigue or arthralgia. Increasingly, AIH is diagnosed after incidental finding of abnormal liver function tests during routine investigations. About a third of the patients present acutely with jaundice, arthralgia, anorexia and fatigue indistinguishable from acute hepatitis due to other causes [9, 10]. Relapsing acute hepatic features are not uncommon. Occasionally, the first symptoms of AIH are complications of portal hypertension like gastrointestinal bleeding or hypersplenism, without previous knowledge of liver disease. At the other end of the spectrum, also AIH type 1 can occasionally present with fulminant hepatic failure.

Whatever the presentation, at least a third of patients are already cirrhotic at the time of diagnosis, indicating that the disease process is longstanding. Even in the group of patients presenting with acute disease, liver biopsy often shows advanced fibrosis or cirrhosis.

An acute presentation is more common in children and young adults, and it is particularly in this age group that fulminant hepatic failure is observed, especially in AIH type 2 [11].

In both types of AIH, 75% of patients are female. A positive family history of autoimmune disease is common, being found in about 40%. Associated autoimmune disorders can be present at diagnosis or develop during follow-up in approximately one fifth of patients [12, 11]

and include thyroiditis, ulcerative colitis, insulin dependent diabetes, vitiligo, nephrotic syndrome, hypoparathyroidism and Addison disease, the latter two being observed in particular in anti-LKM1 positive patients or in patients with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) [13, 14] a monogenic disorder with a variable phenotype that includes in about 20% of the cases AIH [15].

With the exception of a fulminant presentation with encephalopathy, AIH responds satisfactorily to immunosuppressive treatment whatever the degree of liver impairment, with a reported remission (i.e. normal transaminase and IgG levels) rate around 80%. Standard treatment consists of prednisolone usually in association with azathioprine [3]. A combination of budesonide and azathioprine has been recently reported to attain remission in a high proportion of non-cirrhotic patients with fewer side effects than schedules including standard steroids [16]. In difficult-to-treat patients, or frequent relapsers, other drugs that have been used with variable results are micophenolate mofetyl and calcineurin inhibitors [3].

In recent years, it has become apparent that in children and young adults, sclerosing cholangitis is often associated with florid autoimmune features, including elevated titers of autoantibodies, in particular ANA and SMA, elevated IgG, and interface hepatitis [17]. Since these features are shared in common with AIH and are often not accompanied by elevated alkaline phosphatase or gamma glutamyl transpeptidase levels at disease onset, the diagnosis of sclerosing cholangitis relies on cholangiographic studies. In the absence of cholangiographic studies at presentation, many of these patients are diagnosed and treated as AIH, though the diagnosis of sclerosing cholangitis becomes often

apparent during follow up. This condition, referred to as autoimmune sclerosing cholangitis (ASC), is as prevalent as AIH type 1 in childhood, but in contrast to AIH it affects equally boys and girls [17]. ASC responds satisfactorily to immunosuppression, at least in regard to the parenchymal inflammation, if treatment is started early, though bile duct disease progresses despite treatment in about 50% of patients.

Diagnosis

A combination of clinical, biochemical, immunological and histological features is needed for the diagnosis of AIH since there is no single diagnostic test for this condition.

For comparative and research purposes, the International Autoimmune Hepatitis Group (IAIHG) devised a diagnostic system, which includes several positive and negative scores, whose sum gives a value indicative of probable or definite AIH [18, 19]. More recently, the IAIHG published a simplified scoring system, more suitable to clinical application [20]. However, neither scoring system is perfect, in particular in the juvenile form of the disease, where diagnostically relevant autoantibodies often have titres lower than the cut off value considered positive in adults, and in which neither system can distinguish between AIH and ASC.

Key biomarkers for the diagnosis of AIH are serum autoantibodies. These are classically detected by indirect immunofluorescence. Autoantibody detection not only assists in the diagnosis but also allows the differentiation of AIH in type 1, positive for ANA and/or SMA, and type 2, positive for anti-LKM1 and/or anti-LC1. Recognition and interpretation of the immunofluorescence patterns is not always straightforward. Problems do exist between laboratory reporting and clinical interpretation of the results that are partly dependent on insufficient standardisation of the tests, but also partly dependent on a degree of unfamiliarity with the disease spectrum of AIH among clinicians, who do not request the correct investigations with consequent lack of appropriate diagnosis and treatment [21]. Thus, if anti-LKM1 antibody is not tested for, patients with type 2 AIH may go unrecognised and untreated until the development of end-stage liver disease requiring transplantation for 'cryptogenic cirrhosis' [22], while the transplant-free survival of AIH type 2 treated with appropriate immunosuppression is excellent [11]. In regard to standardisation, a lead has been taken by the IAIHG [23].

The basic technique for the routine testing of autoantibodies relevant to AIH is indirect immunofluorescence on a freshly prepared rodent substrate that should include kidney, liver and stomach to allow the detection of ANA, SMA, anti-LKM1 as well as anti-LC1, but also of anti-mitochondrial antibody (AMA), the serological hallmark of

