Oxidative Stress as a Trigger for Cellular Immune Responses in Patients With Alcoholic Liver Disease

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Serum antibodies reactive with neo-antigens generated during ethanol metabolism have been identified in patients with alcoholic liver disease (ALD), although their role in the pathogenesis of disease remains unclear. In this study, we characterized peripheral blood mononuclear cell (PBMC) T-cell and antibody responses to human serum albumin (HAS) adducted with acetaldehyde under reducing conditions (AcA-HSA) or with malondialdehyde (MDA-HSA) in patients with advanced ALD (AALD, $n = 28$), heavy drinkers with no liver disease (NALD, $n = 14$), and mild/moderate drinking controls ($n = 22$). Peak proliferative responses of PBMC were assessed *in vitro* by tritiated thymidine incorporation after the addition of optimized concentrations of antigen or OKT3. Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). MDA-HSA induced PBMC T-cell proliferation was significantly higher in ALD than in NALD or control patients. Moreover, 10 of 28 (36%) of ALD patients had significant T-cell proliferative responses to MDA-HSA compared to 0 of 14 (0%, $P = .02$) of the NALD group and 2 of 22 (9%, $P < .05$) of controls. No significant difference in PBMC T-cell response to Aca-HSA was seen between subject groups. Patients with positive cellular responses to MDA had higher serum anti-MDA antibody titers than those not exhibiting a positive cellular response ($P < .005$). In conclusion, the pattern of cellular and humoral responses to MDA adducts suggests that the development of these responses may be a susceptibility factor for the development of advanced alcoholic liver disease. The apparent importance of T-cell responses to MDA adducts suggests that oxidative stress may represent an important stimulus for the development of cellular immune responses associated with advanced ALD. (HEPATOLOGY 2004;39:197–203.)

The factors that determine in only a minority of heavy drinkers the development of advanced alcoholic liver disease (ALD) are still unclear.[1] They seem likely, however, to include a combination of both endogenous (genetic) and exogenous factors that influence pathways contributing to the various putative mechanisms of alcohol-induced hepatocyte injury.[2] Of these, oxidative stress and cytokine-related mechanisms are currently the most plausible. Reactive oxygen species and other free radicals produced during ethanol metabolism are capable of initiating lipid peroxidation and subsequently hepatocyte apoptosis and necrosis.[3–5] Ethanol also increases gut permeability leading to higher levels of portal endotoxaemia. This endotoxin can then stimulate intrahepatic Kupffer cells to release cytokines, particularly tumor necrosis factor (TNF)α, which can increase the production of reactive oxygen species from hepatocytes, and also directly induce apoptosis.[6,7] As yet, however, with a few notable exceptions,[8–10] studies examining factors potentially influencing these mechanisms have been disappointing in explaining individual susceptibility to advanced disease.

More recently, a growing body of clinical and experimental evidence has suggested that immune mechanisms may play a role in alcohol-related liver injury in at least a subgroup of ALD patients. Variable immune responses to antigens formed as a result of ethanol metabolism, and the resulting oxidative stress, may therefore offer an alternative explanation for inter-individual variability in susceptibility to ALD. Acetaldehyde (AcA) and malondialdehyde (MDA), an end product of lipid peroxidation, are known to bind to

Abbreviations: ALD, alcoholic liver disease; AcA, acetaldehyde; MDA, malondialdehyde; PBMC, peripheral blood mononuclear cell; HSA, human serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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host proteins and to form stable adducts.[11,12] These adducts are found in the livers of patients with ALD and are localized to areas of hepatocyte damage.[13,14] Antibodies to these adducts can be found in the sera of heavy drinkers and in mice fed ethanol.[15–18] Furthermore, sera from patients with ALD can induce antibody dependent cell cytotoxicity in ethanol-treated hepatocytes *in vitro* when co-cultured with normal peripheral blood mononuclear cells.[19] Importantly, these antibodies are found more frequently, and at higher titers in patients with ALD when compared with either heavy drinkers without liver disease[15] or patients with non-alcoholic liver diseases.[18] While it is now clear that they can assist hepatocyte lysis at the correct effector:target cell ratios *in vitro*, it is still not clear how, and if, these antibody responses are involved in the development and perpetuation of ALD *in vivo*.

