十分な効果が得られないことが多く，肝移植を視野に入れた診療が必要となる[14)18)]．現時点では，急性肝炎として発症する症例を厳重に経過観察し，重症化が危惧されれば基幹病院に搬送し，肝炎ウイルス感染の除外，自己抗体の検出と肝生検を早期に行い，ステロイド治療を開始できるような診療連携システム作りが重要ではないかと考えている．2009年末までに本邦でAIHによる急性肝不全が原因で移植を受けたのは26例であり，生体肝移植後の予後は，ほかの成因の急性肝不全と差はない[19)]．しかし，移植前のステロイドを含めた免疫抑制療法が肝移植の予後に影響を及ぼす可能性は高く，感染症誘発の問題もあるため，薬物療法の効果の期待できない症例に，漫然と治療を継続するのは避けるべきである．

予　　後

急性肝不全症例を除くと，ステロイド治療を適切に行うことにより，肝不全による死亡は少なく予後良好と考えられる．Yoshizawaらは，ステロイド治療を行い，寛解が得られたAIH 203例の予後を解析し，適切な治療を行えば同世代の本邦女性の平均余命と差がないことを報告している[20)]．Cox比例ハザードモデルによる多変量解析により，2回以上の再燃のみが予後に寄与する独立した因子であったことから，十分な免疫抑制療法により再燃を極力抑制することが重要と考えられる．現在，「難治性の肝・胆道疾患に関する調査研究」班に所属する施設のAIH症例を対象に予後調査が行われている．

おわりに

AIHは発症機構が不明であるため，診断にあたっては除外診断が必要であるとともに，自己抗体と肝の病理組織学的検索が必要である．また，現状では過剰免疫反応を抑える意味で，副腎皮質ステロイド剤を主体とした非特異的免疫抑制療法が治療手段となっている．治療標的抗原の同定と発症機構の解明により，原因療法の開発が期待される．

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Autoimmune Hepatitis

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Review Article

T Helper 17 Cells in Autoimmune Liver Diseases

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Many autoimmune diseases are driven by self-reactive T helper (Th) cells. A new population of effector $CD4^+$ T cells characterized by the secretion of interleukin (IL)-17, referred to as Th17 cells, has been demonstrated to be phenotypically, functionally, and developmentally distinct from Th1 and Th2 cells. Because the liver is known to be an important source of transforming growth factor-β and IL-6, which are cytokines that are crucial for Th17 differentiation, it is very likely that Th17 cells contribute to liver inflammation and autoimmunity. In contrast, another distinct subset of T cells, regulatory T cells (Treg), downregulate immune responses and play an important role in maintaining self-tolerance. In addition, there is a reciprocal relationship between Th17 cells and Tregs, in development and effector functions, and the balance between Th17 and Treg cells can affect the outcome of immune responses, particularly in autoimmune diseases. In this review, we will focus on the latest investigative findings related to Th17 cells in autoimmune liver disease.

1. Introduction

It has generally been accepted that $CD4^+$ T helper (Th) cells can be categorized into two distinct subsets, that is, Th1 and Th2 cells, based on their cytokine profiles and biological functions [1]. Th1 cells are largely responsible for cellular immunity against intracellular bacteria and viruses and are distinguished by their secretion of interferon (IFN)-γ. Th2 cells are recognized to be integrally involved in the humoral response to parasitic infections and are defined by their characteristic secretion of cytokines of interleukin (IL)-4, IL-5, and IL-13. The pathogenic effects of Th1 cells and the protective contributions of Th2 cells have been recognized as a common feature of autoimmune diseases.

Recently, a new population of effector $CD4^+$ T cells characterized by the secretion of IL-17, identified as Th17 cells, has been demonstrated to be phenotypically, functionally, and developmentally distinct from Th1 and Th2 cells [2, 3] (Figure 1). In addition, another distinct subset of $CD4^+$ T cells, regulatory T cells (Tregs), has been shown to downregulate immune responses through inhibition of effector cells [4]. These two subsets have been shown to have opposing effects in the immune response and may be involved in the pathogenesis of many diseases, including autoimmune diseases [5, 6]. In this review, we will focus on the latest findings related to Th17 cells in autoimmune liver disease.

2. Th17 Cells

Th17 cells have been implicated in host defense, inflammatory disease, tumorigenesis, autoimmune diseases, and transplant rejection, all of which are mediated by the production of several cytokines, including IL-17A, IL-17F, IL-21, and IL-22 [3, 7, 8]. IL-17A and IL-17F possess similar biological functions and bind to the same receptor complex, which is expressed by most cell types in the body. Both IL-17A and IL-17F are key cytokines in the recruitment, activation, and migration of neutrophils and monocytes and can target nonimmune cells (such as fibroblasts, endothelial cells, and epithelial cells) to induce proinflammatory mediators, including cytokines, colony stimulating factors, CC and CXC chemokines, and metalloproteinases [7–10]. IL-21 regulates the differentiation of $CD4^+$ T cells into Th17 cells in an autocrine manner, thereby amplifying the Th17 responses and inducing the autocrine loop [11, 12].

Differentiation of Th17 cells requires the action of various cytokines and transcription factors. In mice, transforming

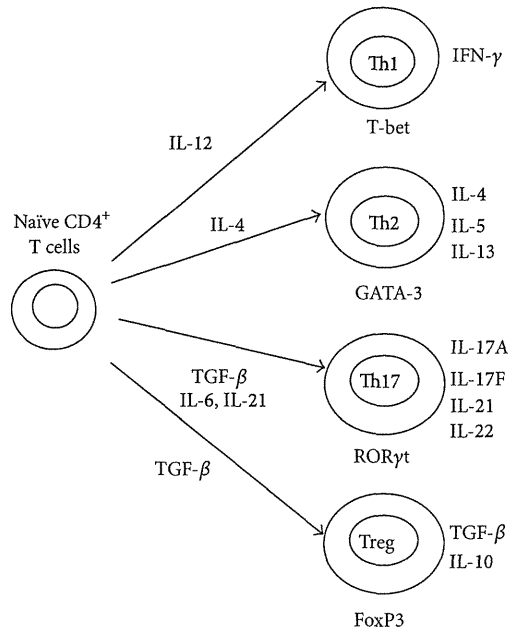

FIGURE 1: Differentiation pathways of naïve $CD4^+$ T cells under different stimulation conditions. Naïve $CD4^+$ T cells can differentiate into different subsets depending on the cytokine milieu. Each subset is characterized by the unique expression of transcription factors and secretion of cytokines.

growth factor (TGF)-β and IL-6 can induce the differentiation of naïve $CD4^+$ T cells into the Th17 phenotype [13–15]. IL-21 also supports the development of Th17 cells [11, 12]. Once Th17 cells have developed, IL-23 is required for the stabilization and further expansion of these cells [13, 14, 16]. Retinoic acid-related orphan nuclear factor (ROR)-γt is a transcription factor that serves as a master regulator to direct the differentiation of Th17 cells in mice [17]. The signal transducer and activator of transcription (STAT) 3 is also critical for the generation of Th17 cells [18].

Although it has been argued that human Th17 differentiation is independent of TGF-β signaling, subsequent studies have confirmed that, as in murine Th17 cells, TGF-β is indispensable for the differentiation of human Th17 cells from naïve T cells [19–21]. While TGF-β is essential for the induction of RORC in naïve T cells at low concentrations, the expression and function of RORC are inhibited at high concentrations of TGF-β [20]. Inflammatory cytokines, such as IL-6, IL-21, IL-23, and IL-1β, initiate human Th17 differentiation [19–21].

3. Relationship between Th17 and Treg Cells

Recently, Th17 and Treg cells have been shown to have opposing immunological effects, and a regulated balance between these two cell types may be crucial for the stability of immune homeostasis. Disruption of the Th17/Treg balance may lead to chronic inflammation and autoimmunity.