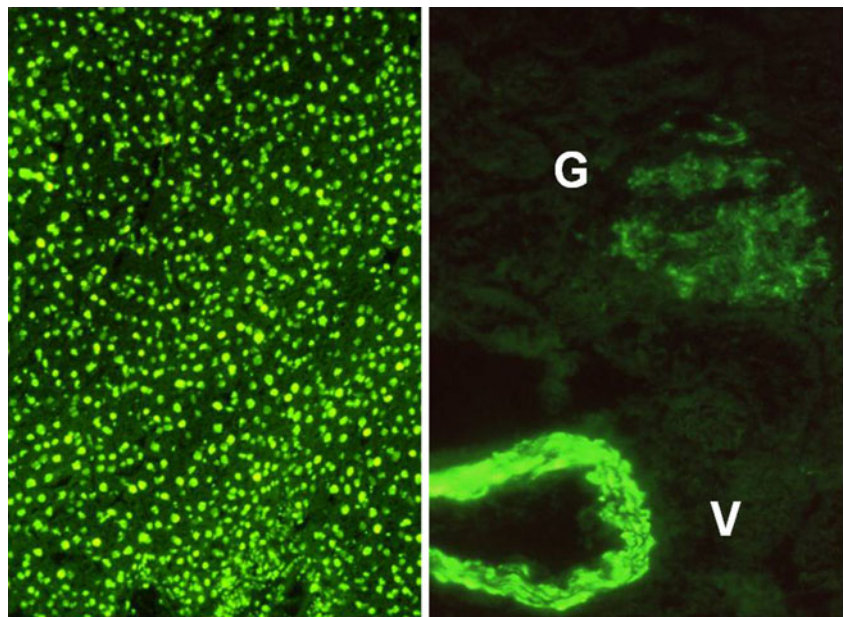
primary biliary cirrhosis. Of particular importance is the plan of section and orientation of the kidney because both anti-LKM1 and AMA stain renal tubules, but with different patterns distinguishable only in the presence of both proximal and distal tubules (see below). The sections of liver, kidney and stomach should be dried in air and used without further fixation. Commercially available sections are of variable quality because, to lengthen shelf-life, they are treated with fixatives, which readily result in enhanced background staining that may hinder the recognition of diagnostic autoantibodies, especially when these are present at low titre.

Since healthy adults may show reactivity at the conventional starting serum dilution of 1/10, the arbitrary dilution of 1/40 has been considered clinically significant by the IAIHG. In contrast, in healthy children autoantibody reactivity is infrequent, so that titres of 1/20 for ANA and SMA and 1/10 for anti-LKM1 are clinically relevant [23]. Hence, the laboratory should report any level of positivity from 1/10, and the attending physician should interpret the result within the clinical context and the age of the patient.

Anti-Nuclear Antibody ANA is readily detectable as a nuclear staining in kidney, stomach and liver (Fig. 2). On the latter in particular, the ANA pattern may be detected as homogeneous, or coarsely or finely speckled. In most cases of AIH, but not in all, the pattern is homogeneous. To obtain a much clearer and easier definition of the nuclear pattern, HEp2 cells that have prominent nuclei should be used (Fig. 3). HEp2 cells, however, should not be used for screening purposes, because nuclear reactivity to these cells is frequent at low serum dilution (1/40) in the normal adult population [24]. For ANA, likely molecular targets include nuclear chromatin, histones, centromere, double- and single-stranded DNA and ribonucleoproteins, akin to lupus, but there are probably several others. The advent of new techniques using recombinant nuclear antigens and immunoassays will enable a better definition of ANA target antigens, an assessment of their specificity for diagnosis and their possible role in the pathogenesis of AIH type 1 [21].

Smooth Muscle Antibody SMA is detected on kidney, stomach and liver, where it stains the walls of the arteries. In the stomach, it also stains the muscularis mucosa and the lamina propria. On the renal substrate, it is possible to visualise the V, G (Fig. 2) and T patterns; V refers to vessels, G to glomeruli, and T to tubules [25]. The V pattern is present also in non-autoimmune inflammatory liver disease, in autoimmune diseases not affecting the liver and in viral infections, but the VG and VGT patterns are more specific for AIH. The VGT pattern corresponds to the so called 'F actin' or microfilament (MF) pattern observed using cultured fibroblasts as substrate. Neither the VGT nor the anti-MF patterns are, however, entirely specific for the

Fig. 2 Anti-nuclear and anti-smooth muscle antibodies: immunofluorescence pattern of anti-nuclear (ANA) and smooth muscle (SMA) autoantibodies on liver and renal rodent sections. ANA (*left*) stains the nuclei in the liver section and SMA (*right*) stains a small arterial vessel (*V*) and a glomerulus (*G*) in the renal section



diagnosis of AIH type 1. Though the VGT–MF pattern has been suggested to be due to a specific antibody uniquely found in AIH type 1, it may just reflect high titre SMA. The molecular target of the microfilament reactivity that is observed in AIH type 1 remains to be identified. While ‘anti-actin’ reactivity is strongly associated with AIH type 1, some 20% of SMA positive patients do not have the F-actin/VGT pattern [26]. The absence, therefore, of anti-actin SMA does not exclude the diagnosis of AIH.

Anti-Liver Kidney Microsomal Antibody Anti-LKM1 stains brightly the liver cell cytoplasm and the P3 portion of the renal tubules (Fig. 4), but does not stain gastric parietal

cells [27]. Anti-LKM1 is often confused with AMA, since both autoantibodies stain liver and kidney. Compared to anti-LKM1, AMA stains the liver more faintly and the renal tubules more diffusely with an accentuation of the small distal ones. In contrast to anti-LKM1, AMA also stains the gastric parietal cells. In the context of AIH, there can be positivity for AMA in a small subset of patients (3–5%), who respond to classical treatment for AIH [28, 29]. The identification of the molecular targets of anti-LKM1, i.e. cytochrome P4502D6 (CYP2D6), and of AMA, i.e. enzymes of the 2-oxo-acid dehydrogenase complexes, has led to the establishment of immuno-assays based on the use of the recombinant or purified antigens. Commercially

Fig. 3 Anti-nuclear antibody pattern on HEp2 cells; *left* homogeneous pattern, typical of autoimmune hepatitis. *Right* speckled pattern, at times found in autoimmune hepatitis, but more frequent in other conditions, such as chronic hepatitis C infection