Lymphocyte infiltration is a frequent (albeit variable) histologic feature in advanced fibrotic or cirrhotic ALD, with a significant proportion of these lymphocytes exhibiting the phenotype of CD8 positive cytotoxic T lymphocytes. This finding raises the possibility that cellular immune responses may, in some individuals at least, contribute directly to hepatocyte damage.[20] This *in situ* evidence led us, in the context of the existing data regarding humoral immune responses to adduct neo-antigens, to hypothesize that adaptive cellular immune responses directed against neo-antigens arising during ethanol metabolism may be important in the initiation and progression of ALD in a proportion of heavy drinkers. In this initial study, we set out to characterize the peripheral blood mononuclear cell (PBMC) CD4+ T-cell responses to AcA and MDA adducted to human serum albumin in heavy drinkers with and without liver disease and mild/moderate drinking controls.

Patients and Methods

Study Groups

Three groups of subjects were studied. The first consisted of heavy drinkers with advanced ALD (AALD group, $n = 28$), all of whom had cirrhosis. The second consisted of heavy drinkers with no evidence of ALD or with simple steatosis only (NALD group, $n = 14$). For inclusion in either of these groups, patients had to have consumed more than 80 g ethanol per day for at least 10 years. The NALD group had no clinical evidence of liver disease; this was defined by having either (i) normal liver serum biochemical testing on 2 occasions (not including an isolated rise in gamma glutamyl transferase), or, for those with abnormal liver blood tests, (ii) liver histology showing either normal liver or steatosis with no evidence of steatohepatitis or fibrosis. Drinkers were excluded if they had serologic evidence of previous hepatitis B virus or hepatitis C virus infection or autoimmune liver disease, histologic evidence of other liver disease, or if they were homozygous for the C282Y mutation in the *HFE* gene. The control group ($n = 22$) consisted of healthy controls who drank between 5 and 21 units per week (men) and 5 and 14 units per week (women). After obtaining informed consent, blood (30 ml) was collected from subjects for PBMC isolation and the preparation of serum. In view of the direct effect of ethanol on antigen presentation,[21] all patients were abstinent for at least 48 h before study. Subjects in the NALD group were enrolled while attending an alcohol dependence psychiatric service that performed regular breathalyzer tests.

Preparation of Antigens

MDA adducted with human serum albumin (MDA-HSA) was prepared by reacting 1 mg/ml HSA with 50 mmol/L MDA for 2 h at 37°C. Acetaldehyde modified HSA (HSA-AcA) was obtained under reducing conditions by incubating 1 mg/ml HSA solution in phosphate-buffered saline (PBS) pH 7.4 with 10 mmol/L acetaldehyde and 10 mmol/L sodium cyanoborohydride for 1 h at room temperature. The unbound aldehydes were removed by overnight dialysis at 4°C against PBS pH 7.4.

Determination of Antibody Titers

Polystyrene microwell plates for enzyme-linked immunosorbent assay (ELISA) were coated for 4 h at 37°C with 0.05 mg/ml of either modified HSA or native protein solubilized in 0.1 M bicarbonate buffer pH 9.6. After incubation, the solutions were removed and replaced by 0.3 ml of coating buffer containing 3% bovine serum albumin in PBS pH 7.4. The plates were further incubated for 1 h at 37°C to block non-specific binding sites. The coated wells were washed 3 times with PBS containing 0.25% Triton X-100. The patients' sera were diluted 1:50 with the coating buffer and added in duplicate as aliquots of 0.2 ml to the appropriate wells and incubated for 1 h at 37°C. After washing 3 times with PBS-0.25% Triton X-100, peroxidase-linked goat anti-human IgG (dilution 1:6,000) was added and incubated for 60 min at 37°C. Antibody binding was revealed by the addition of 0.15 ml of a reaction mixture containing 0.4 mg/ml of 1-phenylendiamine, 0.4μl/ml hydrogen peroxide (30%), 5.1 mg/ml citric acid, and 6.1 mg/ml anhydrous Na_2HPO_4 at pH 5.0. After 15 min, the reaction was stopped by adding 50 μl 2M H_2SO_4 and absorbances were measured at 490 nm using a BioRad microplate reader (Bio-Rad Laboratories, Hercules, CA). The results were corrected by subtracting the background reactivity as assayed on unmodified HSA.

