ANIMAL MODELS

Development of a New Murine Model of Type 2 Autoimmune Hepatitis Using a Human Liver Protein

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Autoimmune hepatitis (AIH) is a chronic inflammatory condition of the liver characterized by parenchymal destruction, hypergammaglobulinemia, specific autoantibody production, and hepatic fibrosis and necrosis. Murine models of AIH have been described; however, little is known about the immunologic mechanisms of tissue destruction. In this study, a new murine model of type 2 AIH was developed using recombinant human cytochrome P450 (CYP) 2D6 emulsified with complete Freund's adjuvant (CFA). BALB/c mice were immunized with 2 µg/mL i.p. of CYP2D6 in CFA. The control group received CFA or phosphate-buffered saline alone. Alanine aminotransferase activity, autoantibody production, IgG concentrations, histologic damage, and specific T-cell response were evaluated. Persistent AIH, characterized by cellular infiltration, hepatic fibrosis, elevated alanine aminotransferase, and the production of anti-liver kidney microsomal antibody type 1 developed in CFA/CYP2D6-immunized mice. These mice presented high levels of IgG and its subclasses IgG1, IgG2a, and IgG2b against liver self-proteins. Interestingly, IL-2⁺ and interferon γ—positive Cyp2d6-specific T cells were present in greater concentrations in mice immunized with CFA/CYP2D6 compared with control. Immunization with CFA, in combination with a natural human autoantigen like CYP2D6, was demonstrated to break tolerance, resulting in a chronic form of autoimmune-related liver damage. This murine model of type 2 AIH is expected to be instrumental in understanding the immunologic mechanisms of the pathogenesis of this autoimmune liver disease. (*Am J Pathol* 2022, 192: 21–30; <https://doi.org/10.1016/j.ajpath.2021.10.006>)

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disorder of unknown etiology, characterized by an immunologic intolerance to hepatic components, that progressively destroys the hepatic parenchyma. Clinical manifestation is commonly characterized by hypergammaglobulinemia, interface hepatitis, aminotransferase elevation, and serum autoantibodies.^{1,2} Two types of AIH—type 1 and type 2—are recognized based on the autoantibody profile of serum samples. Type 1 AIH is defined by seropositivity for anti-nuclear antibodies and/or anti-smooth muscle antibodies, whereas type 2 AIH is characterized by high levels of anti-liver kidney microsomal type 1 antibody (anti-LKM-1) and/or anti-liver cytosol type 1 antibody.^{3–5} The targets of these antibodies

are the cytochrome P450 (CYP) 2D6 isozyme⁶ and formiminotransferase cyclodeaminase (FTCD),⁷ respectively. These enzymes are expressed in hepatocytes, and a previous report demonstrated correlation between the levels of antibodies and AIH activity.⁸ To date, the etiology and pathogenesis of this liver disease are unknown, and more information is needed to clarify which immune mechanism is involved in the pathogenesis of AIH. For this reason, several animal models of liver inflammation have been developed, including transgenic and nontransgenic models,

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which are expected to be useful in the identification of mechanisms of the loss of immunologic tolerance.

Complete Freund's adjuvant (CFA), commonly used in research, acts by prolonging the lifespan of injected autoantigen by stimulating its effective delivery to the immune system, and by providing a complex set of signals to the innate compartment of the immune system, resulting in altered leukocyte proliferation and differentiation.⁹ The use of CFA is necessary for the induction of many experimental models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, thyroiditis, and orchitis.^{10–14} Because of its capacity to stimulate cellular and humoral immune responses, CFA has been used to develop animal models that mimic the liver damage caused by the immune system in patients with autoimmune liver diseases.^{15,16} However, these models have limitations, and more information is necessary for understanding the immune mechanism of this disease. Here, a new murine model was generated using recombinant CYP2D6, a human liver protein target of anti-LKM-1 antibodies, combined with CFA.