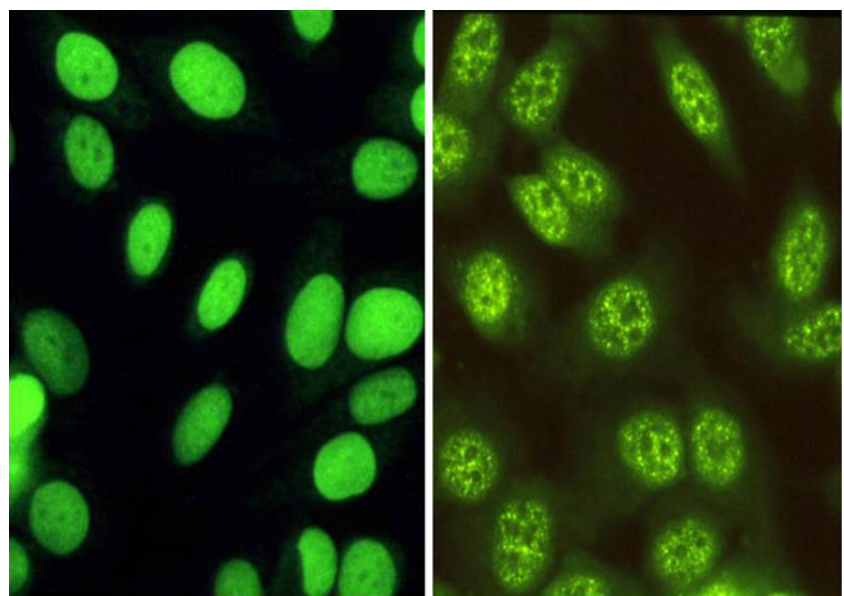
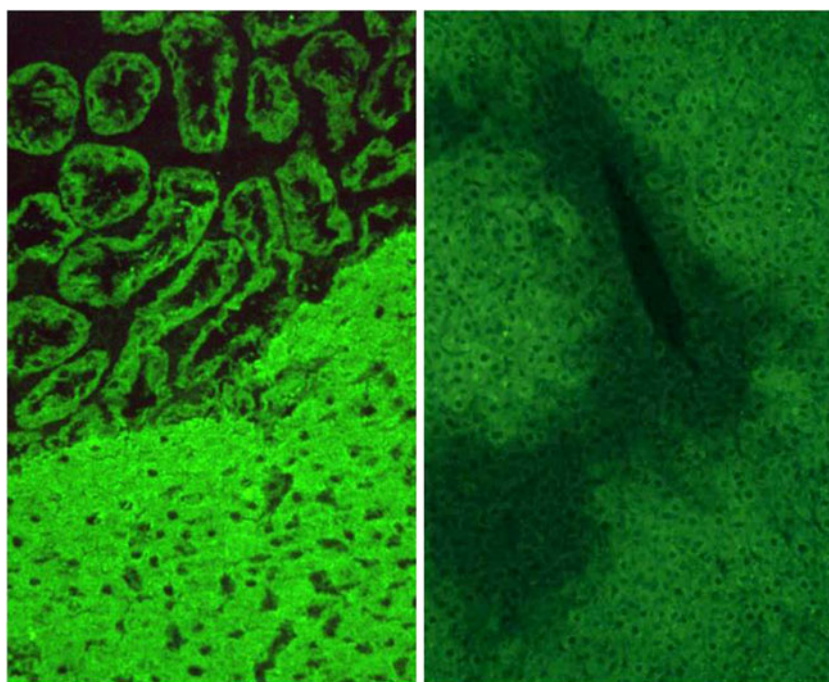


Fig. 4 Anti-liver kidney microsomal type 1 and anti liver cytosol 1 antibodies: *left* immunofluorescence pattern of anti-liver kidney microsomal type 1 (LKM1) autoantibody on renal and liver rodent sections: anti-LKM1 stains the cytoplasm of hepatocytes and proximal renal tubules. *Right* immunofluorescence pattern of anti-liver cytosol 1 (LC1) autoantibody on a rodent liver section: anti-LC1 stains the cytoplasm of hepatocytes with a weakening of the staining around the central vein



available ELISAs are accurate for detection of anti-LKM1, at least in the context of AIH type 2, and reasonably accurate for the detection of AMA [23]. Therefore, if a doubt remains after examination by immunofluorescence, this can be resolved by the use of molecularly based immuno-assays.

Variant Liver Microsomal Antibodies anti-LM, anti-LKM2 and anti-LKM3 [21]. These antibodies are mostly directed against P450 cytochrome isoforms different from 2D6. Anti-LM (liver membrane) antibodies stain only the liver cytoplasm, react with liver specific cytochrome P4501A2 and occur in dihydralazine induced hepatitis and in AIH associated with APECED. An anti-LKM1-like pattern of immunofluorescence is given by autoantibodies to P4502A6 that occur in APECED and occasionally in hepatitis C. The term anti-LKM2 was originally applied to anti-LKM1-like microsomal antibodies produced during hepatitis induced by the no longer marketed anti-hypertensive tienilic acid and are directed against cytochrome P4502C9. Anti-LKM3, which target members of the family 1 UDP glucuronosyltransferases, also give an immunofluorescent pattern similar to anti-LKM1, but occur mainly in hepatitis D (delta).

Anti-Liver Cytosol Type 1 (LC1) This antibody was originally described either in association with anti-LKM1, or in isolation, in both instances defining a clinical entity resembling AIH type 2 [30]. Anti-LC1 can be detected by indirect immunofluorescence using the standard tissue panel composed of rodent liver, kidney and stomach. It

stains the cytoplasm of liver cells with relative sparing of the centrilobular area, but is usually obscured by concurrent presence of anti-LKM1 (Fig. 4). In the presence of anti-LKM1, anti-LC1 can be detected by the use of liver cytosol in double-dimension immunodiffusion, or counterimmunoelectrophoresis, and a positive reference serum [31]. In Western blot, anti-LC1 reacts with a 58–60 kD protein when human liver cytosolic fraction is used as substrate. The molecular target has been identified as formimino-transferase cyclodeaminase [32].