Treg cells produce anti-inflammatory cytokines, such as IL-10 and TGF-β, and suppress functional immune reactions [4]. In addition to naturally occurring, thymus-derived Treg cells, Treg cells can also be differentiated in the periphery under specific conditions. The differentiation of Treg cells may be linked to the differentiation of Th17 cells, depending on the cytokine milieu [22]. The differentiation of both Treg and Th17 cells requires TGF-β. The differentiation of Th17 cells requires low concentrations of TGF-β along with a combination of proinflammatory cytokines (such as IL-6 and IL-21), whereas high concentration of TGF-β in the absence of proinflammatory cytokines induce the differentiation of Th17 cells from naïve T cells [23, 24]. In addition, IL-2 and retinoic acid promote Treg cell differentiation but inhibit Th17 cell differentiation [25, 26]. These data indicate that Th17 cells and Treg cells are reciprocally regulated and can affect the outcome of immune responses, particularly in autoimmune diseases.

Forkhead box P3 (FoxP3) is a transcription factor involved in Treg cell differentiation and has characteristically high expression [27, 28]. However, under certain circumstances, $FoxP3^+$ cells also express RORγt [29]. Cells coexpressing with RORγt and FoxP3 also coexpress C-C chemokine receptor 6 (CCR6), and upon activation, these cells show decreased IL-17 production relative to that of cells expressing RORγt alone, suggesting that FoxP3 antagonizes the expression and function of RORγt, thus leading to inhibition of the Th17 pathway [23, 30]. In contrast, RORα inhibits FoxP3 function [31]. These findings suggest that the relationship between Th17 and Treg cells remains complex and plastic.

4. Th17 Cells in Autoimmune Liver Disease

Many researchers have demonstrated the importance of Th17 cells in the pathogenesis of autoimmune diseases. Specifically, the contribution of Th17 cells in experimental autoimmune encephalomyelitis, arthritis, and inflammatory bowel disease has been investigated [32–35]. In addition, high levels of IL-17 and other cytokines related to the Th17 pathway have been reported in the sera and tissues of patients with several autoimmune diseases, such as psoriasis [36] and multiple sclerosis [37].

Inflammatory responses mediated by a various immune cells play a key role in the development and progression of liver diseases. Among them, T cells are thought to be the primary effector cells contributing to the pathogenesis of many forms of liver diseases. Because the liver is known to be an important source of TGF-β and IL-6, Th17 differentiation may be favored in the liver. In addition, expression of the IL-17 receptor has been detected on the surface of all types of liver cells, including hepatocytes, Kupffer cells, stellate cells, biliary epithelial cells, and sinusoidal endothelial cells [38], which indicates that IL-17 may play an important role in the pathogenesis of many types of liver diseases (Figure 2). Recently, substantial evidence has been accumulated regarding the relationship between Th17 cells and liver diseases [38–41].

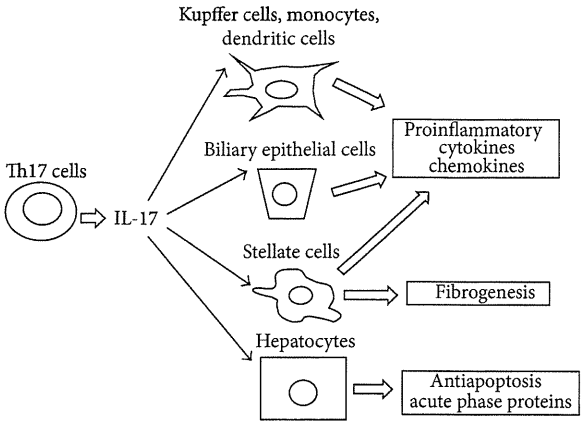

FIGURE 2: IL-17 plays a role in the pathogenesis of liver diseases. IL-17 stimulates multiple types of liver nonparenchymal cells to secrete proinflammatory cytokines and chemokines, thereby inducing and promoting liver inflammation. IL-17 also promotes liver fibrogenesis by hepatic stellate cell activation. In addition, IL-17 may stimulate hepatocytes to produce C-reactive proteins and promote hepatocyte survival.

4.1. Autoimmune Hepatitis. Autoimmune hepatitis (AIH) is defined as a chronic liver disease with unknown etiological factors and is associated with aberrant autoreactivity and a genetic predisposition [42, 43]. The target antigens on the hepatocyte membrane are not known, but it is likely that liver membrane-specific activated T cells are important in the development and/or progression of the disease.

Zhao et al. [44] reported that serum IL-17 levels and the frequency of circulating Th17 cells in patients with AIH are substantially higher than those in healthy controls or patients with chronic hepatitis due to hepatitis B virus. In addition, IL-17$^+$ lymphocytic infiltration (primarily of the CD4$^+$ phenotype) in the liver substantially increases in AIH, and the degree of hepatic IL-17$^+$ cell infiltration is positively correlated with the degree of hepatic inflammation and fibrosis in patients with AIH. IL-17 has also been demonstrated to induce IL-6 expression via the mitogen-activated protein kinase pathway in hepatocytes, thus indicating that Th17 cell proliferation is the key trigger in the pathogenesis of AIH and that the positive feedback loop between Th17 cells and hepatocytes exacerbates the inflammatory process [44].

Functional Treg cell impairment and decreased Treg cell number have been identified in patients with AIH [45–47]. Treg cell impairment in AIH varies with disease stage, appearing worse at presentation than during remission, thereby showing functional restoration potential [47]. Longhi et al. [48] reported that Treg cells can be expanded and generated *de novo* (from CD4$^+$CD25$^-$ cells) in patients with AIH and that the suppressor function and FoxP3 expression levels of these cells are higher than those in freshly isolated Treg cells. However, Treg cells generated from CD4$^+$CD25$^-$ cells in patients with AIH have been found to contain a greater population of IL-17$^+$RORC$^+$ cells and these cells suppressed CD25$^-$ effector cell proliferation with less efficiency than Treg cells from CD4$^+$CD25^{high} cells [49]. Inhibition of IL-17 or Th17 differentiation was found to lead to phenotypic and functionally stable Treg cells, suggesting that the anti-Th17 approach is an important step toward the establishment of new therapeutic strategies in AIH.

4.2. Primary Biliary Cirrhosis. Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by the destruction of small- and medium-sized intrahepatic bile ducts [50, 51]. Although several studies have examined the autoimmune mechanisms underlying biliary damage in PBC, the underlying cause of the disease remains largely unknown. Autoreactive CD4$^+$ and CD8$^+$ T cells have been implicated in the pathogenesis of PBC.

IL-2 receptor (IL-2R)$^{-/-}$ mice spontaneously produce antimitochondrial antibodies, especially against the E2 subunit of pyruvate dehydrogenase, and develop portal inflammation with ductular damage, which is characteristic of PBC [52]. These mice have been found to have a decreased frequency of CD4$^+$FoxP3$^+$ Treg cells. In contrast, they showed increased serum IL-17 levels and marked aggregations of Th17 cells near the portal tracts in the liver [53].

Several studies have demonstrated a close correlation between PBC and Th17 in humans. The number of Th17 cells in peripheral blood has been found to be higher in patients with PBC than in healthy controls [54, 55]. Furthermore, IL-17 and pro-Th17 cytokines, that is, IL-1β, IL-6, and IL-23, were substantially upregulated in terms of both gene expression and serum concentration in patients with PBC relative to healthy controls [53, 56]. Harada et al. [56] demonstrated that liver tissues from patients with PBC have higher counts of IL-17$^+$ cells per portal tract than liver tissues from normal controls, which is consistent with the results obtained for an animal model [52]. Furthermore, biliary epithelial cells possess the ability to produce pro-Th17 cytokines, such as IL-6, IL-1β, and IL-23, in response to pathogen-associated molecular patterns [56], suggesting that periductal IL-17-secreting cells facilitate the migration of inflammatory cells around the bile ducts. These inflammatory cells could be associated with chronic inflammation of the bile ducts in PBC. In contrast, patients with PBC possess reduced counts of Treg cells [54, 55, 57], indicating that an enhanced Th17 response and a weakened Treg response may both play an important role in the pathogenesis of PBC.