Materials and Methods

Animals and Immunization

Four- to six-week-old female mice of the BALB/c strain were purchased from Universidad Nacional Autónoma de México (Mexico City, Mexico). Animals were housed in a 12-hour light/dark cycle with a constant-temperature environment of 22°C, and a standard diet and water were supplied *ad libitum*. A group of 20 mice were immunized with 2 µg/mL i.p. of recombinant human CYP2D6 (Sigma-Aldrich, St. Louis, MO) in 200 µL of emulsion with 1 mL of CFA (Sigma-Aldrich) and 1 mL of sterile phosphate-buffered saline (PBS; 1.5 mmol/L KH₂PO₄, 8 mmol/L Na₂HPO₄, 2.7 mmol/L KCl, 137 mmol/L NaCl). As controls, 20 animals received 200 µL of the emulsion with 1 mL of CFA and 1 mL of sterile PBS (active control), and 20 other animals received only 200 µL of sterile PBS (control). Immunizations were done with a primary and a booster dose, separated by 15 days (Figure 1). The study was conducted according to the guidelines of the Declaration of Helsinki and approved by an independent institutional review board (Instituto de Investigaciones Médico Biológicas, number IIMB-18-009-15).

Preparation of Murine Liver Homogenate

BALB/c normal murine liver samples were homogenized with a tissue grinder in 3 to 6 mL of PBS. The crude homogenate was centrifuged at 7250 × *g* for 30 minutes at 4°C. The supernatant was carefully removed and saved, and the pellet was homogenized with 2 volumes (2 to 4 mL) of PBS and centrifuged at 7700 × *g* for 30 minutes at 4°C. The supernatants were pooled, and then 10 µL of 800 mmol/L

CaCl₂ solution per milliliter of supernatant was added. The supernatant was mixed for 15 to 30 minutes and centrifuged at 4°C for 60 minutes at 16,000 × *g*. The supernatant was saved in a new sterile conic tube and analyzed by SDS-PAGE. The total protein from the liver supernatant was measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Western Blot Analysis

Proteins (100 µg of murine liver homogenate) were separated by electrophoresis on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Merck-Millipore, Burlington, MA). The membrane was blocked overnight with 5% powdered milk in 0.05% Tween 20 in PBS at 4°C. The polyvinylidene difluoride membrane was incubated for 1 hour at room temperature with the mice serum diluted to 1:100 in 0.05% Tween 20 in PBS/5% powdered milk. The secondary antibody (goat anti-mouse IgG) was conjugated to horseradish peroxidase (Invitrogen, Camarillo, CA), diluted to 1:1000 in 0.05% Tween 20 in PBS/5% powdered milk. Bound peroxidase was detected with chemiluminescence blot analysis substrate (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay

Microwell plates were coated with 5 µg/well of CYP2D6 human recombinant protein overnight at 4°C. After blocking with 0.5% bovine serum albumin for 1 hour at 37°C, murine serum samples were serially diluted into the wells and incubated for 2 hours at 37°C. Plates were washed and a secondary antibody (goat anti-mouse IgG, IgG1, IgG2a, and IgG2b, all conjugated with horseradish peroxidase; Jackson ImmunoResearch, West Grove, PA) was added to each corresponding well and incubated for 1 hour at 37°C. The peroxidase reaction was developed by incubation with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich). End point levels were calculated through linear regression equations as the reciprocal of the serum dilutions that produced OD405 nm values of 0.2 above the blank, and the levels were reported in enzyme-linked immunosorbent assay units (EU/mL).