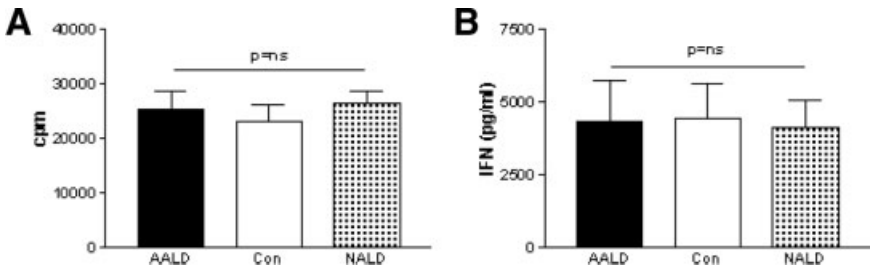

Fig. 1. (A) Proliferative and (B) IFN alfa responses of PBMC from advanced ALD patients (AALD), normal controls and heavy drinkers with no evidence of liver disease (NALD) in response to the pan-T-cell stimulant OKT3

Tissue Culture

PBMC lymphocyte proliferative responses were assayed by measurement of ^{3}H-thymidine incorporation into DNA. Whole blood was diluted 50:50 in RPMI 1640 (Sigma) and mononuclear cells were purified by density gradient centrifugation on Ficoll Histopaque (Sigma). The interfacial cells were recovered, washed 3 times in complete medium (RPMI 1640, 5% fetal calf serum, and penicillin-streptomycin; all from Sigma), and counted. A total of 2×10^5 cells were aliquoted per well in a final volume of 200 μl of complete medium in 96-well round-bottomed plates (Costar, Cambridge, MA). Filter-sterilized HSA, AcA-HSA, or MDA-HSA was added at an optimal range of concentrations established in preliminary experiments (0.1–10 μg/ml). Pentuplicate repeats were used for each antigen concentration and controls. After an optimized period of 6 d in culture at 37°C under 5% CO_2, 100 μl of culture medium was removed from each well and stored at –70°C before interferon gamma (IFN-γ) assay by ELISA. Each well in the culture plate was then pulsed with 1 μCi of ^{3}H-thymidine in 30 μl of complete medium. After a further 16 h in culture, the incorporated radiation was assayed by liquid scintillation counting. The mean value for the antigen-stimulated wells was divided by the mean from the medium-only control wells to give a stimulation index (SI). The cut-off was defined as the medium-only control well (mean + 1 SD)/mean from all experiments. In our case, this gave a value of 1.72. The experimental approach adopted is one that is optimized for the detection of CD4+ T-cell responses.

To confirm that the potential proliferative capacity of the lymphocytes isolated from the 3 groups was similar, OKT3 (a pan-T-cell stimulant) was added to further wells containing 2×10^5 cells at an optimized final concentration of 10 ng/ml and cells were pulsed at day 3 (peak).