4.3. Primary Sclerosing Cholangitis. Primary sclerosing cholangitis (PSC) is a fibrosclerotic disease of the bile ducts, with diffuse structuring of the intrahepatic and extrahepatic biliary tree [58, 59]. The etiological factors and pathogenesis of PSC remain poorly understood, but autoimmune mechanisms are believed to contribute to the development and progression of this disease state. The biliary epithelium appears to be the target for immune-mediated injury. Recently, Katt et al. [60] reported that patients with PSC show increased numbers of Th17 cells in response to heat-inactivated pathogens, which are present in the bile duct of the majority of patients with PSC, relative to healthy controls and patients with PBC. In addition, IL-17$^+$ lymphocytes were detected within the periductal areas of patients with PSC

by immunohistochemical analysis. The Th17 response was induced by the selective stimulation of Toll-like receptor (TLR) 5 and TLR7 but not by stimulation of other pattern-recognition receptors.

One of the histological features of PSC is fibroobliterative sclerosis of intra- and/or extrahepatic bile ducts. Th17 cells may contribute to fibrosis thorough production of IL-17A and other cytokines. Meng et al. [61] demonstrated that the mRNA levels of IL-17A and its receptor increased in animal livers when fibrosis was induced by bile duct ligation and carbon tetrachloride and that serum IL-17A levels were associated with the development of liver fibrosis. These findings indicate that Th17 may contribute not only to inflammation but also to fibrosis in the pathogenesis of PSC. In addition, IL-17RA deletion in mice dramatically inhibits both models of liver fibrosis; therefore, IL-17 may promote liver fibrosis through hepatic stellate cell (HSC) activation or promotion of liver inflammation through the upregulation of proinflammatory cytokines and chemokines in HSC or Kupffer cells. However, the animal models used in these studies do not exhibit all of the attributes of PSC. In particular, the role played by Th17 cells in the pathogenesis of large bile ducts has not yet been clarified. Further studies using other animal models with sclerosing cholangitis and biliary fibrosis [62] are required.

4.4. IgG4-Related Sclerosing Cholangitis. IgG4-related sclerosing cholangitis (IgG4-SC) is a recently described biliary disease that has unknown etiological features and presents with biochemical and cholangiographic features similar to those of PSC and is often associated with autoimmune pancreatitis and other fibrotic conditions [63]. In this condition, the patient's IgG4 serum level is elevated and IgG4-positive plasma cells infiltrate into the bile ducts and liver tissue. Th2-dominant immune responses or Treg cells appear to be involved in the underlying immune reaction [64–66]. Therefore, the immunopathogenesis of IgG4-SC appears to be distinct from that of PBC and PSC. However, the role of Th17 cells in the pathogenesis of IgG4-SC has not yet been clarified, and further studies are required.

4.5. Th17 Cells in Liver Fibrosis. Liver fibrosis is a common outcome of chronic liver diseases, including autoimmune liver disease, and potentially leads to portal hypertension, hepatic failure, and liver cancer. Activated HSCs play a critical role in collagen and extracellular matrix production. In addition, accumulating evidence indicates that IL-17 also plays an important role in promoting liver fibrosis by inducing HSC activation [61, 67–69].

The frequency of Th17 cells in the diseased liver correlates with liver fibrosis in patients with viral hepatitis [67, 70], AIH [49], and alcoholic liver disease [71]. Furthermore, IL-17A and IL-17RA deficiency protects mice from liver fibrosis induced by CCl_4 and bile duct ligation [61, 68, 69]. Tan et al. [68] recently reported that activation of HSC and production of collagen in CCl_4-induced liver fibrosis are IL-17A dependent. Therefore, IL-17A neutralization may be a promising approach for antifibrotic therapy in patients with chronic liver diseases.

5. Conclusion

Unbalanced Th1/Th2 responses in the liver have long been proposed to be associated with perpetuated inflammation and subsequent liver fibrosis. The recently discovered Th17 cells have also been linked to host defense and autoimmunity. Although research on Th17 cells has progressed, several unanswered questions still require clarification, such as the interaction between Th17 cells and other subsets of T cells, especially Treg cells. Th17/Treg imbalance has been implicated in the pathogenesis of many diseases, especially autoimmune diseases. Elucidation of the role of Th17 differentiation and regulation will provide investigators with a novel target for the treatment of autoimmune liver disease.

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Association of *STAT4* Polymorphisms with Susceptibility to Type-1 Autoimmune Hepatitis in the Japanese Population

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Abstract

Background/Aims: Recent studies demonstrated an association of *STAT4* polymorphisms with autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis, indicating multiple autoimmune diseases share common susceptibility genes. We therefore investigated the influence of *STAT4* polymorphisms on the susceptibility and phenotype of type-1 autoimmune hepatitis in a Japanese National Hospital Organization (NHO) AIH multicenter cohort study.

Methodology/Principal Findings: Genomic DNA from 460 individuals of Japanese origin including 230 patients with type-1 autoimmune hepatitis and 230 healthy controls was analyzed for two single nucleotide polymorphisms in the *STAT4* gene (rs7574865, rs7582694). The *STAT4* rs7574865T allele conferred risk for type-1 autoimmune hepatitis (OR = 1.61, 95% CI = 1.23–2.11; $P = 0.001$), and patients without accompanying autoimmune diseases exhibited an association with the rs7574865T allele (OR = 1.50, 95%CI = 1.13–1.99; $P = 0.005$). Detailed genotype-phenotype analysis of type-1 autoimmune hepatitis patients with (n = 44) or without liver cirrhosis (n = 186) demonstrated that rs7574865 was not associated with the development of liver cirrhosis and phenotype (biochemical data and the presence of auto-antibodies).

Conclusions/Significance: This is the first study to show a positive association between a *STAT4* polymorphism and type-1 autoimmune hepatitis, suggesting that autoimmune hepatitis shares a gene commonly associated with risk for other autoimmune diseases.

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Introduction

Autoimmune hepatitis (AIH) is characterized by chronic inflammation of the liver, interface hepatitis, hypergammaglobulinemia and production of autoantibodies [1,2]. The etiology of AIH is unknown, but is thought to have both a genetic and an environmental basis [3]. Although the HLA DRB1 gene is a well-characterized susceptibility gene [4,5], non-HLA susceptibility genes may also contribute to genetic susceptibility to AIH and remain to be elucidated. Recently, with the emergence of genome-wide association studies (GWAS), there has been a dramatic increase in genetic discoveries for many complex genetic autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis (RA) [6]. It is also interesting to note that evaluating the results from the study of one disease in other complex diseases can disclose common risk factors. Thus, there has been a marked overlap of loci between autoimmune diseases [7]. Of those, *STAT4* particularly has been confirmed in several studies and is clearly associated with autoimmune diseases such as RA or systemic lupus erythematosus (SLE) [8–10]. STAT4, a signal transducer and activator of transcription 4, is expressed in activated peripheral blood monocytes, dendritic cells and macrophages at the sites of inflammation in humans [11]. It is activated by interleukin (IL)-12, leading to T helper (Th) 1 and Th 17 differentiation, monocyte activation and interferon (IFN)-α production [12]. Since Th1 and Th17 cells have the capacity to cause autoimmunity [13], STAT4

may play a crucial role in the development of autoimmune diseases, including AIH.

The degree of risk for RA or SLE susceptibility observed with the *STAT4* haplotype was found to be similar in Caucasian and Japanese populations [14–16]. In addition, meta-analysis demonstrated that the *STAT4* rs7574865 T allele conferred susceptibility to various autoimmune diseases, suggesting an association between *STAT4* gene polymorphism and autoimmune diseases [17].

STAT4 is considered important in a mouse model of Th1-dependent liver injury [18]. Therefore, we hypothesized that *STAT4* polymorphisms may overlap in genetic susceptibility between AIH and other autoimmune diseases. To test this hypothesis, we investigated the association of *STAT4* with type-1 AIH susceptibility using a large series of Japan NHO-AIH registry [19]. We also tried to evaluate whether the gene was associated with type-1 AIH outcome measures in a Japanese AIH cohort.

