Immunity, Vol. 21, 539–550, October, 2004, Copyright ©2004 by Cell Press

Osteopontin as a Mediator of NKT Cell Function in T Cell-Mediated Liver Diseases

Hongyan Diao,[1] Shigeyuki Kon,[1] Kazuya Iwabuchi,[2] Chiemi Kimura,[1] Junko Morimoto,[1] Daisuke Ito,[1] Tatsuya Segawa,[3] Masahiro Maeda,[3] Junji Hamuro,[4] Toshinori Nakayama,[5] Masaru Taniguchi,[6] Hideo Yagita,[7] Luc Van Kaer,[8] Kazunori Onóe,[2] David Denhardt,[9] Susan Rittling,[10] and Toshimitsu Uede[1,*]

[1]Division of Molecular Immunology and
[2]Division of Immunobiology
Institute for Genetic Medicine
Hokkaido University
Sapporo, 060-0815
Japan

[3]Immuno-Biological Laboratories Co., Ltd.
Gunma, 375-0005
Japan

[4]Pharmaceutical Research Laboratories
Ajinomoto Co., Inc.
Kanagawa, 210-8613
Japan

[5]Department of Immunology
Graduate School of Medicine
Chiba University
Chiba, 260-8670
Japan

[6]Laboratory of Immune Regulation
RIKEN Research Center for Allergy and Immunology
Yokohama, 230-0045
Japan

[7]Department of Immunology
Juntendo University School of Medicine
Tokyo, 113-8421
Japan

[8]Department of Microbiology and Immunology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

[9]Department of Cell Biology and Neuroscience
Rutgers University
Piscataway, New Jersey 08854

[10]Department of Genetics
Rutgers University
Piscataway, New Jersey 08855

Summary

Both osteopontin (OPN) and natural killer T (NKT) cells play a role in the development of immunological disorders. We examined a functional link between OPN and NKT cells. Concanavalin A (Con A)-induced hepatitis is a well-characterized murine model of T cell-mediated liver diseases. Here, we show that NKT cells secrete OPN, which augments NKT cell activation and triggers neutrophil infiltration and activation. Thus, OPN- and NKT cell-deficient mice were refractory to Con A-induced hepatitis. In addition, a neutralizing antibody specific for a cryptic epitope of OPN, exposed by thrombin cleavage, ameliorated hepatitis. These findings identify NKT cell-derived OPN as a novel target for the treatment of inflammatory liver diseases.

*Correspondence: toshi@igm.hokudai.ac.jp

Introduction

T cell-mediated liver diseases including autoimmune hepatitis and viral hepatitis are associated with significant morbidity and mortality worldwide and remain a serious concern in the clinical setting (Diao et al., 2001; McFarlane, 1999). Concanavalin A (Con A)-induced hepatitis in the mouse is a well-characterized model of T cell-mediated liver diseases (Tiegs et al., 1992). Following Con A administration, liver histology shows massive granulocyte accumulation, CD4 T cell infiltration, influx of a relatively small number of CD8 T cells, and hepatocyte necrosis/apoptosis (Chen et al., 2001; Tiegs et al., 1992). Previous studies have shown that various immunoregulatory cytokines play a role in the pathogenesis of Con A-induced hepatitis. Th1 type cytokines such as interferon (IFN)-γ, interleukin (IL)-12, and tumor necrosis factor (TNF)-α promote liver injury, whereas the Th2 type cytokine IL-10 suppresses liver injury (Kaneko et al., 2000; Louis et al., 1997).

Recent studies have implicated hepatic NKT cells in the development of Con A-induced hepatitis. Both Jα18- and CD1d-deficient mice that lack NKT cells are resistant to Con A-induced hepatic injury (Kaneko et al., 2000; Takeda et al., 2000), indicating that classical CD1d-restricted NKT cells that express the invariant Vα14-Jα18 T cell receptor are critically involved in the process of Con A-induced hepatic injury. Upon activation, NKT cells secrete various cytokines (e.g., IFN-γ) that activate resident Kupffer cells and recruit macrophages to produce TNF-α, which subsequently causes liver injury (Burdin et al., 1998; Takeda et al., 2000). Additionally, the Th2 type cytokine IL-4 causes NKT cells to express Fas ligand (FasL), contributing to Fas/FasL-mediated liver injury in an autocrine fashion (Takeda et al., 2000). However, how activation of NKT cells leads to hepatic injury remains to be fully elucidated.

It is known that a variety of inflammatory and autoimmune diseases (including MS, RA, and atherosclerosis) are critically regulated by NKT cells (Chiba et al., 2004; Jahng et al., 2001; Tupin et al., 2004). Interestingly, each of these diseases is associated with high osteopontin (OPN) expression (Chabas et al., 2001; Matsui et al., 2003; Ohshima et al., 2002; Steinman and Zamvil, 2003). OPN is known not only as an extracellular matrix protein, supporting adhesion and migration of inflammatory cells, but also as an immunoregulatory cytokine (Ashkar et al., 2000; O'Regan et al., 2000). Furthermore, both OPN- and CD1d-deficient mice are resistant to high fat diet-induced atherosclerosis (Matsui et al., 2003; Tupin et al., 2004). Importantly, overexpression of OPN was associated with various inflammatory liver diseases (Morimoto et al., 2004; Tanaka et al., 2004). Therefore, we sought to determine whether there is any mechanistic link between OPN and NKT cells and whether OPN

contributes to Con A-induced fulminant hepatitis. Here, we show that, during Con A-induced fulminant hepatitis, NKT cells regulate the severity of neutrophil infiltration into the liver and the levels of hepatic injury by secreting OPN. Targeting this pathway holds promise for the treatment of inflammatory hepatitis.

Results

Requirement of NKT Cells and OPN in Con A-Induced Hepatic Injury

Since it has been shown that CD1d-deficient mice lacking NKT cells are protected from Con A-induced hepatitis (Takeda et al., 2000), we first compared the liver damage after Con A injection in CD1d-deficient, OPN-deficient, and wild-type mice of the C57BL/6 background. To address the role of CD1d-restricted classical NKT cells, we also injected Con A into Jα18-deficient mice. Liver damage, as reflected by serum ALT levels, peaked at 12 hr and then declined at 24 hr after Con A injection in wild-type mice. In sharp contrast, in CD1d-, Jα18- and OPN-deficient mice, ALT levels were significantly lower than those in wild-type mice. There was no significant difference in ALT levels among Con A-treated CD1d-, Jα18-, and OPN-deficient mice (Figure 1A). Consistent with the ALT levels, histological examination showed diffuse and massive degenerative liver alterations after Con A injection in wild-type mice, whereas only focal and mild liver injury was detected in OPN-, Jα18-, and CD1d-deficient mice (Figure 1B). Importantly, survival was also substantially enhanced in OPN-deficient mice (Figure 1C). We also showed that CD1d-deficient mice were well protected from death after Con A injection (Figure 1C), as previously reported (Kaneko et al., 2000). Thus either OPN deficiency or NKT cell deficiency rendered mice to be resistant to Con A-induced hepatic injury. Importantly, OPN mutant mice have no major defects in NKT cell and conventional T cell development and function, although numbers of NKT cells in the spleen and liver of these animals may be slightly reduced (data not shown).