Cytokine ELISA

Culture supernatant IFN-γ was assayed by sandwich ELISA (Biosource). Briefly, capture antibody (clone 350B10G6) was coated (2.5 μg/ml) overnight at 4°C onto microtiter plates in carbonate-binding buffer, pH 9.6. Detection of bound antigen was performed using a biotinylated detection antibody (0.5 μg/ml of clone 67F12A8) followed by incubation with streptavidin horseradish peroxidase conjugate. Peroxidase reactivity was visualized using the substrate in the presence of H_2O_2. The reaction was terminated with 2 mol/L H_2SO_4 and absorbance measured at 492 nm. The lower limits of the sensitivities of the ELISA used was defined as the mean absorbance +2 SD for control wells. Lower limit of sensitivity for the ELISA used was 10 pg/ml.

Data Analysis and Statistical Calculations

Statistical analysis was performed using the Instat-3 statistical software (GraphPad Software Inc, San Diego, CA, USA) using one-way ANOVA. The degree to which the data were normally distributed in the groups was preliminary evaluated by Kolmogorov and Smirnov test. Significance was taken at the 5% level. Fishers exact test and the Chi squared test were used for frequency analysis.

Results

OKT3 Induced Responses

All subjects in all groups showed a significant PBMC T-cell proliferative response to OKT3 in culture. There was no significant difference in either the magnitude of the proliferative response or the level of culture supernatant IFN-γ between the 3 groups of patients studied (Fig. 1).

CD4+ T-Cell Responses to Adduct Neo-antigens

PBMC T-cells from patients with advanced ALD showed a significantly higher ($P = .01$) mean proliferative response to MDA-HSA (SI 1.45 $\pm$ 0.70) than those from non-ALD heavy drinkers (NALD) (0.97 $\pm$ 0.15) or controls (1.26 $\pm$ 0.30) (Fig. 2). In contrast, no significant differences in proliferative response to AcA-HSA adducts were seen between the 3 subject groups (AALD: 1.35 $\pm$ 0.60; NALD: 1.10 $\pm$ 0.21; control: 1.33 $\pm$ 0.37) (Fig. 3). No responses were seen to native albumin. The frequencies of positive responses to MDA and AcA adducts in heavy drinkers with and without liver disease, and in moderate drinking controls are shown in Table 1. Of 28 patients with advanced ALD, 10 (36%) had an SI of greater than 1.72 (indicative of a positive response to antigen in our experimental system) in response to MDA-

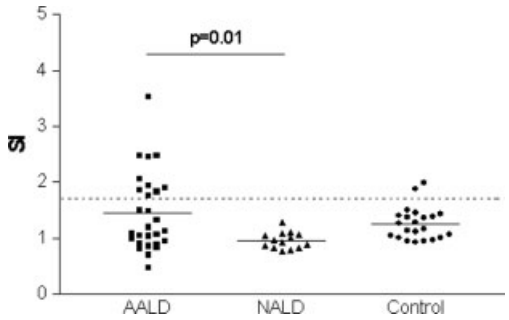

Fig. 2. PBMC T-cell proliferative responses to MDA adducted with human serum albumin. The solid horizontal lines denote the mean value for each subject group. The broken horizontal line denotes the cut-off for a positive proliferative response as defined for this study (defined as the medium-only control well (mean + 1 SD)/mean from all experiments).

HSA adducts as compared to 0/14 (0%, $P = .02$) of the NALD group and 2/22 (9%, $P < .05$) controls. In contrast, only 6/28 (21%) of the AALD group and 4/22 (18%) controls showed a positive response to AcA adducts. Although the presence of AcA adduct responses was not associated *per se* with AALD the presence, in the same individual, of responses to both MDA and AcA adducts did show a unique association with AALD (5/28 AALD patients responding to both antigens compared with 0/22 controls and 0/14 NALD patients; $P < .05$, Chi squared 7.0). It is unclear at present whether the presence of T-cell responses to more than one adduct is associated with any difference in ALD disease phenotype.

The PBMC response to MDA seen in AALD but not NALD patients was functional, as well as proliferative, in nature. Culture supernatant concentrations of IFN-ã were significantly higher in AALD patients demonstrating a positive proliferative response to MDA adduct than in MDA adduct non-responders (Fig. 4).