Histologic Examination

Liver samples were obtained for histologic examination 1 to 6 months after the last immunization. The liver samples were fixed in 10% formalin, dehydrated, embedded in paraffin, sectioned in 4-µm slides, and stained with hematoxylin and eosin. Various organs (spleen, kidney, lung, and pancreas) were histopathologically examined for extrahepatic inflammation. The sections were subsequently scored for inflammation and injury as previously described^{17,18} (grades: 0, no inflammation or necrosis; 1, minor lobular

Figure 1 Immunization protocol. Female BALB/c mice aged 4 to 6 weeks were injected i.p. with CFA, CFA/CYP2D6, or PBS twice with a 15-day interval. Blood specimens were collected 0 to 6 months after the second injection, and mice were euthanized 1, 4, 5, and 6 months after the second immunization. ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; WB, Western blot.

inflammation with no necrosis; 2, lobular inflammation involving <50% of the section; 3, lobular inflammation involving ≥50% of the section; and 4, inflammation with necrosis). Slides treated with trichrome staining for collagen deposition were analyzed for the presence or absence of fibrosis.

Biochemistry Analysis

Biochemistry studies to determine the levels of alanine aminotransferase (ALT) in the serum of the mice were performed using a commercial chemistry systems kit (Bayer Healthcare, Leverkusen, Germany) 1 to 6 months after the last immunization.

ELISpot Analysis

Enzyme-linked immunosorbent spot (ELISpot) assays were performed using a commercial kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, a 96-well polyvinylidene difluoride membrane ELISpot plate (Merck-Millipore, Burlington, MA) was coated with anti-mouse IL-2, interferon (IFN)- γ , and IL-17A overnight at 4°C and was then blocked for 2 hours at room temperature with RPMI-1640 medium (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (Biowest, Riverside, MO) and 1% antibiotic/antimycotic (Sigma-Aldrich). Spleens were removed from CFA- or CFA/CYP2D6-injected mice, placed into RPMI-1640 medium, and passed through a 75- μ m filter. To prepare the splenocytes, red blood cells were lysed with ammonium-chloride-potassium buffer. Cells were suspended in RPMI-1640 and 5×10^6 cells/mL splenocytes were added in duplicate, activated with 2 μ g/mL of CYP2D6 human recombinant protein, and incubated for 24 hours at 37°C in 5% CO₂. Biotinylated anti-mouse IL-2, IFN- γ , and IL-17A were

added and plates were incubated for 2 hours at 37°C. Horseradish peroxidase-conjugated Avidin D was added for 45 minutes at room temperature, and the reaction was developed by the addition of 3-amino-9-ethylcarbazole (Sigma-Aldrich).

Adoptive Transfer

Adoptive transfer was performed as previously described.¹⁶ Briefly, the spleens of the female BALB/c mice were taken 1 month after the second immunization with 2 μ g/mL i.p. of human CYP2D6 in CFA or CFA only. Splenocytes were suspended at 5×10^6 cells/mL and stimulated for 48 hours with 1.25 μ g/mL of the T-cell mitogen concanavalin (Con) A (Sigma-Aldrich) in RPMI-1640 medium. Con A-activated splenocytes (1×10^6 cells/mL) were injected i.p. into naive 6-week-old BALB/c mice. Control mice received the same number of naive Con A-activated splenocytes. Liver specimens were histologically examined 1 month after passive transfer and stained with hematoxylin and eosin.

Statistical Analysis

The Tukey multiple-comparisons test was used for determining the significance of differences between more than two groups. A *P* value of <0.05 was considered statistically significant. The data were generated using GraphPad Prism statistical software version 6.01 (GraphPad, San Diego, CA) and are expressed as means $\pm$ SEM.

Results

Alanine Aminotransferase Activity

ALT is a clinical marker of hepatic damage commonly used to monitor disease activity.¹⁹ Therefore, serum ALT levels

were evaluated in the CFA/CYP2D6-immunized mice and control groups (CFA or PBS). As opposed to the other two groups, the CFA/CYP2D6-immunized mice showed an abnormal ALT level by 4 months after immunization (mean, 77.8 U/L), which increased in subsequent months (means, 84.2 and 55.8 U/L at 5 and 6 months, respectively). The CFA group showed a moderate elevation in ALT level at months 1 and 4 (means, 50.8 and 63 U/L); however, these levels presented a tendency to normalize at months 5 and 6 (means, 59.9 and 42.8 U/L). The PBS group showed normal ALT, considered as nonresponse (Figure 2).