To provide further evidence for the contribution of NKT cells and OPN to Con A-induced hepatic injury, we tested whether adoptive transfer of NKT cells from wild-type animals could render CD1d- or OPN-deficient mice susceptible to Con A-induced hepatic injury. Intrahepatic injection of C57BL/6-derived NKT ($NK1.1^+TCR^+$) cells along with Con A elevated the serum ALT levels in both CD1d- and OPN-deficient mice, whereas injection of NKT ($NK1.1^+TCR^+$) cells derived from OPN-deficient mice did not (Figure 1D). The involvement of CD1d-restricted classical NKT cells in Con A-induced hepatic injury was further tested by adoptive transfer of NKT cells purified with α-galactosylceramide (α-GC)-loaded CD1d dimers and anti-TCR antibodies. Results showed that NKT cells from wild-type, but not OPN-deficient, mice restored Con A-induced hepatic injury in OPN-deficient mice, as judged by the elevation of ALT levels in reconstituted animals (Figure 1E).

NKT Cell-Deficient Mice Are Defective in Con A-Induced OPN Production

To further investigate a mechanistic link between OPN and NKT cells in Con A-induced hepatic injury, we next examined the plasma OPN levels after Con A injection in wild-type, CD1d-, Jα18-, and OPN-deficient mice. In wild-type mice, plasma levels of OPN were significantly increased at 6 hr and further increased thereafter. In CD1d- and Jα18-deficient mice, however, plasma OPN levels were significantly lower than those in wild-type mice (Figure 2A). In addition, we injected mice with the potent NKT cell activator α-GC (Kawano et al., 1997) and found that this treatment induced a significant elevation of plasma OPN levels in wild-type mice, but not CD1d- and Jα18-deficient mice (Figure 2B). Plasma OPN was not detected after Con A (Figure 2A) or α-GC (data not shown) treatment of OPN-deficient mice.

To examine whether NKT cells could be a source of OPN, we isolated intrahepatic leukocytes from normal wild-type mice. Resident intrahepatic NKT ($NK1.1^+$ TCR^+) cells clearly expressed intracellular OPN protein, whereas $NK1.1^-$ conventional T cells did not (Figure 2C). It should be noted that $NK1.1^+TCR^-$ cells, which represent natural killer (NK) cells, also expressed OPN. Since it has been previously shown that depletion of liver NK cells fails to inhibit Con A-induced hepatic injury (Toyabe et al., 1997), we focused on NKT cells as the primary source of OPN. To investigate whether CD1d-restricted classical NKT cells were able to secrete OPN upon activation, we adopted an in vitro system in which $NK1.1^+TCR^+$ NKT cells were stimulated by α-GC in the context of the CD1d molecule. In the presence, but not absence, of α-GC, NKT cells secreted significant amounts of OPN protein, whereas anti-CD3/CD28-stimulated, NKT cell-depleted T cells (i.e., conventional T cells) failed to secrete OPN (Figure 2D). NKT cells also secreted significant amounts of IFN-γ, whereas conventional T cells secreted only a small amount of this cytokine. NKT and conventional T cells secreted similar amounts of TNF-α. To provide further evidence that classical NKT cells are the main source of OPN secretion, we enriched α-GC loaded CD1d-dimer-positive and TCR-positive NKT cells and found that these invariant NKT cells similarly secrete OPN upon stimulation (data not shown). We further tested in vivo production of cytokines and OPN after Con A injection. Liver IFN-γ and TNF-α levels were significantly lower in CD1d- and OPN-deficient mice, as compared with wild-type mice (Figures 2E and 2F). Liver OPN levels were significantly increased after Con A injection in wild-type mice, whereas liver OPN levels remained unchanged in CD1d-deficient mice, in accordance with the plasma OPN levels (Figure 3A). This was confirmed by immunohistochemistry, which showed that liver tissues from wild-type mice indeed express OPN protein after Con A injection (Figure 3B). Collectively, these data indicate that CD1d-restricted classical NKT cells can produce OPN and that these cells are a critical source of plasma OPN in Con A-induced hepatitis.

Role of the Thrombin-Cleaved Form of OPN in Liver Injury

OPN interacts with a variety of cell surface receptors, including αvβ3, αvβ5, α4β1, α8β1, and α9β1 integrins, as well as CD44 (Bayless et al., 1998; Sodek et al., 2000; Yokosaki et al., 1999). Binding of OPN to these cell surface receptors stimulates cell adhesion, migration,

