Adaptive Immune Responses Triggered by Oxidative Stress Contribute to Hepatic Inflammation in NASH

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Previous studies have shown that human nonalcoholic steatohepatitis (NASH) is often associated with the presence of circulating antibodies against protein adducted by lipid peroxidation products. Here we used the methionine-choline deficient (MCD) model of NASH to characterize the possible involvement of adaptive immunity in NASH. In mice fed up to 8 weeks with the MCD diet the extension of liver injury and lobular inflammation paralleled the development of immunoglobulin G (IgG) against malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE)-derived antigens as well as with the hepatic recruitment of CD4+ and CD8+ T-lymphocytes responsive to the same antigens. Moreover, in these animals the individual IgG reactivity against MDA-adducts positively correlated with transaminase release and hepatic tumor necrosis factor alpha (TNF-α) expression. To substantiate the role of immune responses triggered by oxidative stress in the progression of NASH, mice were immunized with MDA-adducted bovine serum albumin (MDA-BSA) before feeding the MCD diet. MDA-BSA immunization did not affect control mice livers, but further stimulated transaminase release, lobular inflammation, and the hepatic expression of proinflammatory cytokine in MCD-fed mice. The increased severity of NASH in immunized MCD-fed mice involved liver recruitment and the T helper (Th)-1 activation of CD4+ T cells that, in turn, further stimulated macrophage M1 responses. Moreover, hepatic fibrosis was also evident in these animals in relation with an IL-15-mediated increase of natural killer T-cells (NKT) and the up-regulation in liver production of osteopontin by NKT cells and hepatic macrophages.

Conclusion: These results indicate that oxidative stress can contribute to the progression of NASH by stimulating both humoral and cellular immune responses, pointing to the possible role of adaptive immunity in the pathogenesis of the disease.

A key issue in understanding the pathogenesis of nonalcoholic fatty liver disease (NAFLD) concerns the identification of the mechanisms responsible for switching from simple steatosis to steatohepatitis (NASH). This aspect is clinically relevant because steatosis does not appear to adversely affect the long-term outcome of NAFLD, whereas parenchymal injury and inflammation are the driving forces for the disease evolution to fibrosis/cirrhosis. Oxidative stress is one of the features of NAFLD/NASH and hepatic oxidative stress markers, such as 4-hydroxynonenal (4-HNE) and 8-hydroxydeoxyguanosine, correlate with the severity of necroinflammation and fibrosis, suggesting that oxidative injury might be involved in triggering steatohepatitis. In this scenario, recent evidence indicates that lipid peroxidation products originating from the oxidation of phospholipids can act as damage-associated molecular patterns (DAMPs) and promote inflammation through the interaction with soluble and cell-associated pattern recognition receptors. A further mechanism by which oxidative stress can stimulate inflammation involves adaptive immunity. Indeed, in atherosclerosis as well as in several autoimmune diseases the interaction of lipid peroxidation products with
cellular proteins leads to the formation of immunogenic adducts that induce both humoral and cellular immune responses.9,10

Previous studies from our laboratory have shown that high titers of immunoglobulin G (IgG) against some of the antigens originating from oxidative stress, namely malondialdehyde (MDA)-derived adducts, are detectable in about 40% of adult NAFLD/NASH patients and in 60% of children with NASH.11,12 In these latter, high antibody titers associated with more severe lobular inflammation and 13-fold increased risk of an NAFLD activity score ≥5,12 while in adults anti-MDA IgG is an independent predictor of fibrosis.11 From this background, we sought to investigate the possible contribution of immune reactions triggered by oxidative stress in modulating hepatic inflammation in NASH. For the experiments, we relied on a rodent model of NASH based on mice feeding with a methionine-choline deficient (MCD).13