B-Cell Response

The role of autoreactive B cells in AIH involves different mechanisms of action, mainly the well-established secretion of autoantibodies.²⁰ To evaluate the B-cell response, levels of total IgG against the recombinant human CYP2D6 protein were measured in serum samples from CFA- and CFA/CYP2D6-injected mice each month during the study. Total IgG anti-LKM-1 levels were elevated at 1 month after the second immunization in the CFA/CYP2D6 group (mean, 26,839.30 EU/mL), and levels of these antibodies were high in the subsequent months (means, 2844.28, 3050.70, and 3180.13 EU/mL at 4, 5, and 6 months, respectively). Levels of antibodies in sera from CFA-immunized mice were low (Figure 3A). To further characterize the B-cell response, levels of IgG subclasses were determined. The CFA/CYP2D6-immunized group showed high IgG1 at 1 month after immunization, which was maintained throughout the 6-month study (means, 5008.90, 3468.40, 4069.98, and 4442.63 EU/mL at 1, 4, 5, and 6 months). IgG1 was low in the CFA-immunized group (Figure 3B). Antibodies of the IgG2 subclasses (IgG2a and IgG2b) were evaluated. As expected, levels of IgG2a and IgG2b antibodies were higher in the CFA/CYP2D6-immunized group than in the CFA-immunized group (Figure 3, C and D). To evaluate the presence of autoantibodies, serum samples from each group of mice were tested against proteins from BALB/c murine liver homogenate in a Western blot analysis. The serum samples from the CFA/CYP2D6-immunized group contained a 48-kDa protein; this molecular weight corresponds to the murine Cyp from the 2d subfamily, Cyp2d9 (Figure 4).

Histologic Characteristics

Mice were sacrificed at 1, 4, 5, and 6 months after the second injection of CFA/CYP2D6, CFA alone, or PBS. The liver, lung, spleen, kidney, and pancreas were obtained for histologic examination. The livers of PBS-injected mice did not show any histologic changes in any month during the study. CFA-injected mice showed a slight portal tract lymphocytic infiltrate by 1 and 4 months after the second immunization, and normal histologic examination in the subsequent months. CFA/CYP2D6-

Figure 2 Serum ALT levels in CFA-, CFA/CYP2D6-, and PBS-injected mice. Mice immunized with CFA present elevated serum ALT levels at the 4th month, with a tendency to normalize in the 5th or 6th month after the last immunization. A trend toward elevated serum ALT levels at the 4th, 5th, and 6th months after the second immunization is observed in mice immunized with CFA/CYP2D6. Mice injected with PBS show no increase in ALT level. The dotted line represents the normal value of the test. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group.

injected mice showed an intense portal tract lymphoplasmacytic inflammatory infiltrate and interface hepatitis (Figure 5). The other organs showed no histologic changes (data not shown). To determine whether the cellular inflammatory infiltrate causes liver damage, hepatic fibrosis formation was characterized at 6 months after the second immunization in the CFA- and CFA/CYP2D6-immunized mice. The administration of CFA was not associated with an accumulation of collagen in the portal tract. In contrast, mice immunized with CFA/CYP2D6 showed a collagen deposition in the portal tract that extended into the lobule (Figure 6).

T-Cell Response

The cellular response plays a central role in the pathogenesis of many autoimmune diseases. Research has shown that autoreactive T cells have a crucial role in the development and establishment of autoimmune diseases.^{21,22} To assess the T-cell response, total splenocytes were isolated from the spleens of CFA/CYP2D6- and CFA-immunized mice, and their response to recombinant human CYP2D6 protein was measured. CFA/CYP2D6-immunized mice presented a response mainly by IL-2⁺/IFN- γ ⁺ T cells compared with CFA-immunized mice (Figure 7). To confirm that these lymphocytes were autoreactive, total splenocytes from animals sacrificed 1 month after the second i.p. immunization with 2 μ g/mL of human CFA/CYP2D6 or CFA only were stimulated over 48 hours with Con A and transferred into wild-type recipient mice, and their autoreactivity response was measured by histologic examination, ALT and aspartate aminotransferase (AST) activity, and the presence of autoantibodies 1 month after the adoptive transfer. Figure 7 shows the results of the adoptive transfer experiment. The CFA/CYP2D6-recipient animals showed high ALT and AST levels (means, 56.20 and 96.40 U/L, respectively),