Materials and Methods

Study population

Consecutive type-1 AIH patients were initially enrolled in the register of the Japanese National Hospital Organization (NHO) liver-network study, contributed to medical facilities in Japan, and prospectively followed since 2009 as a multicenter cohort population. All patients satisfied the 1999 revised criteria of International Autoimmune Hepatitis Group (IAIHG) diagnosis of type-1 AIH [20]. Patients were excluded from the study if there was histological evidence of cholangitis or non-alcoholic steatohepatitis. In addition, patients who were positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA were excluded. Patients with other causes of liver disease, such as excess alcohol or drug use, were excluded based on reviews of their appropriate history and investigations. The control group consisted of 230 gender-matched Japanese healthy subjects (34 men and 196 women). The mean ± SD age was 43.9±13.1 years. Among the cases (AIH) and controls, 156 patients and 163 controls were recruited from West Japan and 74 patients and 67 controls were recruited from East Japan. The study was approved by the Ethics committee of the Nagasaki Medical Center and participating NHO Liver-network hospitals ((NHO Sagamihara National Hospital, Tokyo National Hospital, Yokohama Medical Center, Nagoya Medical Center, Kure Medical Center, Osaka Minami Medical Center, Kyushu Medical Center, Minami Wakayama Medical Center, Shinshu Ueda Medical Center, Kanazawa Medical Center, Higashi Hiroshima Medical Center, Asahikawa Medical Center, Kokura Medical Center, Ureshino Medical Center, Higashi Nagoya National Hospital, Hokkaido Medical Center, Okayama Medical Center, Takasaki General Medical Center, Oita Medical Center, Beppu Medical Center, Osaka Medical Center, Kumamoto Medical Center, Nishigunma National Hospital). Written informed consent was obtained from each individual. This study was conducted with the approval of the ethical committees of Nagasaki Medical Center and participating NHO Liver-network hospitals. Written informed consent was obtained from each individual.

Variables at study entry

Demographic and other characteristics of the 230 retained patients were recorded in a database at the initial assessment. Data included sex, age at diagnosis, time of onset of symptoms or other evidence of liver disease, markers of infection with hepatitis viruses HBV and HCV, alcohol intake, coexisting autoimmune diseases, serum levels of ALT, AST, alkaline phosphatase and bilirubin, platelet count and prothrombin time. Anti-nuclear antibodies (ANA) and anti-smooth muscle antibodies (ASMA) were measured by indirect immunofluorescence on HEp-2 cells and cut-off titers for positivity were 1:40. Liver tissue from percutaneous biopsy performed at the referring facility was available for the majority of patients at the time of entry (192/230, 83.5%), but for only a few at the subsequent follow-up examination (7/230, 3.0%). The histological variables examined included degree of fibrosis (0; absent, 1; expansion of fibrosis to parenchyma, 2; portal-central or portal-portal bridging fibrosis, 3; presence of numerous fibrous septa, 4; multi-nodular cirrhosis). The histological diagnosis of cirrhosis required a loss of the normal lobular architecture, reconstruction of hepatic nodules and presence of regenerative nodules [21]. Liver biopsy was not performed for patients who had apparent biochemical, endoscopic and ultrasound features of liver cirrhosis. All phenotypic data were collected blind to the results of the genotypic data.

DNA extraction and genotyping

Blood samples were taken from all study participants, and genomic DNA was isolated from peripheral blood leukocytes using a DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer's guidelines. STAT4 SNPs (rs7574865, rs7582694) were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [22,23]. The primers used for the PCR reaction were rs7574865, F:5′-AAAGAAGTGGGATAAAAAGAAGTTTG-3′, R:5′-CCACTGAAATAAGATAACCACTGT-3′, and rs7582694, F:5′-ATCCAACTCTTCTCAGCCCTT-3′, R:5′-TCATAAT-CAGGAGAGAGGAGT-3′.

Rs7574865 was a 147-bp PCR product and was digested with restriction enzyme *Hpa*I (New England Biolabs) and electrophoresed on a 2.5% polyacrylamide gel. Rs7574865 was a 338-bp PCR product was digested with restriction enzyme *Hpy*CH4III (New England Biolabs) and electrophoresed on a 3.0% polyacrylamide gel.

HLA-DRB1 genotyping was performed as described previously [24]. Briefly, the HLA-DRB1 genotype was determined by sequence-based typing (SBT) of group-specific PCR products.

Statistical analyses

Results are expressed as mean ± SD. The statistical significance of differences between groups was calculated by either the chi-square test or Fisher's exact test for categorical data and Mann-Whitney's *U*-test for quantitative data. Multivariate logistic regression analysis was performed with SPSS v.18 for windows (SPSS Statistics, Illinois). Deviation from Hardy-Weinberg equilibrium was assessed using the SNPAlyze software ver. 7.0 (Dynacom, Yokohama, Japan). Power calculations were performed by using an online power calculator [25]. A P value of <0.05 was considered significant.

Results

Baseline data at entry

Of the original 240 patients registered in the NHO-AIH study, 10 were excluded from analysis because of overlapping primary biliary cirrhosis (PBC). The remaining 230 patients were eligible for the study. Table 1 shows other demographic data for the cohort at entry. Among the enrolled type-1 AIH patients, 206 (89.6%) were positive for ANA (>1:40) and 96 (41.7%) for ASMA (>1:40). Some patients with lower serum aminotransferase or total bilirubin were managed with ursodeoxycholic acid (UDCA) therapy alone, which was demonstrated to be efficacious in Japanese patients with type I autoimmune hepatitis [26]. Among

Table 1. Baseline characteristics of type-1 AIH patients.

	n = 230
Gender (male/female)	23/207
Age at presentaion (years)	59.6±12.2
Other autoimmune diseases	39(17.0%)
Baseline Laboratory Values	
AST (<40 IU/L)	432.5±444.1
ALT (<40 IU/L)	484.3±490.5
ALP (<112 IU/L)	463.5±210.3
Total Bilirubin (mg/ml)	3.83±6.14
Albumin (3.5–5.0 g/L)	3.85±0.67
IgG (870–1700 mg/dl)	2489.4±931.4
Platelets (15–40×10^4/μl)	18.6±7.1
ANA + (≥1:40)	206(89.6%)
ASMA + (≥1:40)	96(41.7%)
Cirrhosis at presentation	44(19.1%)
Received treatment	
Steroid alone	81(35.2%)
Steroid + UDCA	72(31.3%)
Steroid + Aza	15(6.5%)
UDCA alone	49(21.3%)

Abbreviations: AIH; autoimmune hepatitis, AST; aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphate, IgG; immunoglobulin G, ANA; anti-nuclear antibody, ASMA; anti-smooth muscle antibody, UDCA; ursodeoxy cholic acid, Aza; azathioprine. Data are expressed as number (percentage) or mean ± standard deviations.
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230 eligible patients, 29 (12.6%) had liver cirrhosis at the time of diagnosis, and among the remaining 201 patients without liver cirrhosis, 15 developed liver cirrhosis during the follow-up. Two patients died because of complications (ruptured esophageal varices 1, hepatic failure 1) of liver cirrhosis during follow-up.

Association of *STAT4* polymorphisms with type-1 AIH

The genotype frequencies for *STAT4* rs7574865 and rs7582694 were in HWE (Hardy-Weinberg equilibrium) in both the patient and control populations (data not shown). Because of the strong linkage disequilibrium between rs7574865 and rs7582694 ($R^2 = 0.949$ and $D' = 0.981$), very similar results were observed between rs7574865 (Table 2) and rs7582694 (Table 3). We observed a significant difference in allele frequency and genotype distribution of *STAT4* polymorphisms (rs7574865) between type-1 AIH patients and controls. As shown in Table 2, the minor T allele and TT genotype frequencies at *STAT4* rs7574865 in the type-1 AIH group differed significantly from those in the control group.

To determine whether the observed association of the *STAT4* gene SNPs with disease susceptibility was caused by other autoimmune diseases associated with AIH, we stratified type-1 AIH patients without other overlapping autoimmune diseases. There was a significant association of *STAT4* rs7574865 with susceptibility to type-1 AIH even in the AIH patients without other overlapping autoimmune diseases (Table 4).

Associations between *STAT4* genotype status and type-1AIH phenotype

To examine the associations between HLA-DR and type-1 AIH, HLA-DR allele typing was performed in patients with type-1

Table 2. STAT4 rs7574865 polymorphism in patients with type-1 AIH and controls.