Materials and Methods

Animal and Experimental Protocol. Eight-week-old male C57BL/6 mice were purchased from Harlan-Nossan (Corezzana, Italy) and fed for 4 or 8 weeks with either MCD or control diets (Laboratorio Dottori Piccioni, Gessate, Italy). For immunization experiments mice were injected subcutaneously with 100 μg of MDA-adducted bovine serum albumin (MDA-BSA) in incomplete Freund’s adjuvant and reboosted after 1 week with the same antigen. The control groups received either saline or incomplete Freund’s adjuvant injections. MCD diet feeding was started 2 weeks after the second injection. In some experiments, immunized mice were treated with the anti-CD4 monoclonal antibody GK1.5 (BioXCell, West Lebanon, NH) while receiving the MCD diet to deplete hepatic CD4⁺ T cells (see Supporting materials for further details). The efficiency of cell depletion was preliminarily evaluated by flow cytometry in the liver and the spleen and was >97%. All the experiments were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

Antigen Preparation and Antibody Measurement. Protein adducts with lipid peroxidation products were prepared as before12,14 and used to coat polystyrene microwell enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Roskilde, Denmark). Mouse sera (0.20 mL, 1:50 dilution) were added in duplicate and the antibody binding was revealed using peroxidase-linked goat antimouse IgG or IgM sera as described.12,14 The results were expressed as optical density following the subtraction of background reactivity.

mRNA Extraction and Real-Time Polymerase Chain Reaction (PCR). Liver RNAs were retrotranscribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy). RT-PCR was performed in a Techne TC-312 thermal cycler (Burlington, NJ) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for mouse tumor necrosis factor alpha (TNF-α), interleukin (IL)-12p40, IL-17a, RORγT, interferon-gamma (IFN-γ), T-bet, CCL2, iNOS, CD40, CD40L, osteopontin, z1-procollagen, and β-actin (Applied Biosystems Italia). All samples were run in duplicate and the relative gene expression was calculated as 2ΔΔCt over that of the housekeeping β-actin gene. The results are expressed as fold increase over the control samples.

Histology and Immunohistochemistry. Steatosis and lobular inflammation were scored blind according to Kleiner et al.15 in hematoxylin/eosin-stained liver sections. Hepatocyte apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) using the Apoptags Kit (Intergen, New York, NY). Liver infiltrating T- and B-cells were evidenced in frozen sections using a horseradish peroxidase polymer kit (Biocare Medical, Concord, CA) and anti-mouse CD3 or B220 rat monoclonal antibodies, respectively (R&D System Europe, Abingdon, UK). Polyclonal antibodies against α-smooth muscle actin (α-SMA) (Labvision, Bio-Optica, Milan, Italy) were used to detect activated hepatic stellate cells in formalin-fixed sections.

Intrahepatic Lymphocyte Isolation and Flow Cytometry Analysis. Hepatic mononucleated cells were isolated and purified on a density gradient as before.16 The cells were stained with fluorochrome-
conjugated antibodies for CD45, CD3, CD4, CD8, NK1.1, F4/80, CD69, CD107a, IL-2, and IFN-γ (eBioscience, San Diego CA) and analyzed with a FACScalibur (Becton Dickinson) flow cytometer. Decomplemented mouse serum was used to block unspecific immunoglobulin binding. A polyclonal anti-osteopontin rabbit antiserum (Millipore, Temecula, CA) and phycoerythrin-conjugated antirabbit IgG (Sigma-Aldrich, Milan, Italy) were used for detecting osteopontin-producing cells. The response of intrahepatic lymphocytes to MDA adducts was investigated following an overnight incubation with MDA-adducted or native murine albumin (10 μg/mL) in the presence of brefeldin A (3 μg/mL), anti-CD3e, and CD28 antibodies (1 μg/mL) by detecting IL-2-producing cells with flow cytometry.

**Data Analysis and Statistical Calculations.** Statistical analyses were performed using one-way analysis of variance (ANOVA) test with Tukey’s correction for multiple comparisons or the Kruskal-Wallis test for nonparametric values. Significance was taken at the 5% level. Normality distribution was preliminarily assessed by the Kolmogorov-Smirnov test. Additional methods are described in the Supporting Materials.