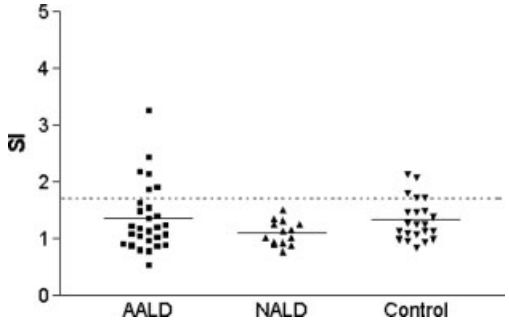

Fig. 3. PBMC T-cell proliferative responses to human serum albumin (HSA) adducted with acetaldehyde (AcA) under reducing conditions. The solid horizontal lines denote the mean value for each subject group. The broken horizontal line denotes the cut-off for a positive proliferative response as defined for this study (defined as the medium-only control well (mean + 1 SD)/mean from all experiments).

Table 1. Frequencies of Positive PBMC T-Cell Responses to MDA and AcA in Advanced ALD Patients (AALD), Normal Controls, and Non-ALD Heavy Drinkers (NALD)

	MDA (%)	AcA (%)
AALD (n = 28)	10 (36)*	6 (21)
Controls (n = 22)	2 (9)	4 (18)
NALD (n = 14)	0 (0)	0 (0)

*$P = 0.02$ versus NALD.

Anti-adduct Antibody Responses

Anti-MDA-HSA IgG titers were higher ($P < .01$) in the ALD group than in both the NALD and control groups (Fig. 5). ALD patients also demonstrated higher anti-AcA-HSA antibody titers than controls ($P < .01$) (the difference in anti-AcA-HSA titers between AALD and NALD patients was not significant) (Fig. 6). Interestingly, the patients demonstrating positive lymphocyte proliferative responses to MDA-HSA also had significantly higher antibody titers to the same antigen compared to T-cell non-responders ($P < .005$) (Fig. 7). No association between T-cell and antibody responses to AcA-HSA was seen (data not shown).

Discussion

A number of studies have demonstrated that ALD patients have an enhanced humoral immune response to neo-antigens formed *in situ* as a result of protein modification by alcohol metabolites or lipid peroxidation products.[15–20] However, despite initial reports showing cytotoxic activity towards ethanol-modified autologous hepatocytes *in vitro*,[22,23] and a close association between the infiltration of CD4 and CD8 positive T-lymphocytes with histologic evidence of intralobular inflammation, regenerating nodules, and central sclerosis,[20] few data have been presented to date regarding the presence of cell-mediated responses towards the same antigens. In this

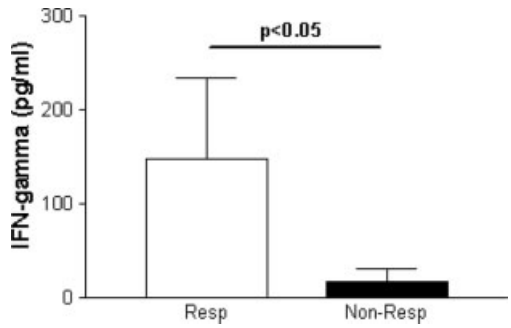

Fig. 4. Culture supernatant concentrations of IFN-ã in response to MDA-HSA for PBMC from AALD patients showing a significant PBMC proliferative response to MDA-HSA in comparison to PBMC from AALD patients showing no PBMC proliferative response to MDA-HSA.

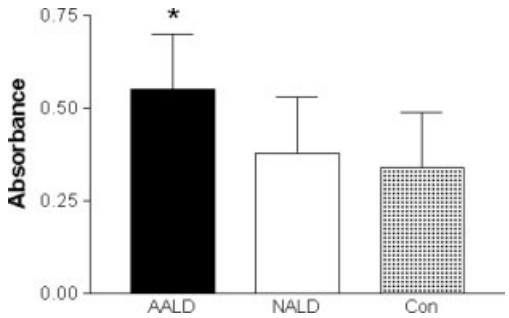

Fig. 5. Antibody responses to MDA adducted with human serum albumin. Data are given as ELISA absorbances at 490 nm. *$P < .01$ versus both control and NALD groups.