		Control (%)	AIH (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 230		
Genotype frequencies				0.001	
	G/G	103(44.8)	77(33.5)		
	G/T	108(47.0)	109(47.4)		
	T/T	19(8.3)	44(19.1)		
Allele				0.001	
	G	314(68.3)	263(57.2)		1
	T	146(31.7)	197(42.8)		1.611(1.230–2.109)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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Table 3. STAT4 rs7582694 polymorphism in patients with type-1 AIH and controls.

		Control (%)	AIH (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 230		
Genotype frequencies				0.001	
	G/G	101(43.9)	80(34.8)		
	G/C	109(47.4)	103(44.8)		
	C/C	20(8.7)	47(20.4)		
Allele				0.001	
	G	311(67.6)	263(57.2)		1
	C	149(32.4)	197(42.8)		1.563(1.195–2.046)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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AIH. In the analysis of HLA-DR alleles, the frequencies of DR *04 allele was significantly increased in type-1 AIH patients as compared with those in controls (Table 5). The STAT4 rs7574865 T allele and HLA-DR *04 allele for the progression to liver cirrhosis were subjected to multivariate logistic regression analysis. Neither HLA-DR *04 allele nor rs7574865 T allele did not contribute to the progression to liver cirrhosis (data not shown). Based on the significant association of the rs7574865 with susceptibility to type-1 AIH, we also performed a detailed genotype-phenotype analysis using the clinical data. However, we found no significant difference in the presence of autoantibodies (ANA or ASMA) and the peak levels of transaminases or total bilirubin (AST, ALT, TB) by laboratory tests among each genotype (data not shown).

Table 4. STAT4 rs7574865 polymorphism in patients with type-1 AIH without other autoimmune diseases.

		Control (%)	AIH without other autoimmune diseases (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 191		
Genotype frequencies				0.008	
	G/G	103(44.8)	68(35.6)		
	G/T	108(47.0)	89(46.6)		
	T/T	19(8.3)	34(17.8)		
Allele				0.005	
	G	314(68.3)	225(58.9)		1
	T	146(31.7)	157(41.1)		1.501(1.131–1.992)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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Discussion

AIH reflects a complex interaction between triggering factors, environmental factors, genetic predisposition and the immune regulatory network [3]. Most knowledge concerning the genetic factors of AIH comes from studies of the HLA genes [4,5]. Although multiple genes are probably involved, HLA genes appear to play a dominant role in the predisposition to AIH [27]. Genetic factors other than HLA genes that can affect the susceptibility of AIH are mainly polymorphisms in genes that encode proteins that affect cytokine pathways responsible for modulating immunity [27–29]. Although autoimmune diseases include a wide array of different organ involvement and symptoms, they all share a common component: the loss of immune tolerance toward "self antigen" [30]. Findings in recent genetic studies support the emerging concept that distinct clinical autoimmune diseases may share genetic susceptibility factors. STAT4 is a critical transcription factor involved in the regulation of Th1/Th2 cytokine balance [12]. *STAT4* polymorphisms have been found to be associated with various autoimmune diseases [8–10].

This study is the first to investigate a detailed correlation between *STAT4* gene polymorphisms and susceptibility to type-1 AIH in a Japanese nationwide AIH cohort study. In the current study, we confirmed an association of *STAT4* polymorphisms with susceptibility to type-1 AIH. Our data suggest that *STAT4* may be an "autoimmune disease susceptibility gene" and support the concept of deregulated pathways across multiple autoimmune diseases. In addition to their influence on autoimmune disease susceptibility, *STAT4* polymorphisms can also influence disease phenotypes. For example, rs7574865 in SLE patients was associated with severe disease manifestations, such as nephritis, high double stranded-DNA antibody production and younger age of disease onset. [31] For patients with systemic sclerosis, this polymorphism was associated with the presence of pulmonary

Table 5. Distribution of HLA-DR alleles distribution in patients with type-1 AIH.

HAL-DR alleles	AIH	Control	*P*	*Pc*	OR (95%CI)
	Alleles, No.(%)	Alleles, No.(%)			
	(n = 460 alleles)	(n = 460 alleles)			
*01	8(1.7)	24(5.2)	0.004	0.052	0.322(0.143–0.723)
*04	189(41.1)	118(25.7)	0.000001	0.000013	2.021(1.528–2.674)
*07	1(0.2)	4(0.9)	0.187	2.431	0.248(0.028–2.231)
*08	67(14.6)	42(9.1)	0.011	0.143	1.697(1.126–2.556)
*09	52(11.3)	70(15.2)	0.080	1.040	0.710(0.483–1.043)
*10	4(0.9)	2(0.4)	0.343	4.459	2.009(0.366–11.021)
*11	7(1.5)	7(1.5)	1.000	13.000	1.000(0.348–2.874)
*12	19(4.1)	26(5.7)	0.285	3.705	0.719(0.392–1.319)
*13	16(3.5)	47(10.2)	0.000052	0.000676	0.317(0.177–0.567)
*14	26(5.7)	28(6.1)	0.779	10.127	0.924(0.533–1.602)
*15	66(14.3)	88(19.1)	0.052	0.676	0.708(0.499–1.004)
*16	4(0.9)	2(0.4)	0.343	4.459	2.009(0.366–11.021)
*17	1(0.2)	2(0.4)	0.500	6.500	0.499(0.045–5.521)

HLA-DRB1 allele was assessed by cis-square test. The probability values were corrected (*Pc*) for multiple testing (Bonferroni correction).
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fibrosis [32]. Therefore, we examined possible associations between *STAT4* and the clinical phenotype of type-1 AIH. However, we did not find evidence of association between *STAT4* polymorphisms and disease progression or phenotype of type-1 AIH.

Regarding the disease-developing effect of genetic variants in the *STAT4* region on type-1 AIH observed in our study, it might be interesting to determine whether the *STAT4* risk alleles have different expression levels or functional effects in different effector cells [33]. The susceptibility SNP rs7574865 is located within intron 3 of *STAT4*, a non-coding region. It is suspected that it may influence the gene expression of STAT4 at the level of transcription or splicing variation [34]. A recent study reported that the expression level of STAT4 in peripheral blood mononuclear cells correlated with the risk allele of STAT4 rs7574865 [33]. This might indicate the effects of different *STAT4* gene variants on STAT4 expression levels. To date, the main alternative spliced isoforms of *STAT4* are STAT4α and STAT4β. STAT4β is a shorter form of the full-length STAT4α and is not as efficient as STAT4α for the direct induction of IFN-γ gene expression activated by IL-12 in Th1 cells [35]. However, expression of STAT4β, lacking the transactivation domain, was not affected by the STAT4 SNPs [33]. Additionally, a significant inverse correlation with T-risk alleles at rs7574865 and the methylation status of the *STAT4* promoter was demonstrated in inflammatory bowel disease [36]. The *STAT1* gene is located adjacent to *STAT4* suggesting it is also a candidate susceptibility gene for autoimmune disease [37]. To examine the role of the STAT1-STAT4 region, 52 tag SNPs encompassing this region in Japanese lupus patients [38]. The SNPs rs11889341 and rs10168266 were in linkage disequilibrium (LD) with rs7574865 and were significantly associated with SLE [38]. In contrast, significant association was not detected for SNPs in the *STAT1* region [38].

AIH pathogenesis are more complex than the traditional dichotomous Th1/Th2 paradigm, where STAT4 represents a transcription factor that induces IL-12, IL-23 and type 1 IFN-mediated signals to Th1 and Th17 differentiation, monocyte activation and interferon-γ production [39]. STAT4 is important for IL-22 production, which plays a pathological role in IL-17-dependent hepatitis [40].

A recent study showed that G allele at rs7574865 was associated with increased risk for HCC, suggesting dual roles of STAT4 in autoimmune diseases and HBV-related HCC [41]. Interestingly, subjects with GG genotype at rs7574865 had the lowest mRNA levels of *STAT4* in both HCC and non-tumor tissues compared with TG and TT genotypes [41]. Considering the role of STAT4 in Th1 immune responses, rs7574865 polymorphisms may affect the hepatic immune response against auto-antigen or viral antigen, contributing to the susceptibility of these related disorders. Further studies will be needed to examine the different possible mechanisms by which the variant haplotypes contribute to AIH.