Figure 3 Anti-liver kidney microsomal type 1 antibody (anti-LKM-1) concentrations. CFA/CYP2D6-immunized mice show elevated levels of total IgG (A) and IgG1 (B), IgG2a (C), and IgG2b (D) subclasses of anti-LKM-1 antibodies compared with CFA-immunized mice or PBS-injected mice. Data are expressed as means $\pm$ SEM [in log₁₀ concentration (EU/mL)]. $n = 5$ mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus CFA (Tukey multiple-comparisons test).

presence of specific autoantibodies against a 48-kDa protein, and mild but distinct histologic changes (Figure 8).

Discussion

AIH is a complex and multifactorial inflammatory disease of the liver involving interactions between genetic background and environmental and immunologic factors that trigger an inflammatory response through autoreactive T cells, inducing the progressive destruction of hepatocytes and leading to liver failure if left untreated. Although AIH was described >50 years ago by Waldenström,²³ little is known about how the immune system acts against self-proteins from the liver. This is partly due to the low incidence and

prevalence of AIH around the world, hindering basic and clinical studies of the disease. To resolve this problem, for the past 30 years, several animal models have been used to reflect the clinical, biochemical, and histologic pattern of the disease in humans. Each model often reflects a particular aspect of AIH, but one that represents complete disease is lacking.

Here, a new murine model of type 2 AIH was developed using CFA in combination with a natural human autoantigen, CYP2D6. Importantly, new evidence regarding how this immunization can break the immune tolerance against liver self-proteins in a nonpermissive genetic background such as the BALB/c strain, resulting in a chronic form of autoimmune liver damage, was presented. In addition, based on the high prevalence of autoimmune disorders associated with sex, the experimental approach used female mice, but not male mice. In particular, in humans, about 70% to 80% of AIH patients are women,^{24,25} and in murine models, liver injury has been reported to be more severe in female mice than in male mice. In models of drug-induced liver injury or Con A-induced liver injury, BALB/c females develop more severe hepatitis and produce more proinflammatory hepatic cytokines and antibodies than do males.^{26,27} Splenocytes cultured from female mice exhibit fewer T-regulatory cells and higher concentrations of IL-1 β and IL-6 than do those from males. However, T-regulatory cell function does not differ by sex.²⁶ Plasma ALT, tumor necrosis factor (TNF)- α , and IFN- γ levels were significantly higher after Con A administration in females than in males. Furthermore, plasma ALT, TNF- α , and IFN-

Figure 4 Determination of autoantibodies in mouse serum samples. Serum samples from mice immunized with CFA/CYP2D6 present positivity against 48-kDa protein at the 1st (line 1), 4th (line 3), 5th (line 5), and 6th (line 7) months after the second immunization. Lines 2, 4, 6, and 8 correspond to mice immunized with CFA. The Western blot technique was used to determine the presence of autoantibodies in the serum samples using mouse liver homogenates.

Figure 5 Histologic examination of livers from BALB/c mice injected with CFA, CFA/CYP2D6, or PBS. **A:** Representative liver sections from CFA-, CFA/CYP2D6-, and PBS-injected mice. Control mice (PBS) do not show any inflammatory infiltrate. CFA-immunized mice show scarce portal inflammation in the 1st and 4th months and normal histologic examination at the 5th and 6th months after the second immunization. CFA/CYP2D6-immunized mice exhibit portal inflammatory infiltrate at the 1st, 4th, and 5th months. Mice in the 6th month present interface hepatitis. **B:** Liver inflammation scores in mice injected with PBS, CFA, and CFA/CYP2D6 at the 1st, 4th, 5th, and 6th months after injection. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group. $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Scale bars = 100 μ m.