Anti-Soluble Liver Antigen/Liver–Pancreas antigen (anti-SLA/LP) Anti-SLA [33] and anti-LP [34], earlier described separately in AIH, target the same antigen and are therefore the same autoantibody [35]. At variance with conventional diagnostic autoantibodies, anti-SLA is not detectable by immunofluorescence. Screening of cDNA expression libraries using high titre anti-SLA serum has allowed to identify the molecular target antigen as UGA tRNA suppressor associated antigenic protein [34, 36, 37] more recently re-named Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase [38]. Molecularly based diagnostic assays have become available, but their full evaluation is still under way. Anti-SLA was thought to identify a third type of AIH in which tests for conventional autoantibodies were negative [33]. However, early reports predated the publication of the IAIHG recommendations and used a cut-off point for conventional autoantibody levels higher than that currently used for the diagnosis of AIH. Though anti-SLA/LP is found occasionally in patients with AIH who are negative for ANA, SMA and anti-

LKM1, and who may be considered to have bona fide AIH type 3, it is also frequently present in typical cases of AIH type 1 and AIH type 2, and also in AIH/sclerosing cholangitis overlap syndrome. Anti-SLA appears to be highly specific for the diagnosis of AIH. Its detection at the time of diagnosis identifies patients with more severe disease and worse outcome [7].

Antineutrophil Cytoplasmic Antibodies Most antineutrophil cytoplasmic antibodies (ANCA), described originally in liver unrelated diseases, are directed at cytoplasmic components of neutrophils and give either a perinuclear (p-ANCA) or a cytoplasmic (c-ANCA) pattern. c-ANCA mainly react with proteinase 3 and are found in Wegener's granulomatosis; p-ANCA react with myeloperoxidase and are frequently detected in microscopic polyangiitis. In AIH type 1, akin to primary sclerosing cholangitis and inflammatory bowel disease, p-ANCA are frequently detected, but they are atypical, since they react with peripheral nuclear membrane components (perinuclear anti-nuclear neutrophil antibodies, p-ANNA) [37]. In contrast to AIH type 1, p-ANNA are virtually absent in AIH type 2. Detection of atypical p-ANCA can act as an additional pointer towards the diagnosis of AIH, particularly in the absence of other autoantibodies [23].

Pathogenesis

AIH is a 'complex trait' disease, i.e. a condition not inherited in a Mendelian autosomal dominant, autosomal recessive or sex-linked fashion. The mode of inheritance of a complex trait disorder is unknown and involves one or more genes, operating alone or in concert, to increase or reduce the risk of the trait, and interacting with environmental factors.

Susceptibility to AIH is conferred by genes within the human leukocyte antigen (HLA) region on the short arm of chromosome 6, especially those encoding DRB1 alleles [39, 40]. Since the role of class II major histocompatibility complex molecules (HLA in man) is to present peptide antigens to CD4 T cells, this suggests the involvement of HLA class II antigen presentation and T cell activation in the pathogenesis of AIH. In Europe and North America, susceptibility to AIH type 1 is conferred by the possession of HLA DR3 (*DRB1*0301*) and DR4 (*DRB1*0401*) [39, 40]. In Japan, Argentina and Mexico, susceptibility is linked to *DRB1*0405* and *DRB1*0404* [41]. These HLA associations may be the molecular footprints of the prevailing environmental triggers that precipitate AIH type 1 in different environments. In this context, it is of interest that in South America possession of the HLA *DRB1*1301*

allele, which predisposes to paediatric AIH type 1 in that population, is also associated with persistent infection with the endemic hepatitis A virus [42]. Susceptibility to AIH type 2 is conferred by the possession of HLA DR7 (*DRB1*0701*) and DR3 (*DRB1*0301*), patients positive for *DRB1*0701* having a more aggressive disease and severe prognosis [43].

The mechanisms leading to autoimmune liver damage have been a focus of intense investigation over the past 30 years. The initial trigger of liver centred autoimmunity, however, remains elusive. The histological picture of interface hepatitis (Fig. 1), with its striking infiltrate of lymphocytes, plasma cells, and monocytes/macrophages was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of AIH. Immunohistochemical studies, focused on the phenotype of the inflammatory cells infiltrating the liver parenchyma, have revealed a predominance of $\alpha\beta$ T cells [44]. Amongst these cells, a majority was found to be positive for the CD4 helper/inducer phenotype and a sizeable minority for the CD8 cytotoxic/suppressor phenotype. Lymphocytes of non-T cell lineage included NK cells, monocytes/macrophages and B-lymphocytes [32]. Recently, it has been shown that a considerable number of cells producing IL-17, a potent pro-inflammatory cytokine, are present in the AIH inflammatory infiltrate (M.J. Hussain, M. Baker, MS Longhi, Y Ma, G. Mieli-Vergani, D. Vergani, unpublished data).