The current study was limited because there were relatively small numbers of patients, and because some of the phenotypes examined were related to disease activity, and therefore may have fluctuated naturally or as a result of treatment. Additionally, it was difficult to perform a replication study due to the very low prevalence of type-1 autoimmune hepatitis and limited numbers of enrolled patients. In the current study, the power to detect a 1.6-fold increased risk, assuming an alpha value of 0.05, was 0.627 for rs7574865 T allele. Another limitation is the lack of complete information regarding the causal polymorphisms and their exact functional roles.

In summary, our results identified *STAT4* SNP rs7574865 as a disease-susceptible gene variant in type-1 AIH. Further studies on the expression and regulation of *STAT4* in the liver will be required to investigate the functional consequences of *STAT4* gene variants in more detail.

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Author Contributions

Conceived and designed the experiments: KM M. Nakamura H. Yatsuhashi HI. Performed the experiments: YJ MY. Analyzed the data: KM M. Nakamura MY. Contributed reagents/materials/analysis tools: SA SN AK SH SB K. Yamasaki TK MS HK TH M. Kohjima M. Nakamuta M. Kato K. Yoshizawa HO YN ET HN TS KA NH YO AN TM HS EM KS H. Yamashita FM. Wrote the paper: KM M. Nakamura MY HI.

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CASE REPORT

Granulocytapheresis for the Treatment of Severe Alcoholic Hepatitis: A Case Series and Literature Review

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Abstract Severe alcoholic hepatitis has a high mortality rate due to limited therapeutic methods. Although corticosteroids have been used to control the inflammatory response, the outcomes vary and no standardized therapy has been established. Novel therapeutic approaches, such as anti-TNF-α, pentoxifilline, and others have been tested clinically on the basis of their cytokinemic pathophysiology with limited success. However, treatment of leukocytosis that causes cytokinemia and hepatic inflammation in patients via granulocytapheresis and leukocytapheresis showed promising results in a number of reports. Here, we report two cases of severe alcoholic hepatitis treated with granulocytapheresis. The liver function and inflammation recovered after the therapy. A review of 35 cases treated with granulocytapheresis and leukocytapheresis demonstrated their efficacy in treating alcoholic hepatitis by controlling leukocytosis as well as cytokines such as IL-8. Multidisciplinary treatment for severe alcoholic hepatitis should be considered case by case on the basis of the complexity and severity of the condition.

Keywords Severe alcoholic hepatitis · Granulocytapheresis · Leukocytapheresis · IL-6 · IL-8

Notice The clinical course of case 1 is from Kamimura K et al. *Kanzo* (2002) 43:316-321 with permission from the Japan Society of Hepatology.

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Introduction

Severe alcoholic hepatitis (SAH) is an acute inflammatory response to the endotoxin results in leukocytosis, neutrophil infiltration in the liver, severe hepatic injury, renal failure, hepatic encephalopathy, pneumonia, and others [1, 2]. This inflammation causes increased plasma levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8. In addition, increased neutrophil elastase enhances the production of pro-inflammatory cytokines by macrophages. To date, corticosteroids [1, 3] have been used in cases with high cytokinemia and anti-TNF-α antibody [4], and pentoxifilline [5] has been considered as a new therapeutic option; however, the results of clinical trials have not revealed any significant therapeutic effects. In cases that demonstrate a poor response to corticosteroids, liver transplantation is often considered [6]; however, due to ethical issues, including alcoholic relapse, these patients are not considered as good candidates for the procedure [7]. Granulocytapheresis (GCAP) and leukocytapheresis (LCAP) have been used in the treatment of ulcerative colitis, and since our first report in 2002 [8], they have been considered as a therapeutic option for SAH to control leukocytosis, neutrophil infiltration in the liver, and the cytokinemia [8–18]. The use of this strategy has improved the prognosis of SAH from 32.6 % in the 1990s to 62.9 % during 2004–2008 in Japan on the basis of an analysis conducted in 1,234 medical institutes [19]. In this report, we reviewed two cases of SAH treated with GCAP and demonstrated the recovery of their liver function, as well as 35 cases reported to date, treated with GCAP or LCAP for further understanding of this disease and the efficacy of these therapeutic options. On the basis of the available data, we

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showed that GCAP and LCAP yield a prognosis of approximately 60 % mortality, which is consistent with the recent rate of SAH in Japan [2, 19]. Because the etiology of SAH results from an increase in the endotoxin due to the infection, the administration of corticosteroid should be carefully considered. Therefore, the treatment of leukocytosis by GCAP or LCAP, supporting hepatic function by plasma exchange (PE), inhibiting systemic cytokinemia by hemodiafiltration (HDF), corticosteroid, anti-TNFα antibody, and pentoxifilline will contribute to improve the prognosis of this disease. We conclude that multidisciplinary therapy is necessary depending on the complexity and severity of the patients' condition.

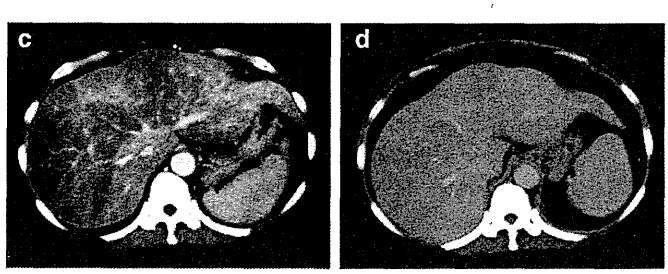
Case Reports

Case 1

A 59-year-old Japanese woman presented at our hospital with loss of appetite, jaundice, abdominal distention, and pretibial edema in January 2000. She had been taking alcohol every day, equivalent to approximately 150 mg of ethanol for 40 years and her daily consumption had increased to approximately 200 mg 3 months prior to admission. Physical examination revealed fever, jaundice, ascites, pretibial edema, and marked hepatomegaly. Laboratory test results revealed significantly increased levels of white blood cell [WBC; 20,160/μL (neutrophils 90.5 %)], direct bilirubin (D-Bil; 23.4 mg/dL), aspartate aminotransferase (AST; 81 IU/L), lactate dehydrogenase (LDH; 483 IU/L), γ-glutamyl transpeptidase (GGTP; 282 IU/L), creatinine (Cre; 1.3 mg/dL), and c-reactive protein (CRP; 7.8 mg/dL). The decrease in albumin (Alb) and prothrombin time (PT) to 2.2 g/dL and 40 %, respectively, was revealed. No other marker of viral hepatitis and autoimmune hepatitis was notably changed. Importantly, significant increase in IL-6 (61.2 pg/mL), IL-8 (608 pg/mL), and neutrophil elastase (122 μg/L) was observed (Fig. 1a, b). There was no increase in tumor markers, including carcinoembryonic antigen, carbohydrate antigen 19-9, alpha-

a

Hematology		Biochemistry		Serum Marker	
WBC	20.2 x10^3/μl	TP	5.5 g/dl	IL-6	61.2 pg/ml
RBC	202 x10^4/μl	Alb	2.2 g/dl	IL-8	608 pg/ml
Hb	7.9 g/dl	BUN	27 mg/dl	Neutrophil elastase	122 μg/l
Ht	23.2%	Cre	1.3 mg/dl		
PLT	23.3 x10^4/μl	T-Bil	23.4 mg/dl		
Coagulation		D-Bil	19.6 mg/dl		
PT%	40%	AST	81 IU/l		
		ALT	24 IU/l		
		ALP	485 IU/l		
		LDH	483 IU/l		
		GGTP	282 IU/l		
		CRP	7.8 mg/dl		