**Results**

**Immune Responses Against Oxidative Stress-Related Antigens Associates With NASH Progression.** NASH induced in mice by feeding an MCD diet is characterized by a time-dependent worsening of liver injury. Accordingly, in C57BL/6 mice receiving the MCD diet for up to 8 weeks we observed a progressive increase in hepatic triglyceride content, transaminase release, and circulating TNF-α levels that paralleled the histological severity of hepatic inflammation (Supporting Fig. 1). Liver oxidative stress, as measured by thiobarbituric acid reactive compounds (TBARs), was also evident in the animals with NASH at the fourth week (Fig. 1). As in humans, oxidative stress was associated with the development of IgG against adducts originating from lipid peroxidation products, such as MDA and 4-HNE (Fig. 1). The individual IgG reactivity against MDA adducts, but not liver TBARs, positively correlated with alanine aminotransferase (ALT) release and hepatic TNF-α mRNA expression (r = 0.61, P = 0.04; r = 0.66, P = 0.03, respectively). Immunohistochemistry of NASH livers revealed that hepatic inflammatory infiltrates were also enriched by T- and B-lymphocytes (Supporting Fig. 1), the number of which positively correlated with the individual IgG reactivity against MDA adducts (r = 0.68, P = 0.02; r = 0.75, P = 0.006, respectively). Flow cytometry analysis of hepatic mononucleated cells confirmed a progressive recruitment of T-lymphocytes in NASH livers that involved effector CD8+ T cells and CD4+ helper T (Th) cells (Fig. 1). Furthermore, the proportion of CD3+ T cells expressing the CD69 activation marker was higher in the livers of MCD-fed mice as compared to controls (Fig. 1). Intrahepatic CD4+ T cells from mice with NASH also showed an enhanced IFN-γ expression (Fig. 1), suggesting that lipid peroxidation-derived antigens might contribute to the development of cell-mediated immune responses. Supporting this view, we observed that CD8+ and CD4+ T cells obtained from NASH, but not from healthy livers, produced IL-2 when incubated in vitro with MDA-modified murine albumin (Fig. 1).

**Induction of Immunity Against MDA-Adducts Enhances NASH Severity in Mice.** To substantiate the possible role of adaptive immunity induced by lipid peroxidation-derived antigens in promoting hepatic inflammation in NASH, we stimulated immune reactions against MDA adducts by injecting mice with MDA-modified BSA in incomplete Freund’s adjuvant before the administration of the MCD diet. In preliminary experiments, this immunization protocol led to appreciable humoral and cellular reactivity against MDA adducts (not shown). In the animals receiving the control diet MDA-BSA immunization did not affect liver histology and ALT release, nor significantly modified the hepatic expression of inflammatory mediators, such as TNF-α and CCL2 (Supporting Fig. 2). However, following 4 weeks on the MCD diet, ALT release and the hepatic mRNA expression of TNF-α and CCL2 were higher in MDA-BSA-immunized than in naïve mice (Fig. 2). No appreciable changes in liver injury and inflammation were observed in mice injected with incomplete Freund’s adjuvant before receiving the MCD diet (Fig. 2). The enhanced severity of NASH was further supported by histology that showed higher scores for lobular inflammation and an increased frequency of necroinflammatory foci and apoptotic cells in MCD-fed immunized mice (Fig. 3). Furthermore, these latter had circulating TNF-α levels 3-fold higher than similarly treated naïve mice (Fig. 2). Bieghs et al. recently reported that the induction of IgM antibodies crossreacting with oxidized phosphatidylycerine ameliorated NASH caused by feeding low-density lipoprotein (LDL) receptor-deficient C57BL/6 mice with a high-fat/cholesterol diet. In our hands, the immunization with MDA-BSA adducts did not influence IgM reactivity towards MDA-derived antigens, while it moderately stimulated...
that against oxidized phosphatidylcholine (Supporting Fig. 3), indicating that different mechanisms were involved. Thus, we sought to further investigate the role of oxidative stress-driven immunity in promoting liver inflammation in NASH.