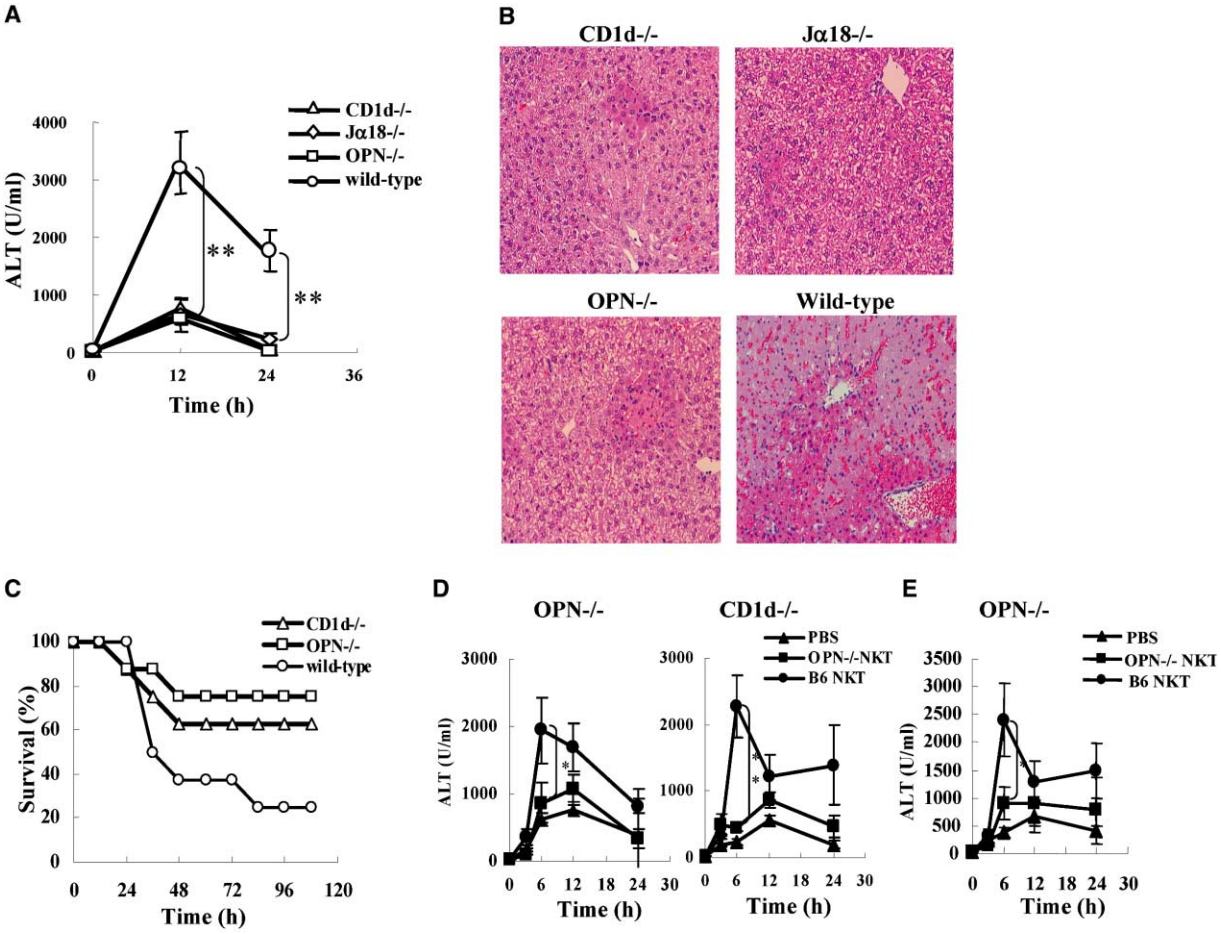

Figure 1. OPN- and NKT Cell-Deficient Mice Fail to Develop Severe Con A-Induced Hepatitis

(A) Serum ALT levels in OPN$^{-/-}$, CD1d$^{-/-}$, Jα18$^{-/-}$, and wild-type mice were assessed after Con A injection. n = 8 per group (Jα18$^{-/-}$; n = 4). **p $<$ 0.001.

(B) Representative liver histology. Livers were obtained at 24 hr after Con A injection, and sections were stained with H&E. Original magnification, 200×.

(C) Survival advantage of OPN$^{-/-}$ and CD1d$^{-/-}$ mice as compared to wild-type mice after 20 mg/kg Con A injection. n = 8 per group.

(D) Restoration of Con A-induced hepatic injury by adoptive transfer of NK1.1^{+}TCR^{+} NKT cells. NKT cells (2 $\times$ 10^{6}) from wild-type and OPN$^{-/-}$ mice or PBS were injected into recipient OPN$^{-/-}$ and CD1d$^{-/-}$ mice. 1 hr after cell transfer, mice received 10 mg/kg Con A. n = 3 per group. *p $<$ 0.05 and **p $<$ 0.001.

(E) Restoration of Con A-induced hepatic injury by adoptive transfer of α-GC-loaded CD1d dimer^{+}TCR^{+}NKT cells. NKT cells (2 $\times$ 10^{6}) from wild-type and OPN$^{-/-}$ mice or PBS were injected into recipient OPN$^{-/-}$ mice. 1 hr after cell transfer, mice received 10 mg/kg Con A. n = 3 per group. *, p$<$0.05 and **, p$<$0.001.

and specific signaling functions. The major integrin binding domain within OPN is the arginine-glycine-aspartate (RGD) integrin binding motif. However, cleavage of human OPN by thrombin exposes an additional integrin binding motif, the SVVYGLR (SLAYGLR in mice) sequence, which promotes the adherence of cells expressing α4 and α9 integrins. Importantly, this thrombin-cleaved form of OPN has been implicated in the pathogenesis of RA (Yamamoto et al., 2003). Therefore, we examined the molecular nature of OPN in the liver of Con A-injected mice. We found that in addition to the noncleaved form of OPN, a low molecular form of OPN, corresponding to the thrombin-cleaved product, was present in the liver of wild-type mice after Con A injection (Figure 4A). Both noncleaved and cleaved forms of OPN were absent in OPN-deficient mice, even after Con A stimulation (Figure 4A). We then examined the expression of integrins on NKT and conventional T cells. We found that normal intrahepatic NKT cells expressed both α9 and α4 integrins (Figure 4B). In contrast, conventional T cells only expressed α4 integrin. Both NKT and conventional T cells expressed β1 and β3 integrins. We also quantitated integrin expression, which revealed that α9 expression on NKT is twenty times higher than that on conventional T cells, whereas α4 expression on conventional T cells is two times higher than that on NKT cells (Figure 4B). Next, we asked whether the thrombin-cleaved form of OPN, expressed in the liver after Con A injection, plays a role in the development of hepatitis. In vitro migration assays showed that infiltrating leukocytes purified from the liver after Con A injection migrated toward the thrombin-cleaved form of OPN more