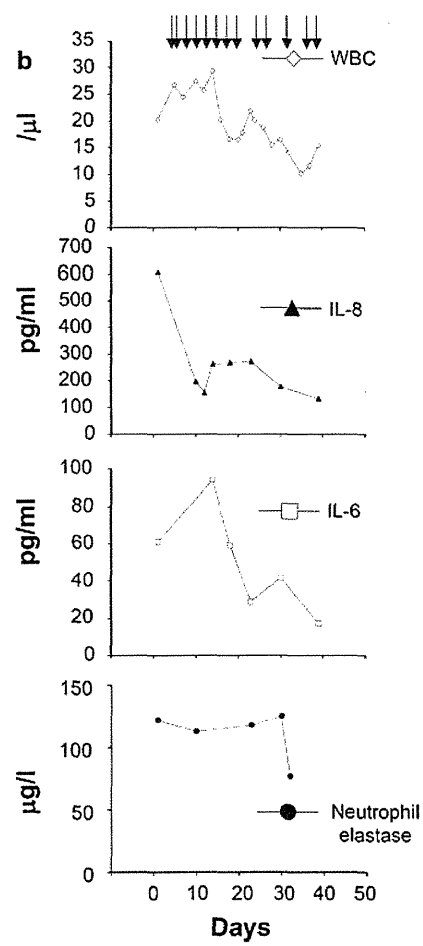


Fig. 1 Summary of case 1. **a** Laboratory data upon admission. **b** Time courses of WBC, IL-8, IL-6, and neutrophil elastase. *Black arrows* represent granulocytapheresis (GCAP) treatment. **c** Computed tomography (CT) before GCAP treatment. **d** CT after 11 sessions of GCAP treatment

fetoprotein, and prostate-specific antigen. Ultrasonography (US) revealed severe fatty infiltration of the liver with ascites, and contrast-enhanced computed tomography (CT) revealed inflammation of the liver with a diffuse low density area indicating severe fatty infiltration (Fig. 1c).

Clinical Course

On the basis of the above mentioned findings, a diagnosis of severe alcoholic hepatitis (SAH) was made for case 1 according to the Diagnostic Criteria for Alcoholic Liver Disease set by Takada et al. [20]. A possible bacterial infection was evidenced by elevated WBCs, high grade fever, and elevated CRP. Thirteen sessions of GCAP were performed to treat granulocytosis (Adacolumn, JIMRO, Takasaki, Japan), plasma exchange (PE) and hemodiafiltration (HDF) to treat the activated cytokines, along with administration of ulinastatin and antibiotics instead of corticosteroids. The WBC counts, cytokine levels, and neutrophil elastase gradually decreased (Fig. 1b) followed by a decrease in ascites and recovery of her appetite. The CT image showed marked improvement of the fatty infiltration (Fig. 1d). She showed severe ventricular fibrillation on day 37 after admission, probably due to alcoholic cardiomyopathy that caused cerebral ischemia, and she expired on day 68.

Case 2

A 45-year-old Japanese woman presented at our hospital with fever, loss of appetite, diarrhea, jaundice, abdominal distention, and pretibial edema in November 2011. She had been taking alcohol every day, equivalent to approximately 230 mg of ethanol for 25 years. Her appetite significantly decreased in October 2011, and she was unable to drink any alcohol since that time. Physical examination revealed fever, jaundice, ascites, pretibial edema, and marked hepatomegaly. No neurological findings were seen. Laboratory test results revealed significantly increased levels of WBC [39,880/μL (neutrophils 89.5 %)], D-Bil (14.9 mg/dL), AST (96 IU/L), and CRP (11.1 mg/dL). Decreased platelet counts (33,000/μL), Alb (1.7 g/dL), and PT (42 %) were also revealed. No other marker of viral hepatitis and autoimmune hepatitis was notable. As in case 1, a significant increase in IL-6 (79 pg/mL), IL-8 (279 pg/mL), and neutrophil elastase (290 μg/L) was observed (Fig. 2a, b). There was no increase in tumor markers. The US and CT showed significant hepatic inflammation along with severe fatty infiltration (Fig. 2c).

Clinical Course

Case 2 was diagnosed with SAH according to the Diagnostic Criteria for Alcoholic Liver Disease set by Takada et al. [20], similar to case 1. However, her fatigue was significant, which prevented GCAP. Therefore, she received intravenous infusion of the neutrophil elastase inhibitor, sivelestat sodium hydrate, along with the administration of ulinastatin, antibiotics, and fresh frozen plasma on days 2–16 after admission. However, when no improvement was seen in the physical findings, WBC counts, and cytokines (Fig. 2b), GCAP and HDF were initiated on days 21–26 with the informed consent of the patient and her family. Her fatigue and WBC counts improved, and the level of IL-6, IL-8, and neutrophil elastase significantly decreased after the treatment. The CT image showed significant improvement of the fatty infiltration in the liver (Fig. 2d). However, the patient condition took a sudden turn for the worse and she expired on day 30. Bleeding from the pleural wall was evident upon autopsy.

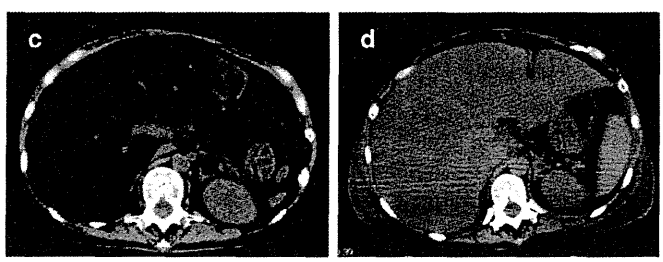
Discussion

Alcoholic hepatitis (AH) is an acute manifestation of alcoholic liver injury and the majority of cases recover following basic treatment including the abstinence from alcohol, nutritional support, and others [1]. Among the various pathophysiologies of AH, SAH demonstrates poor response to these basic treatments resulting in a poor prognosis [1, 2]. Due to the lack of an effective treatment, SAH has a high mortality rate, reportedly up to 35 % at day 28 without effective treatment [21]. Horie et al. also reported that the survival rate within 100 days of hospitalization in Japan was 32.6 and 23.8 % in the 1990s and the 1980s, respectively, on the basis of Takada's criteria [20]. However, an increased understanding of the pathophysiology of this disease has led to the clinical testing of various therapeutic options [2–5, 19].

The key etiology of SAH is the malfunction of the Kupffer's cells due to chronic alcoholic liver damage, which causes penetration of the endotoxin into the systemic circulation through the hepatic parenchyma followed by the inflammatory response, leukocytosis with extensive neutrophil infiltration in the liver, severe hepatic cell necrosis, multiple organ failure including renal failure, pneumonia, encephalopathy, and others [22, 23]. This inflammation causes increased plasma levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8. In addition, increased neutrophil elastase enhances pro-inflammatory cytokine production by macrophages. Therefore, although limited, treatment options for patients with SAH are often multitargeted to treat the inflammatory response, leukocytosis, and cytokinemia. Corticosteroids have been used to treat the inflammatory response in cases with high levels of Maddrey's discriminant function (DF) [24]; however, no significant improvement has been seen in long-term prognosis [25, 26].

a

Hematology		Biochemistry		Serum Marker	
WBC	39.8 x10^3/μl	TP	5.0 g/dl	IL-6	79 pg/ml
RBC	245 x10^4/μl	Alb	1.7 g/dl	IL-8	279 pg/ml
Hb	7.4 g/dl	BUN	5 mg/dl	Neutrophil elastase	291 μg/l
Ht	23.5%	Cre	0.43 mg/dl		
PLT	3.3 x10^4/μl	T-Bil	19.2 mg/dl		
Coagulation		D-Bil	14.9 mg/dl		
PT%	42%	AST	96 IU/l		
		ALT	33 IU/l		
		ALP	318 IU/l		
		LDH	208 IU/l		
		GGTP	252 IU/l		
		CRP	11.05 mg/dl		



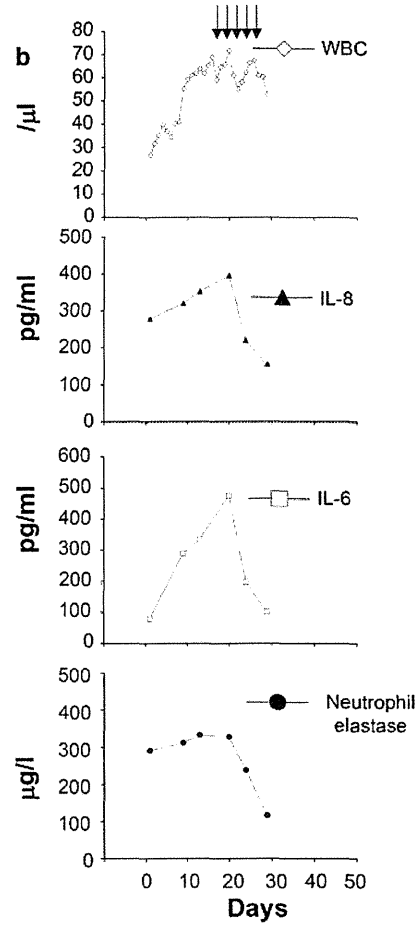


Fig. 2 Summary of case 2. **a** Laboratory data upon the admission. **b** Time courses of WBC, IL-8, IL-6, and neutrophil elastase. *Black arrows* represent granulocytapheresis (GCAP) treatment. **c** Computed tomography (CT) before GCAP treatment. **d** CT after the four sessions of GCAP treatment