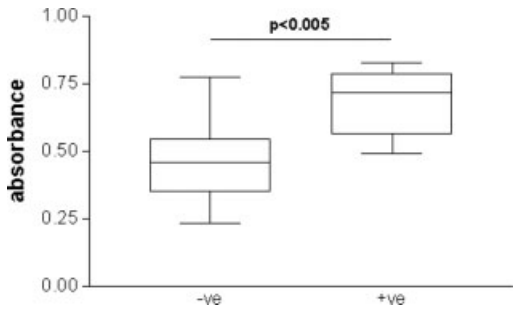

Fig. 7. Antibody responses to MDA adducted with human serum albumin in AALD patients demonstrating (+ve) and not demonstrating (–ve) a PBMC T-cell proliferative response to MDA-HSA.

initial study of the cellular immune response in ALD, we set out to characterize CD4+ T-cell responses to AcA- and MDA-derived adducts. Antibody responses against these neo-antigens have been extensively characterized in heavy drinkers, with antibody titers showing a significant correlation with severity of liver disease.[15,17,18] Future studies, guided by the findings of the current study, will address the important question of the nature of the CD8+ T-cell response to these antigens in ALD.

In this study, we demonstrated that a significant proportion (36%) of ALD patients have peripheral blood CD4+ T-cell responses specific for the MDA-HSA adduct. Such responses were absent from all heavy drinkers without liver disease, and from the vast majority of normal drinking controls. In contrast, T-cell responses against AcA in isolation showed no statistically significant disease association. The absence of MDA-HSA-specific PBMC T-cell responses from the NALD subject group did not reflect a global lack of T-cell proliferative capacity, as responses to OKT3 (a pan-T-cell stimulating agent) were similar in all subject groups. Parallel studies in the same subject groups demonstrated, as previously, significantly higher levels of anti-MDA-HSA antibody responses in AALD patients than in either NALD patients or normal drinking controls. T-cell and antibody responses to MDA-HSA were associated, with anti-MDA-HSA antibody levels being significantly higher in patients demonstrating a positive T-cell response to the neo-antigen than in apparent T-cell non-responders. Taken together, our findings indicate that oxidative damage is able to promote both humoral and cellular immune responses supporting the concept that oxidative stress represents a key steps in neo-antigen generation in ALD.[24–27]

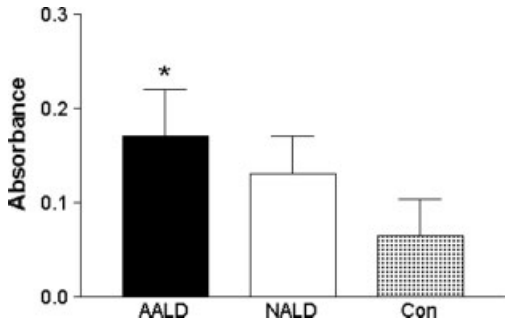

Fig. 6. Antibody responses to human serum albumin adducted with acetaldehyde (AcA) under reducing conditions. Data are given as ELISA absorbances at 490 nm. *$P < .01$ versus control group. Difference between NALD and AALD groups was not statistically significant.

It is noteworthy that, despite the appreciable increase in antibodies specific for AcA seen in AALD patients, the T-cell response to this antigen is not enhanced. The explanation for this apparent paradox may lie in the fact that the true specificity of the antibodies detected using AcA adducts prepared under reducing conditions as antigens remains uncertain.[28] Recent studies have shown that 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde is one of the main immunogenic adducts generated by the reaction of proteins with high MDA concentrations.[29] The same adduct can also be generated *in vivo* by the condensation of MDA and acetaldehyde and elicits a specific humoral immune response in both alcohol-fed animals and ALD patients.[26,30] Thus, it is possible that the T-cell response against MDA-HSA observed in AALD patients might actually be directed towards mixed MDA-acetaldehyde adducts.