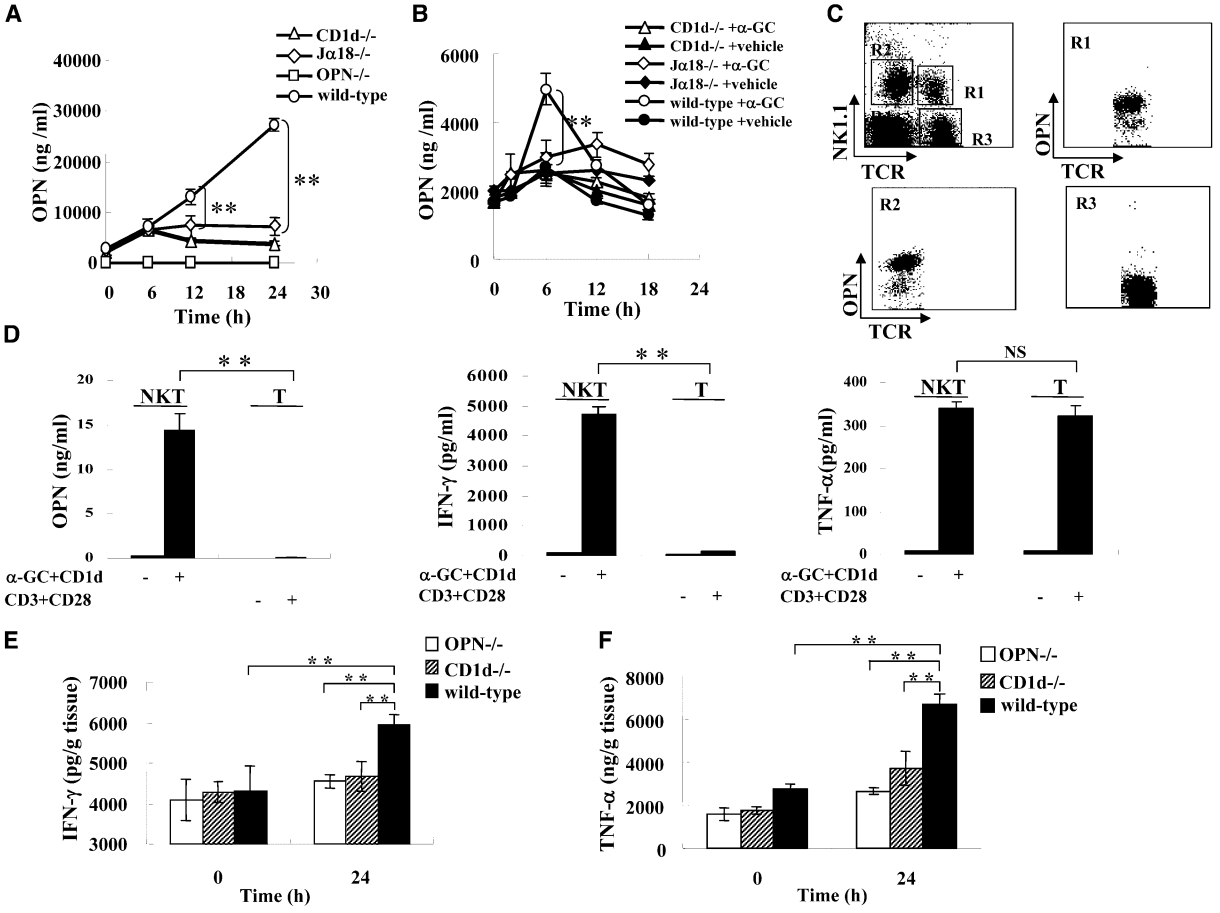

Figure 2. Secretion of OPN and Cytokines Is Defective in NKT Cell-Deficient and OPN$^{-/-}$ Mice

(A and B) Plasma OPN levels in C57BL/6 wild-type, CD1d$^{-/-}$, Jα18$^{-/-}$, and OPN$^{-/-}$ mice were measured by ELISA after Con A (A) or α-GC (B) injection. n = 4 per group. **p < 0.001.

(C) Intracellular OPN expression in intrahepatic leukocytes from wild-type mice. The expression of NK1.1, TCR, and intracellular OPN was analyzed by flow cytometry (upper left). NKT cells as defined by NK1.1$^+$TCR$^+$ (R1) and NK cells as defined by NK1.1$^+$TCR$^-$ (R2) clearly expressed intracellular OPN (upper right and lower left, respectively), whereas conventional T cells as defined by NK1.1$^-$TCR$^+$ (R3) did not (lower right).

(D) Secretion of OPN by NKT cells. NKT and conventional T cells from normal mice were stimulated with α-GC in the presence of CD1d-RBL cells or anti-CD3/CD28 mAbs, respectively, for 3 days. OPN production in the culture supernatant was measured by ELISA. Data are representative of three independent experiments. **p < 0.001.

(E) IFN-γ levels in the liver before and after Con A injection. n = 4 per group. **p < 0.001.

(F) TNF-α levels in the liver before and after Con A injection. n = 4 per group. **p < 0.001.

efficiently than toward the full-length form of OPN (Figure 4C). In sharp contrast, leukocytes obtained from normal livers migrated toward the cleaved form of OPN only marginally. We also examined the type of cells migrating toward the thrombin-cleaved form of OPN by morphological analysis and found that migrating cells were predominantly neutrophils (Figure 4C). We have used antibodies with specificity toward different integrins and specific forms of the OPN molecule to explore the role of OPN cleavage products in Con A-induced hepatic injury. Antibodies directed against β1 and α4 integrins, but not β3 and αv integrins, inhibited the migration of liver-infiltrating cells toward the thrombin-cleaved form of OPN (Figure 4D). Most compellingly, the antibody M5, which was raised against the SLAYGLR peptide (Yamamoto et al., 2003), inhibited cell migration induced by the thrombin-cleaved form of OPN (Figure 4D). The M5 antibody specifically inhibits interaction of this cryptic epitope within the OPN molecule with its receptors, the α4β1 and α9β1 integrins (data not shown). A control anti-OPN antibody M1, raised against the amino-terminal portion of OPN, had no effect on cell migration. Importantly, in vivo M5 antibody treatment significantly reduced serum ALT levels (Figure 4E) and liver necrosis (evaluated by histology) (Figure 4F) in Con A-injected wild-type mice. These data provide strong evidence that the interaction of the thrombin-cleaved form of OPN with its integrin receptor is involved in neutrophil infiltration and liver injury.

OPN Induces Neutrophil Infiltration and Activation during Liver Injury

We then tested whether the absence of OPN or neutralization of OPN by the M5 antibody influences the pro-

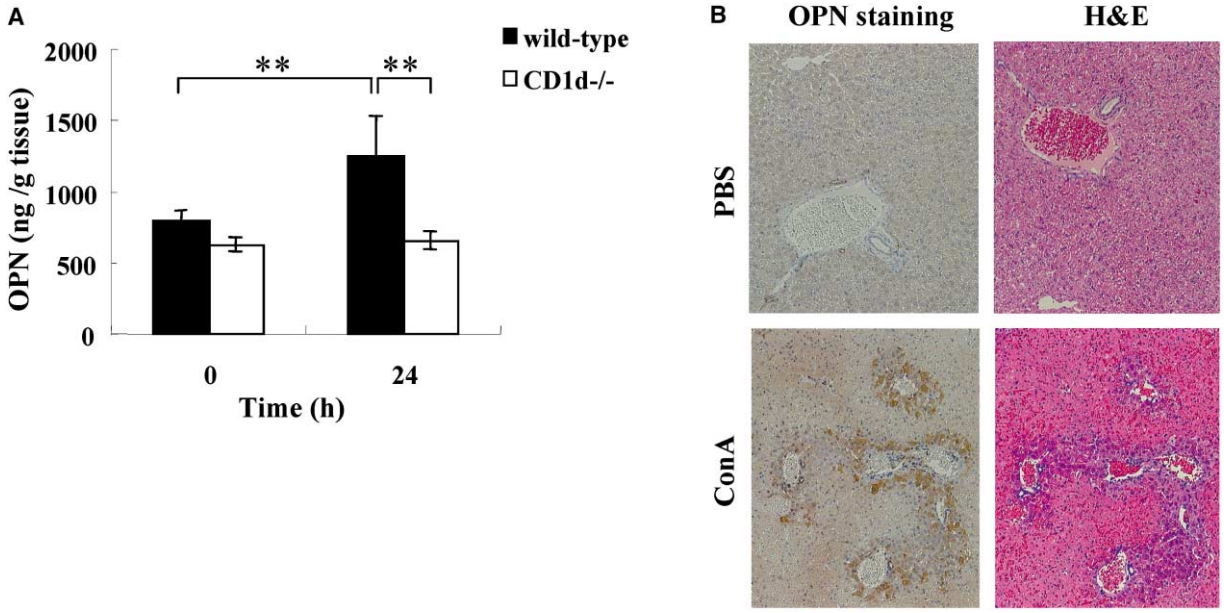

Figure 3. Expression of OPN Protein in the Liver

(A) OPN protein levels in the liver of C57BL/6 wild-type and CD1d-deficient mice after Con A injection. n = 8 per group. **p < 0.001.