Liver damage is believed to initiate with the presentation of a self-antigenic peptide to the T cell receptor of an uncommitted T helper (T_H0) lymphocyte by professional antigen presenting cells (APCs), such as macrophages, dendritic cells and B-lymphocytes, in the presence of co-stimulating signals, induced by the interaction of CD28 on T_H0 and CD80 on APC. Once exposed to the antigen embraced by an HLA class II molecule on the APC, the T_H0 lymphocytes become activated and, according to the presence in the milieu of IL-12 or IL-4, they differentiate into T_H1 or T_H2 cells and initiate a series of immune reactions determined by the cytokines they produce. T_H1 cells predominantly secrete IL-2 and IFN- γ , the latter being the main orchestrator of tissue damage because of its ability to stimulate cytotoxic T cells (CTL), to enhance the expression of HLA class I molecules on APCs and of HLA class II molecules on hepatocytes [45, 46] and to activate monocytes/macrophages, which in turn release IL-1 and tumour necrosis factor alpha (TNF- α). The expression of HLA class II on hepatocytes, that normally do not express it, enables them to present the autoantigen to T_H1 lymphocytes and hence the perpetuation of the autoimmune process. The function of T_H1 cells is counter-balanced by that of T_H2 cells, arising in the presence of IL-4 and mainly producing IL-4, IL-10 and IL-13. These cytokines induce also the maturation of B cells into plasma cells, with consequent production of autoantibodies. If regulatory cells are

numerically deficient and/or impaired in their suppressor function, these effector responses are perpetuated with ensuing persistent liver cell destruction by the direct action of CTL, cytokines released by T_H1 cells and monocytes/macrophages, complement activation or engagement of natural killer cells by the autoantibodies bound to the hepatocyte surface. The role of the recently described T_H17 cells, which produce IL-17, IL-22, TNF- α , and CCL20, and have been suggested to play an important role in both murine and human autoimmune disorders [47, 48] is currently under investigation in AIH. They derive from naive T cells exposed to IL-1 β , IL-6, IL-23, and, in mice, TGF- β [48–50], cytokines secreted mainly by innate immune cells [51].

Mechanisms leading to and/or perpetuating autoimmune attack in AIH are depicted in Fig. 5. The contribution of both humoral and cellular immune responses to AIH pathogenesis is reviewed below.

Impairment of T-Regulatory Cells

The mechanisms underlying the breakdown of self-tolerance in autoimmune disease have not been fully elucidated, though there is mounting evidence that a defect in homeostatic processes, normally keeping the response to self-antigens under control, is

involved. Early studies have shown that patients with AIH have impaired ‘suppressor’ cell function, which could be corrected following in vitro exposure to therapeutic doses of steroids [52]. Later, a defect specifically involving a subpopulation of T-lymphocytes controlling immune responses directed against a liver-specific membrane autoantigen was reported [53]. In line with these observations, evidence provided over the last few years has indicated that impairment in regulatory T cells is central to the loss of immune tolerance in AIH and to the emergence of uncontrolled effector autoimmune responses [54–59]. Among recently described regulatory T cell subpopulations, namely natural killer T (NKT) cells, T-helper 3 (T_H3), T-regulatory 1 (Tr1), CD8+CD28- and $\gamma\delta$ T cells, the CD4 T-lymphocytes constitutively expressing high density IL-2 receptor (IL-2R) α chain (CD25) are central to immune-tolerance maintenance [60]. CD4+CD25+ regulatory T cells (T-regs), which in health constitute 5–10% of the total population of peripheral CD4 T-lymphocytes control innate and adaptive immune responses by preventing proliferation and effector function of autoreactive T cells. In addition to CD25—also present albeit at a lower density on activated T cells—T-regs express a number of defining markers, including the glucocorticoid inducible tumour necrosis factor receptor, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the forkhead

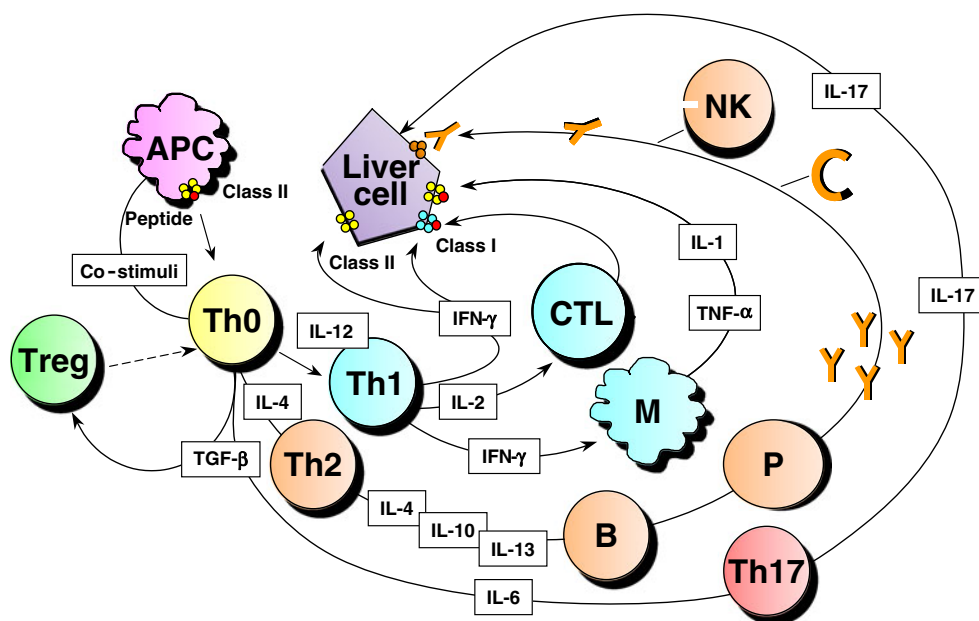


Fig. 5 Autoimmune attack to the liver cell. A specific autoantigenic peptide is presented to an uncommitted T helper (T_H0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). T_H0 cells become activated and, according to the presence in the microenvironment of interleukin (IL)-12 or IL-4 and the nature of the antigen, differentiate into T_H1 or T_H2 and initiate a series of immune reactions determined by the cytokines they produce: T_H1 secrete IL-2 and interferon-gamma (IFN- γ), which stimulate cytotoxic T-lymphocytes (CTL), enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis

factor alpha (TNF- α). T_H2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B-lymphocytes. If regulatory T cells (Treg) do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of CTL; cytokines released by T_H1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described T_H17 cells, which arise in the presence of transforming growth factor beta (TGF- β) and IL-6, is under investigation

winged helix transcription factor box P3 (FOXP3), the best marker for murine T-regs and still the most specific human T-reg marker, being associated with the ability of these cells to suppress [61]. Another major feature of T-regs is low or absent expression of the IL-7 receptor [60, 62–64].