γ levels were decreased significantly after ovariectomy.²⁷ Castrated males developed more severe hepatitis than did intact males in a murine model of drug-induced liver injury.²⁶

The possible explanation for this sex-based disparity remains unknown, but it has been theorized that sex hormones have a crucial role in autoimmunity. There is a general consensus to use female over male mice in animal models. While it is true that, in humans, AIH occurs predominantly in women, men have been reported to have a

higher relapse rate and a younger age of disease onset. Despite this, men with AIH have significantly greater long-term survival than do women with AIH.²⁸

Consistent with the findings from the present study, other studies have reported that chronic hepatitis is produced by i.p. immunizations with liver proteins in CFA. One study reported extensive periportal infiltration in most mice treated with consecutive i.p. immunizations with allogeneic liver homogenate. In the same study, liver inflammation could be adoptively transferred to syngeneic recipients by splenocytes

Figure 6 Liver fibrosis after immunization in mice injected with CFA/CYP2D6 but not CFA alone. Representative liver sections from CFA- and CFA/CYP2D6-injected mice at 6 months after the second immunization (trichrome staining). The liver in CFA-immunized mice does not show the presence of collagen fibers in the portal tract. CFA/CYP2D6-immunized mice exhibit the presence of collagen fibers (arrows) in the portal tract that extend into the lobule. Scale bars = 100 μ m.

Figure 7 Characterization of the T-cell response. **A–C:** Frequency of T-cell populations producing IFN- γ (**A**), IL-17A (**B**), and IL-2 (**C**) in splenocytes from mice immunized with CFA or CFA/CYP2D6. The splenocytes were obtained at the 1st, 4th, 5th, and 6th months after the last immunization. **D:** Representative wells of specific T-cell-producing IFN- γ , IL-17A, and IL-2 from mice immunized with CFA or CFA/CYP2D6. Data are expressed as mean $\pm$ SEM spots-forming cells (SFC)/10⁶ splenocytes. $n = 5$ mice per group. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$ versus CFA.

or lymphocytes.²⁹ In other work, Lohse et al¹⁶ demonstrated that hepatitis was induced for up to 6 months with a single i.p. immunization in C57BL/6 mice with the 100,000 $\times$ g supernatants of syngeneic liver homogenate (S-100) in CFA. Additionally, the ALT level was moderately increased. These results are similar to those observed in the present study, in which CFA/CYP2D6-immunized mice presented an increase in ALT level by 4 months after immunization (mean, 77.8 U/L). Interestingly, these levels were elevated in the subsequent months (means, 84.2 and 55.8 U/L at 5 and 6 months, respectively) (Figure 2). The measurement of ALT in serum is commonly used to evaluate liver damage in AIH patients. AIH may also be identified by recognition of mild to moderate elevations of ALT activity.³⁰ Therefore, the results from the present study suggest that this animal group presented progressive hepatic damage that could be comparable to that in human disease.

Elevated serum total IgG level is another important parameter characteristic of human AIH and is used as a criterion for its diagnosis.³¹ In the present study, the CFA/CYP2D6-immunized group showed an elevated IgG level. High serum IgG levels were mainly of the IgG1 subclass. Interestingly, the other IgG subclasses, IgG2a and IgG2b, were present in the serum of the CFA/CYP2D6-immunized group (Figure 3). Elevations of these IgG subclasses are representative of a Th1-type response, which has been

reported to have a role in complement activation and antibody-dependent cell-mediated immunity.³² These results are similar to those reported in another murine model in which the presence of the subclasses IgG2a and IgG2b was observed.³³ Anti-LKM-1 is known to be present in the serum of patients with type 2 AIH and in a subset of patients with chronic hepatitis C. The autoantigen to anti-LKM-1 has been identified to be CYP2D6.^{34,35} In the present experiment, the murine serum samples contained a 48-kDa protein. This molecular weight corresponds to the murine Cyp isozyme from the 2d subfamily, Cyp2d9. The foregoing is due to the high amino acid sequence homology between the murine Cyp isozymes Cyp2d9, Cyp2d11, Cyp2d22, and Cyp2d26 and human CYP2D6.³⁶ Thus, Cyp2d6-specific B cells react to human CYP2D6 peptides with homology to the murine Cyp isozymes, indicating that molecular mimicry breaks tolerance and subsequently causes severe persistent autoimmune-related liver damage.