(B) Immunohistochemical detection of OPN protein in BALB/c mice at 24 hr after PBS (top, left) and Con A (bottom, left) injection. The right panels represent H&E staining of respective sections. Original magnification, 100×. Data are representative of several independent experiments.

duction of a known chemotactic factor for neutrophils, macrophage inflammatory protein-2 (MIP-2) (Feng et al., 1995). We found that intrahepatic NKT cells, but not conventional T cells, from normal wild-type mice expressed MIP-2 (Figure 4B). In wild-type mice serum MIP-2 levels were significantly increased after Con A injection, whereas serum MIP-2 levels were only marginally increased in both OPN- and CD1d-deficient mice (Figure 5A). In addition, in vivo M5 antibody treatment significantly reduced MIP-2 production in the liver in wild-type mice after Con A injection (Figure 5B). These results suggested that OPN might be involved in the regulation of MIP-2 production by NKT cells.

To further address the role of neutrophils in Con A-induced liver injury, we examined the number of liver-infiltrating cells. It should be noted that strong neutrophil infiltration into the liver occurs at 6 hr in Con A-induced hepatitis, preceding the liver tissue damage. In both OPN- and CD1d-deficient mice that were protected from Con A-induced hepatic injury, neutrophil infiltration into the liver was significantly reduced as compared with other cell populations (Figure 6A). At 12 hr, macrophage numbers increased not only in livers from wild-type mice, but also in livers from OPN- and CD1d-deficient mice, indicating that the contribution of macrophages to hepatic injury is not critical. Moreover, neutralization of the thrombin-cleaved form of OPN with the M5 antibody significantly reduced the Con A-induced infiltration of neutrophils, but not that of CD4 T cells or macrophages in wild-type mice (Figure 6B). These results suggested that the thrombin-cleaved form of OPN induces neutrophil infiltration. We also noted that the infiltrating neutrophils expressing myeloperoxidase (MPO) were mainly located in the degenerative area of the liver in Con A-injected wild-type mice (Figure 6C). Interestingly, the amino-terminal half fragment of OPN, which binds to both $\alpha4\beta1$ and $\alpha9\beta1$ integrins (Bayless and Davis, 2001; Smith et al., 1996), efficiently activated neutrophils to secrete MPO. In sharp contrast, both the full-length form of OPN, which binds to $\alpha4\beta1$ (Barry et al., 2000), and the carboxy-terminal fragment of OPN, which binds neither $\alpha4\beta1$ (Barry et al., 2000) nor $\alpha9\beta1$ (Smith et al., 1996), failed to efficiently induce secretion of MPO (Figure 6D). Consistent with these findings, we found that expression of $\alpha9$ and $\alpha4$ integrins was significantly augmented in the liver after Con A injection, but this was substantially reduced after M5 antibody treatment (data not shown). These results indicate that NKT cell-derived OPN contributes to the development of Con A-induced hepatitis through recruitment and activation of neutrophils.

Discussion

NKT cells play a critical role in several models of inflammatory diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), and atherosclerosis (Chiba et al., 2004; Jahng et al., 2001; Tupin et al., 2004). Interestingly, OPN has also been implicated in the same inflammatory diseases (Chabas et al., 2001; Matsui et al., 2003; Ohshima et al., 2002; Steinman and Zamvil, 2003; Yumoto et al., 2002). However, until now, it has remained unclear whether there is any mechanistic link between OPN and NKT cells for the development of inflammatory diseases. To search for a possible link between OPN and NKT cells in inflammatory disease, we used Con A-induced fulminant hepatitis, a well-characterized murine model of inflammatory liver disease. We found that both NKT- and OPN-deficient mice are resistant to Con A-induced hepatic injury. More importantly, we demonstrated that adoptive

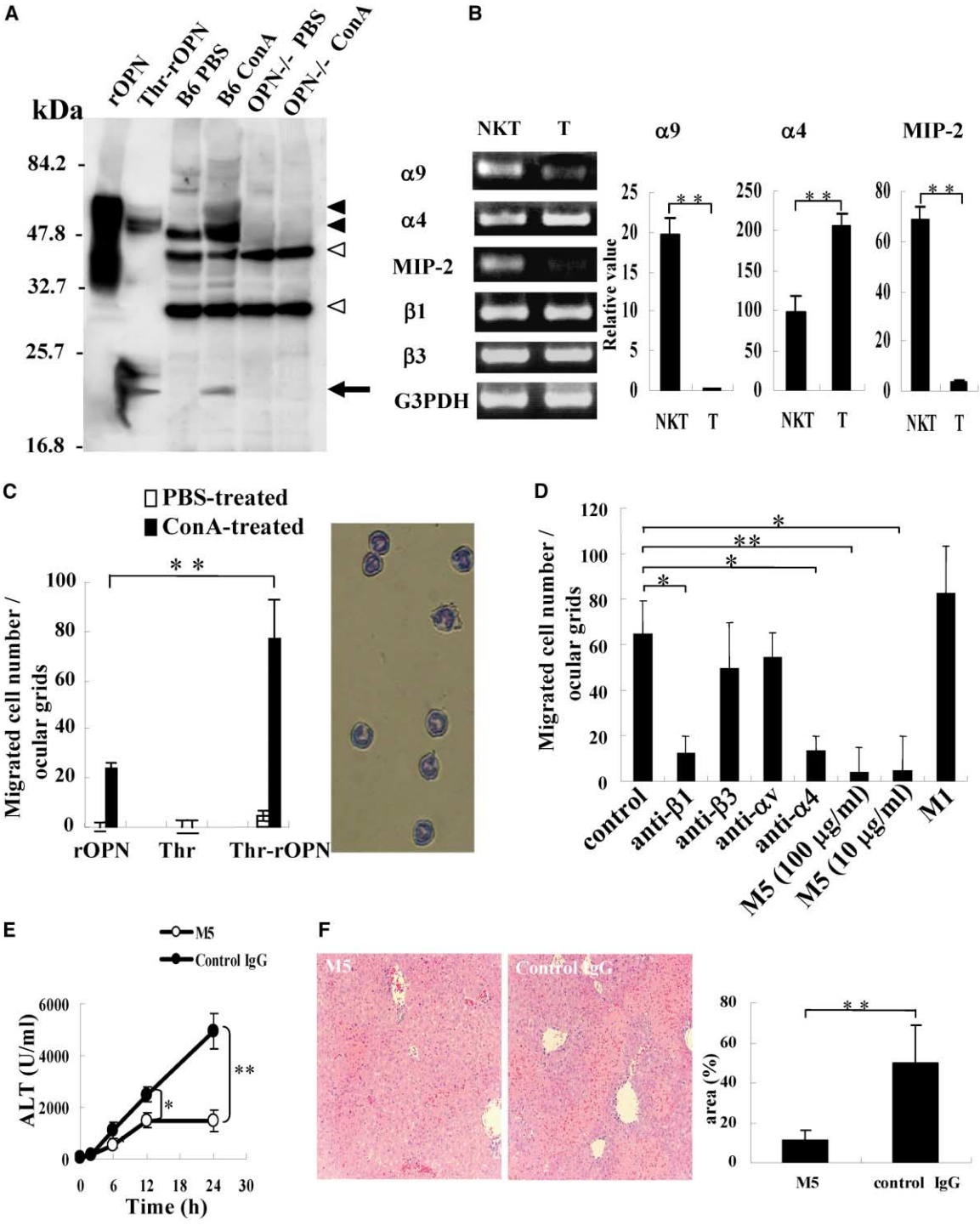

Figure 4. Critical Involvement of the Thrombin-Cleaved Form of OPN in Con A-Induced Hepatitis

(A) Western blot analysis of liver extracts. Liver extracts were prepared from wild-type and OPN$^{-/-}$ mice 24 hr after PBS or Con A injection. Recombinant OPN (rOPN) and thrombin-treated rOPN (Thr-rOPN) were used as controls. A low molecular form of OPN is indicated by the arrow. Closed arrowheads indicate the full-length OPN and open arrowheads indicate nonspecific bands.