Characterization of Immune Response Associated With the Development of NASH in Immunized Mice. Flow cytometry of intrahepatic lymphocytes showed that MDA-BSA immunization did not modify the liver T-cell profile in mice receiving the control
diet (Supporting Fig. 3). However, immunization further promoted the recruitment of CD3$^+$ T cells in MCD-fed mice, increasing both the CD8$^+$ and CD4$^+$ pools (Fig. 4). However, the proportion of CD8$^+$ T cells expressing the CD107a activation marker was unchanged (Fig. 4). Th-1 and Th-17
activation of CD4^+ T-lymphocytes are regarded as important proinflammatory stimuli. We observed that the expression of the Th-1 transcription factor T-box transcription factor (T-bet) as well as the liver IFN-\(\gamma\) content were selectively increased in MCD-fed immunized animals (Fig. 4), in parallel with a stimulation of the macrophages M1 activation markers IL-12p40 and inducible NO synthase (iNOS) (Fig. 2). Among immunized MCD-fed mice there was also a positive correlation between the individual expression of IFN-\(\gamma\) and that of TNF-\(\alpha\), IL-12p40, and iNOS \((r = 0.82, 0.75, \text{and} 0.88, \text{respectively}; P < 0.02)\). No changes were evident in the hepatic mRNAs for the Th-17 transcription factor retinoic acid-related orphan receptor-\(\gamma\)t (ROR-\(\gamma\)t) and IL-17a (not shown). Furthermore, MCD-fed immunized mice also showed upregulated mRNAs for CD40 ligand (CD40L; CD154) and its receptor CD40, a pair of costimulatory receptor-ligand molecules involved in macrophage activation by CD4^+ T cells.\(^1\)\(^9\) To further verify the role CD4^+ T-lymphocytes in promoting NASH, MCD-fed immunized mice were depleted of CD4^+ T cells by using an anti-CD4 monoclonal antibody. As shown in Fig. 5, CD4^+ T-cell depletion significantly lowered the hepatic mRNA expression of IFN-\(\gamma\) and CD40L as well as that of the macrophage M1 markers iNOS and IL-12p40. Histology in these animals confirmed an improvement of lobular inflammation and focal necrosis (Fig. 5).
Fig. 4. The immune responses against oxidative stress-derived antigens promote the liver recruitment of T-lymphocytes and Th-1 responses. Liver mononucleated cells were isolated from the livers of either naive controls (Cont), naïve mice fed 4 weeks an MCD diet, or mice preimmunized with MDA-modified BSA before the administration of the MCD diet (Imm-MCD). (A-D) Representative dot blots of T-lymphocytes staining for CD45 and CD3 and the percent distribution of total CD3⁺ and CD8⁺ or CD4⁺ T-cells subsets. The values refer to 5-6 animals in each group and the bars represent medians ± SD. (E) Representative dot blots of CD8⁺ T cells displaying the CD107b activation marker. (F-J) Th-1 activation of CD4⁺ T-cells was evidenced by the intrahepatic production of IFN-γ and the mRNA expression of the Th-1 transcription factor T-bet, CD40, and CD40 ligand (CD154). The RT-PCR values were normalized to those of the β-actin gene and presented as fold increase over control values. The data refer to 8-12 animals in each group and the boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the 80% values.
Progression of NASH in Immunized Mice Involves Natural Killer T (NKT) Cells. Unexpectedly, the worsening of NASH occurring in immunized mice was also associated with changes in liver NKT cells. According to previous observations, the development of NASH in naïve mice was characterized by the lowering of liver natural killer (NK) (CD3\(^{-}\), NK1.1\(^{+}\)) and NKT (CD3\(^{+}\), NK1.1\(^{+}\)) pools. On the contrary, MCD-fed immunized animals did not show NK cell depletion, while the NKT fraction was significantly higher than in control mice (Fig. 6). Recent findings in animal models of NAFLD/NASH have implicated macrophage production of IL-15 in controlling liver NKT cell differentiation and survival. In turn, by
producing osteopontin (OPN), NKT cells have been proposed to favor NASH progression to fibrosis.\textsuperscript{16,23} In our hands, the expansion of NKT cells observed in MCD-fed immunized mice paralleled with an increase of the hepatic IL-15 content (Fig. 6). Such an effect was likely mediated by CD4\textsuperscript{+} T-cell activation, as CD4\textsuperscript{+} T-cell depletion significantly lowered intrahepatic IL-15 mRNA in MCD-fed immunized mice (Fig. 5). We also observed that, while the development of NASH in naïve mice did not affect liver OPN, OPN production was significantly up-regulated in MCD-fed immunized animals and such an increase involved an expansion of OPN-expressing NKT cells and hepatic macrophages (Fig. 6). Furthermore, in line with the OPN capacity to stimulate hepatic stellate cells (HSCs),\textsuperscript{24} Sirius Red staining for collagen and α-SMA positive HSCs were more evident in immunized than in naïve MCD-fed mice (Fig. 2).