Histologic findings of the liver in AIH patients are usually characterized by interface hepatitis mainly composed of mononuclear and plasma cells.^{25,37} Recently, several experimental works have demonstrated that liver-infiltrating T cells from patients with type 2 AIH recognize CYP2D6.^{22,38} Thus, the CYP2D6-specific T-cell response in AIH may contribute to the pathogenesis of the disease. In a recent murine model, CFA/CYP2D6-immunized mice presented a response mainly by

Figure 8 Adoptive transfer. **A:** The spleen cells to be transferred were obtained 1 month after the second immunization. Serum and liver sections were prepared from recipient mice 1 month after transfer. **B:** Serum levels of ALT and AST in CFA cells or CFA/CYP2D6 cells from a transferred mouse. The **dotted line** represents the normal value of the test. **C:** Hematoxylin and eosin staining of a liver section from a transferred mouse with CFA cells. Note the intact structures of the hepatic parenchyma. A section from a transferred mouse with CFA/CYP2D6 cells. Note the scarce amount of inflammatory cell infiltration in the portal tracts. **D:** The Western blot technique is used to determine the presence of autoantibodies in the serum samples from mice recipient of CFA cells or CFA/CYP2D6 cells using mouse liver homogenates. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group. Scale bars = 50 μ m.

IL-2⁺/IFN- γ ⁺ T cells, compared with CFA-immunized mice, in total splenocytes stimulated with recombinant human CYP2D6. Another interesting observation was that these groups of animals presented a characteristic portal and periportal (interface hepatitis) inflammatory infiltrate mainly characterized by lymphocytes, as occurs in human disease.^{35,36} Furthermore, CFA/CYP2D6-immunized mice showed an accumulation of collagen fibers in the portal tract that extended into the lobule at 6 months after the second immunization with the antigen. These results indicate that the presence of a murine Cyp family in the liver plays an important role in the development of chronic hepatic inflammation and fibrosis.

However, some limitations should be noted. First, serum ALT was the only biochemical marker of liver damage that was measured. Although the level of serum ALT activity reflects damage to hepatocytes and is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of hepatotoxicity, an increase in serum ALT activity level has also been associated with other organ toxicities.^{39,40} To assess the

risk for false-positives due to extraneous sources of serum ALT activity, biomarkers in addition to serum ALT activity are sometimes used for determining liver function.⁴¹

In conclusion, the results of the study in the current model of AIH suggest a loss of tolerance to murine liver proteins, causing with it the development of an autoimmune process. Loss of immunologic tolerance is induced by the molecular mimicry established in the presence of a foreign antigen to conserved regions of murine liver proteins. Therefore, the experimental model developed could be used as an important tool for investigating and characterizing, in additional detail, the immunopathologic mechanisms of loss of tolerance to self-proteins leading to chronic liver parenchymal destruction.

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Author Contributions

H.V.-C. conceptualized the study; P.T.-D. and I.Y.I.-H. curated data; P.T.-D., J.M.R.-T., and H.V.-C. formally analyzed data; H.V.-C. acquired funds; H.V.-C. performed investigation; P.T.-D., I.Y.I.-H., K.G.H.-F., E.E.S.-M., A.D.C.-C., and P.G.-P. developed methodology; H.V.-C. performed project administration; H.V.-C. supervised the study; P.T.-D. and H.V.-C. wrote the original draft; P.T.-D., K.G.H.-F., and H.V.-C. reviewed and edited the draft; all authors read and agreed to the published version of the manuscript.

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