(B) RT-PCR (left) and quantitative real time PCR (right) analyses of gene expression of integrins and MIP-2 in NKT and conventional T cells of normal C57BL/6 mice. In quantitative real-time PCR analyses, the relative value of gene expression was normalized against the gene expression levels of G3PDH. Data are representative of three independent experiments.

(C) Migration of liver-infiltrating leukocytes toward the thrombin-cleaved form of OPN. Infiltrating leukocytes prepared from the liver of BALB/c mice 6 hr after Con A or PBS injection were tested for their migratory activity against 10 μg/ml of full-length OPN (rOPN), 1 U/ml of thrombin (Thr), or 10 μg/ml of thrombin-treated rOPN (Thr-rOPN). Background cell migration toward medium only was subtracted. Migrated cells were recovered and stained with Diff-Quik. Original magnification, 400×. Data are representative of three independent experiments.

(D) Migration assays were performed in the presence of various antibodies. Antibody concentrations used were 100 μg/ml unless specifically

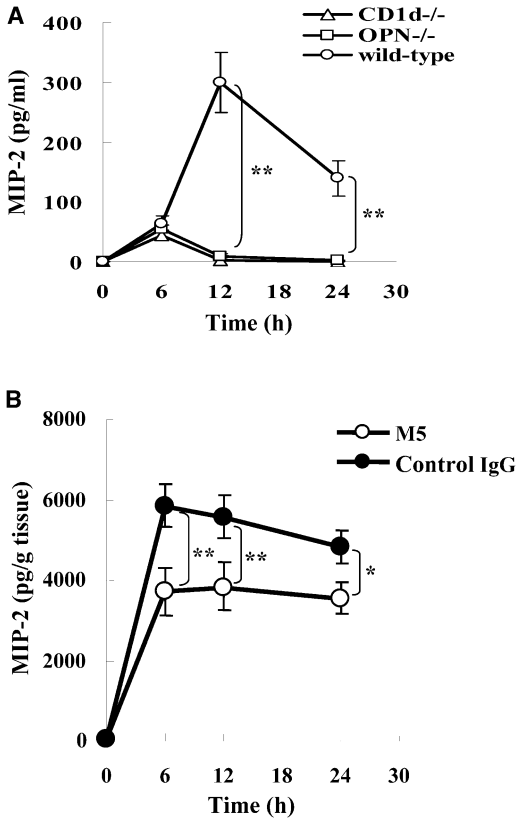

Figure 5. The Absence of OPN or Neutralization of OPN by M5 Antibody Influence the Production of MIP-2

(A) MIP-2 protein levels in the serum of $OPN^{-/-}$, $CD1d^{-/-}$, and wild-type mice after Con A injection. n = 4 per group. **p < 0.001.

(B) Inhibitory effect of M5 antibody on liver MIP-2 levels. n = 6 per group. *p < 0.05 and **p < 0.001.

transfer of $NK1.1^{+}TCR^{+}$NKT cells from wild-type, but not OPN-deficient, mice restores Con A-induced hepatic injury in both OPN- and CD1d-deficient mice. We also found that normal intrahepatic NKT cells express OPN and that purified NKT cells secrete substantial amounts of OPN upon activation. Consequently, OPN levels were significantly increased after Con A injection in wild-type mice, but remained unchanged in CD1d-deficient mice. Consistent with those findings, plasma OPN levels were very low after Con A injection in CD1d- and Jα18-deficient mice. Thus NKT cells are an important cellular source of plasma OPN after Con A injection. In addition, we found that plasma OPN levels are significantly elevated after treatment of wild-type mice with α-GC, a potent NKT cell activator, whereas plasma levels in OPN- and CD1d-deficient mice were not significantly increased. These findings demonstrate a critical link between OPN and NKT cells in the pathogenesis of Con A-induced hepatitis.

We noted that Con A administration resulted in a progressive increase and high levels of plasma OPN, whereas α-GC induced only transient and low levels of OPN in wild-type mice. A likely explanation for these findings is that Con A stimulates not only secretion of stored OPN but also de novo synthesis of OPN, whereas α-GC only stimulates secretion of stored OPN. An alternative possibility is that Con A remains in the liver for an extended timeperiod, therefore stimulating NKT cells chronically, whereas the half-life of α-GC in the blood is relatively short. In addition, we showed that hepatic resident NK cells as defined by $NK1.1^{+}TCR^{-}$ expressed OPN. Thus it is possible that in addition to NKT cells, hepatic NK cells also contribute to the production of OPN in Con A-induced hepatitis. However, deletion of NK cells did not inhibit the development of Con A-induced hepatic injury (Toyabe et al., 1997), indicating that NK cell involvement in Con A-induced hepatic injury is not critical. In the case of IFN-γ production in response to α-GC treatment of mice, the initial burst of IFN-γ was derived from NKT cells, which stimulated IFN-γ production by NK cells, thus contributing to sustained serum IFN-γ levels (Smyth et al., 2002). In contrast, α-GC induced only a sharp and transient, but not sustained, OPN production, indicating that NK cells are not involved in α-GC-induced OPN production. We also found that hepatic NKT cells expressed both OPN and its receptors, α9 and α4 integrins. Importantly, liver tissues expressed both the noncleaved and thrombin-cleaved forms of OPN after Con A injection. We demonstrated that the thrombin-cleaved form of OPN was critically involved in the pathogenesis of Con A-induced liver injury since the M5 antibody that specifically recognizes the cryptic epitope exposed by thrombin digestion and interferes with the binding of a cryptic epitope to α9β1 and α4β1 integrins inhibited Con A-induced hepatic injury. In this regard, it has been shown that the ratio of the thrombin-cleaved form of OPN to the noncleaved form was significantly increased in the plasma and synovial fluid of RA patients compared with plasma from healthy control and osteoarthritic patients (Ohshima et al., 2002). Nevertheless, M5 antibody treatment also resulted in amelioration of disease in a murine model of RA (Yamamoto et al., 2003).

Our findings support a recent study showing the importance of thrombin in Con A-induced hepatic injury. Inhibition of thrombin activity by antithrombin III significantly attenuated the degree of liver injury as indicated by ALT levels and neutrophil infiltration (Nakamura et al., 2002). However, thrombin is generated during tissue damage in various organs, including the liver (Esmon,

indicated. Data are representative of three independent experiments. Background cell migration toward medium only was subtracted. *p < 0.05 and **p < 0.001.