**Discussion**

Recent studies have implicated the contribution of adaptive immunity in promoting fat inflammation in obesity, since CD4\textsuperscript{+}/CD8\textsuperscript{+} T cells are recruited into
the adipose tissue and provide stimulation for the macrophage production of proinflammatory mediators. Lymphocytes are often detected in the lobular infiltrates of NASH, but the actual role of adaptive immunity in the pathogenesis of the disease is still poorly understood. We previously reported that subsets of adult and pediatric NAFLD/NASH patients show antibody responses against oxidative stress-related antigens, such as MDA-derived adducts, that associate with an increased severity of lobular inflammation or fibrosis. Similar antibodies are also detectable in rats with NASH induced by enteral nutrition with a high-fat diet, while preventing oxidative stress with N-acetylcysteine attenuates both the IgG formation and the severity of steatohepatitis. In the present study, we observed that the progression of MCD-induced NASH parallels with the development of IgG against lipid peroxidation-derived adducts and the liver recruitment of CD4+ and CD8+ T-lymphocytes recognizing the same antigens. The possible contribution of adaptive immunity to the progression of experimental NASH is further substantiated by the observation that stimulating immune responses against MDA-protein adducts, one of the antigens recognized by the antibodies detected in both human and rodent NASH, promotes parenchymal injury and inflammation in mice fed the MCD diet. We are well aware that NASH induced by the MCD diet does not reproduce some of the key features of the human disease such as obesity and insulin resistance; however, in this study we exploited the capacity of this model to cause oxidative stress and extensive steatohepatitis rapidly progressing to fibrosis. Our data are not in contrast with a recent report Bieghs et al. showing that IgM targeting oxidized LDLs reduce NASH in LDL receptor-deficient mice receiving a high-fat/cholesterol diet. These discrepancies, in fact, can be explained considering that the two experimental settings are quite different for the immune responses involved and the mechanisms leading to NASH. In Bieghs et al.’s work mice were immunized with heat-inactivated pneumococci leading to the production of natural IgM against bacterial antigens that crossreact with oxidized phosphatidylcholine in LDLs. Feeding a high-fat/cholesterol diet to LDL receptor-deficient mice causes Kupffer cell engulfment by oxidized LDLs that, in turn, promotes Kupffer cell activation and hepatic inflammation. In this scenario, the IgM interaction with oxidized LDLs reduces their uptake by Kupffer cells, lowering the proinflammatory stimuli. These conditions are quite different from those occurring in MCD-induced NASH, where parenchymal injury, oxidative stress, and inflammation result from the impairment of hepatocyte lipid secretion. Furthermore, our data indicate that the immunization with MDA adducts mainly stimulates IgG production and T-cell responses and that these latter are mainly responsible for promoting inflammation.