In AIH, T-regs are defective in number, this defect being more marked at disease presentation than during drug-induced remission, when a partial T-reg restoration is observed [54, 55, 58]. The frequency of T-regs is inversely correlated with markers of disease severity, such as levels of anti-SLA and anti-LKM1 antibodies, suggesting that T-reg reduction favours the serological manifestations of autoimmune liver disease [54].

Functional studies have demonstrated that, at variance with healthy subjects, T-regs isolated from patients with AIH are unable to regulate proliferation and IFN- γ production by CD4 and CD8 T cells, both involved in the autoimmune liver attack [54, 55]. Akin to cell number, T-reg suppressor function improves during drug-induced remission, though never reaching the levels seen in health. T-regs from AIH patients are defective at promoting regulatory cytokine secretion by their targets, which in health create a regulatory environment supporting and enhancing the function of T-regs themselves [56]. In addition to impaired regulation of effector T cell function, T-regs in AIH are unable to restrain the activation of monocytes, cells of the innate immune system abundantly present in the portal-periportal inflammatory infiltrate [65].

Because of their ability to control autoaggression, T-regs represent an attractive candidate for immune intervention aiming at reconstituting self-tolerance in autoimmune conditions. The immunotherapeutic use of CD4+CD25+ T-regs, however, has been so far hindered by their limited ability to proliferate and by their propensity to apoptosis [66], making it difficult to obtain numbers adequate for treatment. The observation of a partial T-reg restoration in patients during remission [54, 55] indicates that T-regs in AIH, though impaired, have the potential to expand and regain their function. Following in vitro exposure to a polyclonal T cell stimulation, T-regs can be expanded not only in healthy subjects, but also in patients with AIH [57]. While maintaining the phenotypic features of original CD4+CD25+ T cells, expanded T-regs increase their FOXP3 expression alongside their suppressor function.

Studies in animals show that T-regs with autoantigen specificity suppress more efficiently than their non-antigen-specific counterparts. In this regard, AIH type 2 would be an excellent model for attempting reconstitution of self-tolerance by specific immunological intervention since not only is the key autoantigen, CYP2D6, known [67] but also the antigenic regions (CYP2D6_{217–260} and CYP2D6_{305–348}), target of B, CD4 and CD8 T cell immune responses well characterised [43, 68, 69]. In a recent report, antigen-specific

T-regs were obtained in patients with AIH type 2 and shown to exert a more powerful immunosuppressive effect than polyclonally expanded T-regs [59]. The most efficient suppression of autoreactive T cells was achieved when T-regs exposed to CYP2D6 peptides were co-cultured with semi-mature dendritic cells loaded with the same peptide.

Among other cells with suppressive potential, in AIH NKT cells behave similarly to CD4+CD25+ T-regs, being numerically reduced, especially during active disease; moreover, they produce lower quantities of the immunoregulatory cytokine IL-4 than in healthy subjects. In contrast, $\gamma\delta$ T-cells in AIH are more numerous than in health, have an inverted V δ 1/V δ 2 ratio and a higher IFN- γ and granzyme B production, the latter being correlated to biochemical indices of liver damage, suggesting a prevailing effector over regulatory function for these cells in this condition [58].

Engagement of Effector Mechanisms

Humoral Immunity The role of autoantibodies in the pathogenesis of autoimmune liver damage has been suggested by the finding that hepatocytes, isolated from patients with AIH, are coated with immunoglobulins and are susceptible to cytotoxicity when exposed to autologous Fc receptor bearing mononuclear cells [70, 71]. It has been also shown that titres of anti-liver-specific membrane lipoprotein (LSP) antibody, as well as antibodies to the LSP components asialoglycoprotein receptor (ASGPR) and alcohol dehydrogenase (ADH), correlate with biochemical and histological indices of disease severity [72, 73]. The finding that CYP2D6 is expressed on the surface of hepatocytes, and therefore susceptible to be recognised by anti-LKM1 autoantibodies, has led to the hypothesis that these autoantibodies could be directly involved in the pathogenesis of autoimmune liver damage in AIH type 2 [74]. Following the identification of CYP2D6 as the target antigen of anti-LKM1, the search for B cell epitopes on CYP2D6 has been the focus of intensive investigation. In AIH type 2 anti-LKM1 antibodies recognise linear regions (autoepitopes) of CYP2D6 in a hierarchical manner. Thus, the principal linear B cell epitope, CYP2D6_{193–212} is recognised by 93% of patients, CYP2D6_{257–269} by 85%, CYP2D6_{321–351} by 53%, and two additional minor epitopes CYP2D6_{373–389} and CYP2D6_{410–429} are recognised by 7% and 13%, respectively [68].

Using two complementary strategies, consisting in the expression of both the full-length and a series of truncated CYP2D6 proteins in a eukaryotic system, and in the characterization of the identified epitope by mutagenesis, Ma et al. [75] have provided a conformational (i.e. highly dependent on the three-dimensional structure of the protein) epitope mapping of CYP2D6, showing that the antigenicity

is confined to the C terminal portion of the molecule where it increases stepwise towards the C terminal. In addition, a short amino acid sequence, CYP2D6_{316–327}, further characterised by molecular modelling, is located on the surface of the molecule, suggesting that it may be directly involved in autoantibody mediated liver cell damage [75].