Recently, anti-TNF-α antibody and pentoxifylline have been used in the United States of America and Europe to control TNF-α; however, the clinical outcomes vary among the institutions [4, 5]. GCAP and LCAP may control the activated leukocytes both in the systemic circulation and in the liver tissue. Activated leukocytes release inflammatory cytokines and therefore it is a reasonable hypothesis to state that the control of the number of cells may result in the treatment of cytokinemia and the inflammatory response. A number of cases have been treated with GCAP or LCAP, especially in Japan [8–18] (Table 1) since our first report in 2002 [8]. A literature review of 35 cases shown in Table 1 clearly revealed that the control of leukocytosis improved survival to approximately 60 % compared with 32.6 % in Japan in the 1990s before the introduction of GCAP or LCAP, and currently the survival rate in Japan is 62.9 % since 2004 [2, 19, 20]. Horie et al. [2] reported the survival and mortality rates with and without the different therapeutic options, including corticosteroid, plasma exchange, hemodialysis, and granulocytapheresis, and also showed that GCAP is statistically associated with improved survival. In addition, the patients with leukocytosis evidenced by WBC counts higher than 10,000 showed a statistically higher survival rate with GCAP ($p < 0.0007$) [2]. On an average the WBC counts decreased approximately 29 % after 2.95 sessions of GCAP or LCAP. Morris et al. [17] reported less effective results in six cases in Europe; however, the patients received only one session of GCAP in their course and leukocytosis was not adequately treated. The serum level of IL-8 reported in 11 cases showed a 71 % decrease after the treatment, indicating that controlling leukocytosis improved cytokinemia. Due to the limited number of cases reported, there is no significant correlation with the prognosis based on statistical analysis. However, because the serum IL-8 has been reported to be correlated with the severity of liver injury and leukocytosis in AH patients and it may serve as a predictor of survival in the patients [27], the results support the efficacy of GCAP and LCAP for treatment of SAH. The level of IL-6 also showed a tendency to decrease; however, the values varied and the serum level of TNF-α was not available. No major adverse

Table 1 Summary of reported cases treated with GCAP and LCAP

Case no. [Ref. no.]	GCAP/ LCAP	Session	PE	HDF	Corticosteroid	Silvelestat	Ulinastatin	Outcome	Before WBC	After WBC	Decrease (%)	Before IL-8	After IL-8	Decrease (%)	Before IL-6	After IL-6	Decrease (%)	Cause of death
1 [9]	LCAP	2	+	−	+	−	−	D	71,600	20,300	71.6	492	87.9	82.1	49.1	8.1	83.5	Pneumonia
2 [8]	GCAP	13	+	+	−	−	+	D	20,160	10,200	49.4	608	104	82.9	61.2	9.5	84.5	Stroke
3 [10]	GCAP	2	−	−	+	−	−	A	26,900	~8,000	70.3	70.4	48.4	31.3	19.3	8.9	53.9	
4 [11]	GCAP	2	+	+	−	−	+	A (T)	12,040	~10,000	16.9	205	45.6	77.8	21.1	57.5	−172.5	
5 [12]	GCAP	5	−	−	+	+	+	A	38,700	20,000	48.3	300	~50	83.3	65	~10	84.6	
6 [13]	LCAP	5	+	+	−	−	−	A	14,800	5,000	66.2	31.8	3.7	88.4	355	8.7	97.5	
7 [14]	GCAP	4	+	+	+	−	+	A	72,700	5,000	65.5	658	/	/	53.3	/	/	
8 [15]	GCAP	3	−	−	+	−	+	D	42,600	~70,000	−64.3	~250	~130	48.0	~5	~25	−400.0	Pancreatitis, Pneumonia
9 [15]	GCAP	2	−	−	+	−	+	A	27,000	~5,000	81.5	~70	~20	71.4	~20	~5	75.0	
10 [15]	GCAP	2	−	−	+	−	−	A	23,800	~12,000	49.6	~85	~20	76.5	~50	~5	90.0	
11 [15]	GCAP	2	−	+	+	−	−	D	16,000	~10,000	37.5	~80	~20	75	~300	~500	−66.7	Cerebral hemorrhage
12 [15]	GCAP	3	−	−	+	−	−	A	18,600	/	/	/	/	/	/	/	/	
13 [15]	GCAP	1	+	+	+	−	−	D	11,600	/	/	/	/	/	/	/	/	SMA thrombosis, GI tract perforation
14 [16]	GCAP	4	+	+	+	−	−	A	27,400	18,400	32.8	/	/	/	/	/	/	
15 [17]	GCAP	1	−	−	+	−	−	D	16,900	~20,000	−18.3	/	/	/	/	/	/	Variceral bleeding
16 [17]	GCAP	1	−	−	+	−	−	D	12,900	~6,000	53.5	/	/	/	/	/	/	Multiple organ failure
17 [17]	GCAP	1	−	−	+	−	−	D	25,800	~25,000	3.1	/	/	/	/	/	/	Pneumonia
18 [17]	GCAP	1	−	−	+	−	−	D	24,700	~30,000	−21.5	/	/	/	/	/	/	Multiple organ failure
19 [17]	GCAP	1	−	−	+	−	−	A	16,700	~17,000	−1.8	/	/	/	/	/	/	
20 [17]	GCAP	1	−	−	+	−	−	D	14,500	~13,000	10.3	/	/	/	/	/	/	Multiple organ failure
21 [18]	GCAP	/	+	+	+	/	/	A	27,400	/	/	/	/	/	/	/	/	
22 [18]	GCAP	/	−	−	−	/	/	A	17,600	/	/	/	/	/	/	/	/	
23 [18]	GCAP	/	−	−	−	/	/	A	23,300	/	/	/	/	/	/	/	/	
24 [18]	GCAP	/	−	−	−	/	/	A	39,300	/	/	/	/	/	/	/	/	
25 [18]	GCAP	/	+	−	−	/	/	A	31,800	/	/	/	/	/	/	/	/	
26 [18]	GCAP	/	+	−	+	/	/	A	33,700	/	/	/	/	/	/	/	/	
27 [18]	GCAP	/	−	−	−	/	/	A	17,500	/	/	/	/	/	/	/	/	
28 [18]	GCAP	/	−	−	−	/	/	A	12,300	/	/	/	/	/	/	/	/	
29 [18]	LCAP	/	−	−	+	/	/	A	39,100	/	/	/	/	/	/	/	/	
30 [18]	GCAP	/	+	+	+	/	/	A	31,600	/	/	/	/	/	/	/	/	
31 [18]	GCAP	/	−	−	+	/	/	A	78,000	/	/	/	/	/	/	/	/	
32 [18]	GCAP	/	−	−	−	/	/	D	32,000	/	/	/	/	/	/	/	/	
33 [18]	GCAP	/	+	+	+	/	/	D	25,900	/	/	/	/	/	/	/	/	
34 [18]	GCAP	/	+	+	+	/	/	D	20,100	/	/	/	/	/	/	/	/	
35	GCAP	6	−	+	−	+	−	D	39,880	14,000	64.9	396	156	60.6	478	102	78.7	Pleural bleeding

GCAP granulocytapheresis, *LCAP* leukocytapheresis, *PE* plasma exchange, *HDF* hemodiafiltration, *WBC* white blood cell, *Before* before GCAP/LCAP, *After* after GCAP/LCAP, *D* died, *A* alive, *T* transplantation