(E) Effect of M5 antibody on serum ALT levels in BALB/c mice after Con A injection. Mice were treated with M5 antibody or control IgG. *p < 0.05 and **p < 0.001. The discrepancy in the levels of ALT between Figures 1 and 4 was due to the strain difference; B6 mice were used in Figure 1 and BALB/c mice in Figure 4.

(F) Representative liver histology of BALB/c mice treated with M5 antibody or control IgG. Livers were obtained at 24 hr after Con A injection and liver sections were stained with H&E. Original magnification, 100×. Degenerative area per liver section was quantitated by using NIH image 1.62. **p < 0.001.

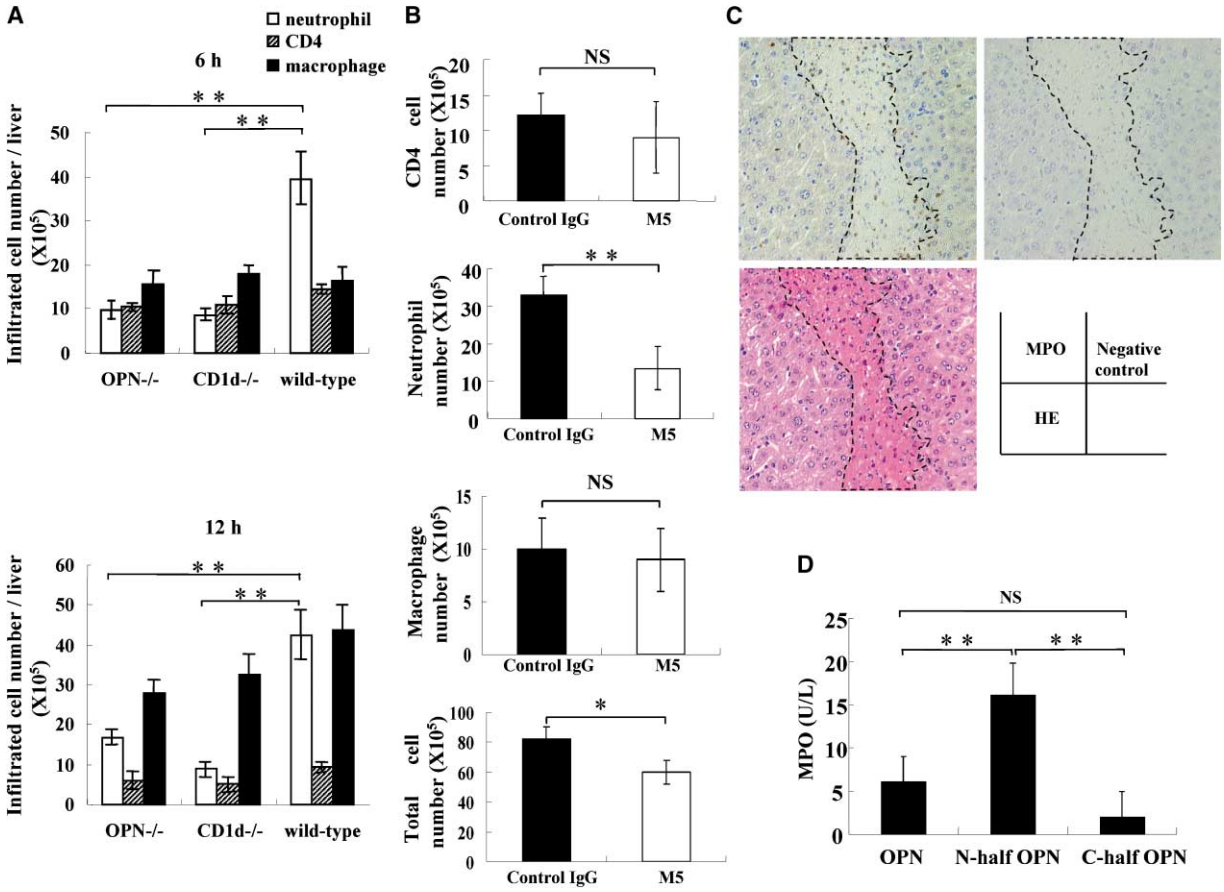

Figure 6. The Predominant Liver-Infiltrating Leukocytes in Con A-Induced Hepatitis Are Neutrophils

(A) Liver-infiltrating leukocytes were prepared from C57BL/6 wild-type, $CD1d^{-/-}$, and $OPN^{-/-}$ mice at 6 and 12 hr after Con A injection. The number of $Gr1^+$ $CD11b^+$ neutrophils, $CD4^+$ T cells, and $F4/80^+$ macrophages was determined by flow cytometry. n = 3 per group. **$p < 0.001$.

(B) Effect of M5 antibody on infiltration of neutrophils. Con A-injected mice were treated with control IgG or M5 antibody. At 6 hr, liver-infiltrating leukocytes were analyzed for $CD4^+$ T cells, $Gr1^+CD11b^+$ neutrophils, and $F4/80^+$ macrophage number by flow cytometry. n = 3 per group. **$p < 0.001$.

(C) Immunohistochemical detection of neutrophils. Livers were obtained from BALB/c mice at 24 hr after Con A injection, and sections were stained with anti-MPO or H&E. Sections were also stained with secondary antibody only as a negative control. Dotted lines indicate the area of liver degeneration. Original magnification, 200×.

(D) OPN-induced production of MPO. Neutrophils obtained from BALB/c mice were stimulated with 20 μg/ml of GST fusion proteins containing full-length OPN (OPN), N-terminal half of OPN (N-half OPN), or C-terminal half of OPN (C-half OPN) for 36 hr. Background production of MPO by neutrophils stimulated with GST was subtracted. Data are representative of three independent experiments. **$p < 0.001$.

1993). Nevertheless, thrombin could contribute to Con A-induced hepatic injury in many other ways. For example, thrombin may activate coagulation to increase hepatic sinusoidal hemostasis, which in turn results in liver tissue injury (Arai et al., 1996). Alternatively, thrombin may induce expression of IL-8 and leukocyte adhesion molecules including P-selectin, E-selectin, and ICAM-1 on endothelial cells (Leirisalo-Repo, 1994), promoting leukocyte activation (Copple et al., 2003). However, we favor the interpretation that at the earliest stage of Con A-induced hepatitis, activated NKT cells secrete OPN, which is subsequently cleaved by thrombin to expose the cryptic OPN epitope SLAYGLR, thereby activating receptors for this epitope, such as the α9β1 and α4β1 integrins expressed by NKT cells. This interaction of OPN with its receptor could further activate NKT cells and contribute to liver cell injury in Con A-induced hepatitis.