At present, it is unclear what role the identified T-cell responses play, if any, in the pathogenesis of ALD. One interpretation of our findings would be that oxidative stress is the key etiologic process, the presence of which distinguishes AALD from NALD patients. In this model, the generation of T-cell responses to MDA-adducts would represent "immunologic noise;" a consequence of the presence of neo-antigens generated by the key etiologic process of lipid peroxidation. The alternative interpretation would be that T-cell responses to neo-antigens generated as a result of oxidative stress represent, in a sub-group of patients, a key pathogenetic factor, with immune-mediated cellular responses playing a significant

role in hepatocyte damage. There is some evidence to support the latter view. Limited *in vitro* studies have demonstrated cytotoxic potential of lymphocytes from ALD patients against alcohol treated autologous hepatocytes,[22,23] while the histologic studies alluded to previously suggest the presence of activated T-cells with an effector phenotype in livers from patients with ALD.[20,31]

Intriguingly, the largest study of the *in situ* histologic phenotype of ALD has suggested that the presence of significant lymphocytic infiltrates is a feature seen in only a minority (40%) of patients.[31] This proportion of patients with an *in situ* immune phenotype is similar to the proportion of patients in the current study found to have an MDA-hsa specific peripheral blood T-cell response. The design of the current study did not allow us to address the obvious question as to the extent to which these 2 phenotypes overlap. If, however, the phenotype is indeed shared, and the same group of patients who have peripheral blood T-cell responses to neo-antigen have liver lymphocytic infiltrates, it would argue quite strongly in favor of a role for cellular immune responses in disease pathogenesis in a subgroup of patients with ALD.

The current study demonstrated that the majority of ALD patients do not exhibit MDA-HSA-specific T-cell responses. Moreover, the Colombat study demonstrated that the majority of ALD patients do not have liver lymphocytic infiltrates.[31] These observations would support the view that even if immune mediated mechanisms are important in some patients, other pathogenetic mechanisms are likely to be predominant in other ALD patients. We would, therefore, suggest that the pathogenesis of ALD involves a variety of mechanisms, and that patients may be susceptible through one or more pathways, including the direct sequelae of oxidative stress, endotoxin mediated cytokine-release, and immune-mediated liver injury. These mechanisms are, however, not mutually exclusive, as suggested by the apparent inter-relationship between oxidative stress and immune-mediated mechanisms highlighted by the current study and the recent demonstration that endotoxin can stimulate immune induction by inhibiting T regulatory cells through a Toll-like receptor/interleukin-6 dependent pathway.[32]

Defining the primary pathogenetic mechanism in each individual ALD patient may, however, become more important if the putative disease variants are found to be associated with different patient prognoses and patterns of response to therapy. Identification of patients with a primarily immune-mediated disease variant may assist with selection of patients who might be expected to show the best response to immunosuppressive agents, such as corticosteroids, when acutely unwell,[33] or who may benefit from long-term immunosuppression. This may also prevent the use of corticosteroids in patients that have little or no immune element to their disease, and therefore increase the risk/benefit ratio of this controversial treatment.

Advanced alcoholic liver disease is associated with a poor prognosis, and progress in our understanding of its pathogenesis is badly needed if the goal of improved therapy is to be realized. The identification of apparently discrete groups of AALD patients with immune and non-immune disease phenotypes raises the interesting possibility that different pathogenetic processes may be at work in different subgroups of patients (with the implication that different therapeutic modalities may be appropriate in different ALD patients). Further work is required to address this hypothesis. Information regarding the stability over time of the apparent immune and non-immune disease phenotypes, differences in prognosis of different disease variants, and response patterns to currently used therapeutic approaches would be of particular value.

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