Concerning the mechanisms by which adaptive immunity contributes to the evolution of NASH, we observed that hepatic CD4+ T cells are increased in MCD-fed immunized mice in parallel with a stimulation in the liver expression of IFN-γ and CD40L (CD154). CD40L is a costimulatory molecule predominantly expressed by CD4+ T cells and activated platelets that, by the interaction with its receptor CD40 on macrophages and lymphocytes, has a key role in orchestrating inflammation and immunity in several diseases, including atherosclerosis and obesity. In line with this, CD4+ T-cell depletion prevents the up-regulation of IFN-γ and CD40L and ameliorates lobular inflammation, indicating that Th-1 activation of CD4+ T-lymphocytes plays a major role in promoting NASH. It is noteworthy that Th-1 activation characterizes CD4+ T-cell responses to LDL-derived oxidation antigens in atherosclerosis. In this setting, CD4+ T cell or IFN-γ deficiency have been shown to ameliorate plaque inflammation and the disease progression. Interestingly, an increase in circulating IFN-γ-producing CD4+ T-cells has been observed in either pediatric and adult NASH patients in conjunction with an enhanced liver IFN-γ production, suggesting the possible relevance of these mechanisms to the human disease. A recent report indicates that an increase in hepatic CD8+ T cells also characterizes pediatric NASH. In our hands, CD8+ T-cell recruitment is evident in NASH livers and is further promoted by preimmunization. However, immunization does not affect the expression CD8+ T-cell activation markers, suggesting that in our experimental setting effector T cells do not significantly contribute to hepatic inflammation. Nonetheless, the involvement of CD8+ T cells in NASH requires further investigations. In a similar manner, more studies are needed to clarify the role of B-cell responses. Indeed, the presence of circulating anti-MDA IgG might not be just a hallmark of immune activation against oxidative stress-derived epitopes, but might influence the disease evolution by causing antibody-mediated injury. Furthermore, B cells have been shown to drive CD4+ T-cell activation and cytokine production in the adipose tissue during obesity and modulate the progression of liver injury to fibrosis.
Accumulating data indicate that NKT cells can orchestrate inflammation in autoimmune liver diseases and modulate hepatic fibrogenesis. In line with these observations, recent reports point to an involvement of NKT cells in NASH. Indeed, while steatosis is characterized by the lowering of the liver NKT pool as a consequence of IL-12 production and Tim-3/ galectin-9 signaling, NK T cell expansion is a feature of advanced NASH in either rodents and humans. The former, NKT depletion prevents hepatic inflammation and fibrosis. We observed that an increase in NKT cells characterizes the enhanced severity of NASH in immunized MCD-fed mice, as opposed to NKT cell depletion present in similarly treated naïve animals, further supporting the contribution of NKT cells to NASH progression. Changes in the hepatic levels of IL-15 have been proposed to modulate the NKT pool in NASH. IL-15 is a pleiotropic cytokine responsible for macrophage, T, NK, and NKT cell survival and maturation. In healthy livers, hepatocyte constitutively produce IL-15 to create a T-cell favorable environment, while an increased hepatocyte and macrophage IL-15 expression in response to injury is critical for driving both innate and adaptive immunity. We observed that IL-15 is selectively up-regulated in immunized, but not in naïve MCD-fed mice concomitantly with NKT cell recruitment, suggesting that parenchymal damage and inflammation resulting from Th-1 responses promote an IL-15-mediated expansion of NKT cells, which, in turn, might participate in the evolution of NASH.

According to Syn et al., OPN generated by NKT cells contributes to fibrosis in NASH. OPN is a cytokine produced by either immune and parenchymal cells that modulates both inflammation and tissue healing. In the liver, OPN production by NKT cells drives concanavalin A-induced hepatitis, but OPN can also stimulate collagen synthesis by HSCs through a transforming growth factor beta (TGF-β1)-independent pathway. An up-regulation in liver OPN is evident in either humans and rodents with advanced NASH, while OPN-deficient A/J mice are protected against steatohepatitis and fibrosis induced by feeding the MCD diet. In our hands, hepatic OPN is specifically up-regulated in MCD-fed immunized mice concomitant with the recruitment of OPN-expressing NKT cells. Moreover, we observed that hepatic macrophages also contribute to OPN production. This is consistent with the capacity of Th-1 cytokines to stimulate OPN synthesis in macrophages. However, we cannot exclude that other liver cells, such as cholangiocytes, might also generate OPN. Differently from that reported by Sahai et al. using A/J mice, we did not observe changes in hepatic OPN expression in naïve C57BL/6 mice receiving the MCD diet for 4 weeks. This discrepancy might reflect strain differences in the mice susceptibility to steatohepatitis and further support the importance of OPN in NASH evolution. Furthermore, the capacity of OPN to stimulate HSC might account for the increase in collagen deposition observed in MCD-fed immunized mice in spite of the fact that these animals have a high hepatic IFN-γ production that should antagonize fibrogenesis.

In conclusion, the results presented indicate that immune responses triggered by oxidative stress-derived antigens contribute to hepatic inflammation in experimental NASH by promoting the Th-1 activation of CD4⁺ T-lymphocytes. NASH in immunized animals is also associated with an increase in liver NKT cells that likely participate in the disease evolution by generating osteopontin. Altogether, these data support recent observations in humans about the possible involvement of adaptive immunity in the mechanisms leading to NAFLD evolution.

References


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