Cellular Immunity Early investigations of cellular immune mechanisms involved in the pathogenesis of autoimmune liver damage led to the conclusion that patients suffering from AIH had circulating lymphocytes ‘sensitised’ to liver antigens and able to kill hepatocytes in vitro. Studies performed using separated T and non-T cell subsets from the peripheral blood of AIH patients and autologous hepatocytes as targets demonstrated that cytotoxic cells were present in the non-T cell subpopulation [70]. The authors also observed high cytotoxic activity in all untreated patients with AIH but in only 40% of those in remission induced by immunosuppressive treatment [70]. The findings that the cytotoxic cells were present in the non-T cell compartment and that aggregated IgG were able to block the cytotoxic activity led to the suggestion that an antibody-dependent-cell-mediated cytotoxicity is involved in the pathogenesis of the autoimmune liver damage, an hypothesis confirmed later by the observation that hepatocytes from AIH patients carrying IgG on their surface were susceptible to damage by lymphocytes from healthy subjects [71]. Subsequent studies of clonal analysis, performed in the early 1990s, showed that cytotoxicity against liver-specific antigens could also be detected within the T cell compartment [76]. Studies conducted later to identify liver-specific T cells in peripheral blood of patients with AIH demonstrated that they have a tenfold higher frequency of liver antigen-specific precursors in their circulation than normal subjects [77]. Experiments of clonal analysis to investigate the function of activated T-lymphocytes in AIH, revealed a high frequency of CD4 T cell clones expressing the HLA-DR molecule. Inhibition of proliferation with anti-HLA-DR and anti-CD4 monoclonal antibodies led to the conclusion that these clones follow the classical rules of immune recognition, being able to recognise antigens in the context of class II HLA molecules [76]. Subsequent investigations showed that in AIH the largest number of clones generated from the peripheral blood are CD4⁺ T cells bearing the $\alpha\beta$ T cell receptor, while the highest proportion of clones obtained from liver biopsies, are CD4-CD8⁻ T cells bearing the $\gamma\delta$ T cell receptor or CD8⁺ $\alpha\beta$ T cells. Both types of liver derived clones were able to proliferate in response to liver-specific antigens, such as ASGPR and ADH; clones that proliferated in the presence of ASGPR were also able to induce the production of ASGPR-specific autoantibodies from B-lymphocytes in vitro [77].

Autoreactive CD4 T Cells T cell epitopic targets have been mainly studied in AIH type 2, where the autoantigen is known. The observation that autoantibodies to CYP2D6 belong to the IgG isotype, implicating a CD4-dependent class switch, and that the majority of T cells infiltrating the liver in AIH are CD4 lymphocytes has led to studies investigating CD4 T cell immune response against CYP2D6 in anti-LKM1 positive patients. CYP2D6_{262–285}-specific T cell clones generated from liver tissue and peripheral blood express a T_H1 CD4⁺ phenotype [78, 79]. At variance with these studies, which focused on a short antigenic sequence of CYP2D6, a systematic approach based on the construction of overlapping peptides covering the whole CYP2D6 molecule was adopted to define the specificity of ex vivo CYP2D6 reactive T cells in patients with AIH type 2 [43]. This study showed that T cells from patients positive for the predisposing HLA allele *DRB1*0701* recognise in a proliferation assay seven regions of CYP2D6, four of which are also partially recognised by T cells of *DRB1*0701* negative patients. While distinct peptides induce production of IFN- γ , IL-4 or IL-10, peptides that induce IFN- γ and proliferative responses overlap. There was also an overlap between sequences inducing T and B cell responses. The number of epitopes recognised and the quantity of cytokine produced by T cells were directly correlated to biochemical and histological markers of disease activity. These results indicate that the T cell response to CYP2D6 in AIH type 2 is polyclonal, involves multiple effector types targeting different epitopes, and is associated with hepatocyte damage [43].

Autoreactive CD8 T Cells In the early 1990s, CD8 T cell clones specific for ASGPR were described in patients with AIH [76]. More recent studies in AIH type 2 have identified CYP2D6-specific CD8 T cells capable of secreting IFN- γ and of exerting cytotoxicity after recognition of CYP2D6 epitopic sequences in an HLA class I-restricted fashion [69]. The possibility that CYP2D6-specific CD8 T cell immune responses are directly involved in the liver damage is suggested by the association of their strength with indices of disease activity and by their presence within the portal tract cellular infiltrates [69]. The CYP2D6_{245–254} sequence, which flanks a major B cell epitope (CYP2D6_{254–271}) [80] and overlaps with CYP2D6_{225–260}, a T cell epitope able to induce CD4 T cell proliferation and IFN- γ production [43], was identified as the HLA-A2-restricted sequence most effective at inducing CD8 T cell functions [69]. The colocalisation of B, CD4 and CD8 T cell epitopes suggests functional interrelations between immune responses to this particular region. B cells can function as antigen presenting cells to CD4 T helper lymphocytes that in turn provide help to cytotoxic T-lymphocytes. The fact that B and T cell

immune responses converge on the same antigenic region indicates that this part of the CYP2D6 molecule may play a key role in the initiation and perpetuation of the autoimmune process in AIH type 2, by promoting the activation of different arms of the immune system. This region could therefore be an appropriate candidate for potential antigen-specific immune intervention.

Monocytes In addition to CD4 and CD8 T-lymphocytes, monocytes/macrophages represent a major component of the portal/periportal cellular infiltrate in AIH. A recent study has demonstrated that compared to health, peripheral blood monocytes, isolated from patients with AIH, have a more vigorous spontaneous migration, which cannot be further augmented by exposure to migration-inducing stimuli [65]. In addition, monocytes in AIH are skewed towards a pro-inflammatory phenotype, as indicated by the higher TNF- α over IL-10 production and by the elevated level of expression of toll-like-receptor-4, whose engagement is key to the initiation of adaptive immune responses. The finding of a marked monocyte activation during active disease, a time when also CD4 and CD8 T cell autoimmune responses are at their highest in terms of proliferation and IFN- γ production, suggests a monocyte participation in the pathogenesis of liver damage in AIH, possibly promoted by autoreactive cells belonging to the adaptive arm of the immune system [65].

Taken together, the data presented above indicate an involvement of both humoral and cellular immune responses in the pathogenesis of AIH, involvement which is likely to be permitted by a failure of immune homeostatic processes.

Molecular Mimicry

A mechanism that has been implicated in the initiation of autoimmune damage is molecular mimicry whereby immune responses to external pathogens become directed towards structurally similar self-components. Several studies have shown that autoimmunity is a common feature during chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, where up to 50% of patients become seropositive for autoantibodies such as ANA and SMA [81, 82]. It is also well documented that positivity for LKM1 is present in up to 10% of patients chronically infected with HCV and correlates with disease severity and adverse reactions to interferon treatment [83, 84]. These findings have led to postulate a role for HBV and HCV infection in the initiation of the autoimmune process, though a strong link between autoimmunity and viral hepatitis has been shown only for HCV. Interestingly, reactivity against CYP2D6₁₉₃₋₂₁₂, identified as major B cell

epitope in AIH type 2, being recognised by 93% of the patients, is also found in 50% of HCV infected patients with seropositivity for anti-LKM1 autoantibodies [85]. HCV positive/anti-LKM1 positive patients have antibodies that cross-react with homologous regions of HCV (NS5B HCV₂₉₈₅₋₂₉₉₀) and CYP2D6 (CYP2D6₂₀₄₋₂₀₉), and also of cytomegalovirus (exon CMV₁₃₀₋₁₃₅) [68]. Cross-reactive mechanisms to explain the emergence of CYP2D6 specific autoimmunity have also been suggested for other sequences of CYP2D6 which share homologies with HCV and herpes simplex virus (HSV), such as the sequence spanning aa 310–324 of E1 HCV and aa 156–170 of IE175 HSV1, which share homology with the CYP2D6 region comprising aa 254–271. As anti-LKM1 antibodies cross-react with homologous regions of CYP2D6, HCV, HSV, and CMV, a ‘multi-hit’ mechanism for the generation of these antibodies and possibly of AIH type 2 may be envisaged. In this model, multiple exposures to CMV or HSV, common viral pathogens, may establish permissive immunological conditions, by priming a cross-reactive subset of T cells, in a genetically predisposed host. Depending on the degree of immunological priming, i.e. level of exposure and the degree of genetic susceptibility (particularly at the HLA locus and coding regions for innate components of immunity), a minority of recurrently infected individuals may progress to autoimmune disease. It is therefore conceivable that an as yet unsuspected virus infection may be part of the origin of the autoimmune attack in AIH. Evidence for such a mechanism has been provided by an interesting case report describing a 10-year-old girl who acquired HCV infection after liver transplantation for end-stage liver disease caused by α 1-antitrypsin deficiency [86]. Two weeks after HCV infection IgM anti-LKM1 appeared, followed by IgG anti-LKM1, a finding suggesting HCV as a trigger of a primary anti-LKM1/anti-CYP2D6 autoimmune response.

Animal Models

Research on the pathogenesis of AIH has been hampered by the lack of animal models reproducing faithfully the human condition. The ideal model for AIH should have a well-defined initiating event followed by chronic inflammation leading to fibrosis. Recently, researchers have focused on animal models of AIH type 2, since in this condition the autoantigen is well defined. The model produced by the group of Alvarez [87] is based on immunising every 2 weeks for three times C57BL/6 female mice with a plasmid containing the antigenic region of human CYP2D6, the target of anti-LKM1, and formiminotransferase cyclodeaminase, the target of anti-liver cytosol-1, an additional marker for AIH type 2 [32], together with

the murine end terminal region of CTLA-4. The latter was added to facilitate antigen uptake by antigen presenting cells. In a parallel set of experiments a plasmid containing the DNA encoding IL-12, a T_H1 skewing pro-inflammatory cytokine, was also used. When autoantigens and IL-12 were used to break tolerance, antigen-specific autoantibodies were produced, a relatively modest elevation of transaminase levels at 4 and 7 months was observed, and a portal and periportal inflammatory infiltrate composed of CD4 and CD8 T cells and, to a lesser extent, B cells was demonstrated 8–10 months after the third immunisation. When the same immunisation protocol was used in different mouse strains, either a mild hepatitis or no inflammatory changes were observed indicating the importance of a specific genetic background. A recent paper from the same group shows that severity of AIH in female C57BL/6 mice depends on the age at xenoinmunisation, 7-week-old mice having a more severe disease both biochemically and histologically, and a higher specific T cell cytotoxicity and autoantibody production than younger or older females. In contrast, vaccinated males developed minimal liver inflammation and higher percentages of Tregs in the circulation, spleen, and liver than females. Tregs were virtually absent in liver lymphocyte infiltrates of females. Manipulation of sex hormones and of thymic control did not alter this pattern. Antigen specific B cell responses correlated with disease activity, suggesting a role for B cells in AIH pathogenesis [88]. Another model of AIH type 2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an adenovirus-CYP2D6 vector [89]. While focal hepatocyte necrosis was seen in both mice treated with the adenovirus-CYP2D6 vector and control mice treated with adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated to a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T cell response to adenovirus CYP2D6 vector-infected target cells. Though these experimental approaches provide useful information on the possible pathogenic mechanisms leading to AIH type 2, a model mimicking closely AIH in humans is still missing.

Conclusion

AIH is an important organ-specific autoimmune disease, as it allows investigating mechanisms of damage not only in the circulation but also within the liver itself, which is easy to biopsy. The intense research into mechanism of liver damage in this fascinating disease over the past 30 years have led to exciting new findings that are likely to provide the basis for revolutionary, specific modes of treatment and possible cure.

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