We found that strong cellular infiltration into the liver occurred at an early stage of Con A-induced hepatitis, preceding the liver tissue damage. We further showed that among these infiltrating cells, neutrophils were abundant. The appearance of neutrophils in Con A-induced hepatitis was demonstrated in several previous studies (Bajt et al., 2001; Jaruga et al., 2003; Miyazawa et al., 1998). Our data strongly indicate that neutrophils play a pivotal role in liver injury after Con A injection. The distribution of neutrophils in the liver, as defined by MPO activity, correlated well with the area of liver degeneration. Hydrogen peroxide, produced by activated neutrophils, is transformed by MPO into an array of potentially damaging reactants and causes acute liver injury (Brown et al., 2001). In both OPN- and CD1d-deficient mice that were protected from Con A-induced hepatic injury, neutrophil infiltration into the liver was significantly reduced as compared to other cell populations. Importantly, it has been shown that depletion of neutrophils by anti-Gr1 antibody prevents the development of hepatitis

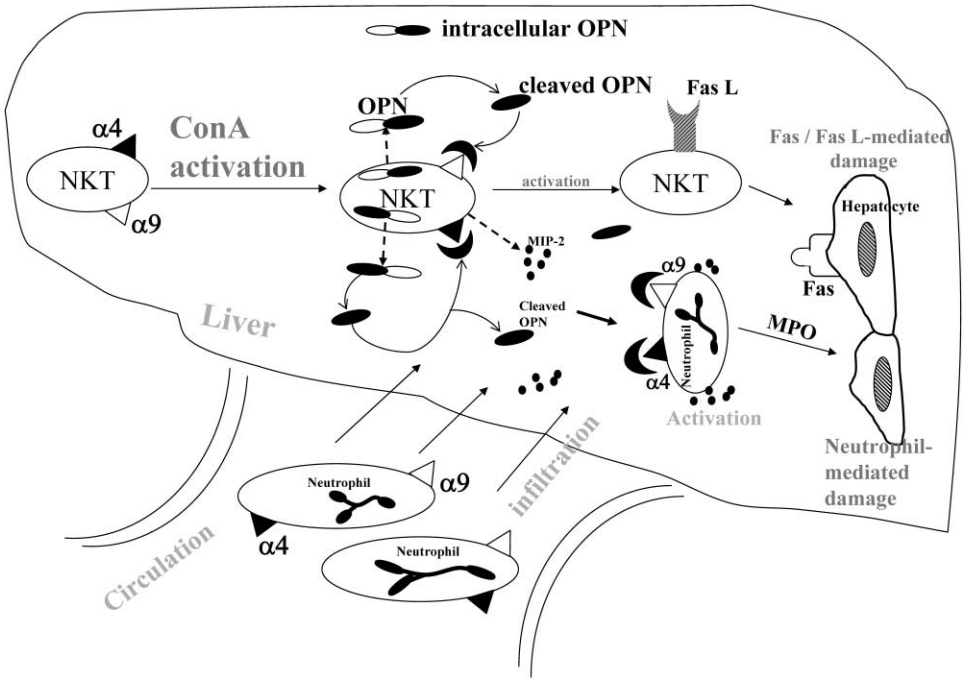

Figure 7. Schematic Representation of the Link between OPN, NKT Cells, and Neutrophils in Con A-Induced Hepatic Injury

Intrahepatic resident NKT cells express α9 and α4 integrins, receptors for the thrombin-cleaved form of OPN. After Con A-induced activation, NKT cells secrete OPN, which is presumably cleaved by thrombin in the liver. The interaction of NKT cells and the thrombin-cleaved form of OPN through its receptors further activates NKT cells. MIP-2, a known chemotactic factor, is produced in the liver upon Con A stimulation. Activated NKT cells express FasL and contribute to Fas/FasL-mediated liver cell injury. On the other hand, the thrombin-cleaved form of OPN, together with MIP-2, recruits neutrophils into the liver. Upon interaction of the thrombin-cleaved form of OPN with its receptors on neutrophils, the latter cells become activated, secrete MPO, and contribute to additional liver cell damage.

and reduces the liver damage in Con A-induced hepatitis (Bonder et al., 2004). Therefore, we explored how OPN regulates neutrophil function. We demonstrated that the amino-terminal half of OPN, which contains the cryptic epitope SLAYGLR, could activate neutrophils to release MPO. We also found that the thrombin-cleaved form of OPN can induce migration of neutrophils in vitro. The M5 antibody, as well as anti-β1 and anti-α4 integrin mAbs, was able to inhibit this cell migration. Furthermore, the infiltration of neutrophils into the liver was significantly inhibited by the M5 antibody treatment. Neutrophils are known to express the α9β1 integrin and, to a lesser extent, the α4β1 integrin, and lymphocytes do not express the α9β1 integrin (Bayless et al., 1998; Yokasaki and Sheppard, 2000). Thus, it is possible that neutrophils, through interaction of both α9β1 and α4β1 with the thrombin-cleaved form of OPN, are involved in neutrophil infiltration and activation, leading to liver damage. Consistent with our data, it was previously shown that blocking either the α4 or α9 integrin by antibodies inhibited neutrophil transendothelial migration (Taooka et al., 1999).

We also found that Con A induced, in an NKT cell-dependent manner, production of MIP-2, which is chemotactic for neutrophils. However, we were unable to detect significant levels of MIP-2 production by purified NKT cells after Con A stimulation in vitro (data not shown). This may indicate that there is crosstalk between NKT and other cell types that produce MIP-2 in response to NKT cell activation, as has been described for other cytokines (Kitamura et al., 1999; Smyth et al., 2002). It is tempting to speculate that initial activation of NKT cells by Con A may lead to MIP-2 production by dendritic cells. In this regard, it has been known that MIP-2 not only recruits neutrophils but also NKT cells (Faunce et al., 2001). In addition, OPN recruits dendritic cells (Tanaka et al., 2004). Importantly, MIP-2 levels in the liver were upregulated by Con A injection, which was significantly inhibited by M5 antibody. Thus, in addition to the thrombin-cleaved form of OPN, MIP-2 may also contribute to the infiltration of neutrophils into the liver and the subsequent liver injury.

In conclusion, during the course of Con A-induced hepatitis, activated NKT cells secrete OPN, which also leads to production of MIP-2, two cytokines that are chemotactic for neutrophils. We propose that the interaction of a cryptic epitope of OPN, SLAYGLR with the α9β1 and α4β1 integrins on NKT cells, and neutrophils contributes to the hepatic injury induced by Con A as illustrated in Figure 7. Targeting this OPN epitope could represent a new modality for the treatment of inflammatory hepatitis. A similar mechanism may also contribute to the development of other inflammatory diseases in which NKT cells and OPN have been implicated.

Experimental Procedures

Animals

Specific pathogen-free female BALB/c and C57BL/6 (B6) (6-week-old) mice were purchased from Japan SLC (Shizuoka, Japan). OPN-deficient ($OPN^{-/-}$) mice (Rittling et al., 1998) backcrossed 11 times to B6 mice at the Institute for Genetic Medicine, Hokkaido University were used. CD1d-deficient ($CD1d^{-/-}$) and Jα18-deficient

($J\alpha18^{-/-}$) mice of B6 origin were generated as described (Cui et al., 1997; Singh et al., 1999). All mice were maintained under specific pathogen-free conditions and used according to the institutional guidelines.

Antibodies

Antibodies used for blocking studies were as follows: polyclonal M5 antibody that specifically recognizes SLAYGLR (Yamamoto et al., 2003), polyclonal M1 antibody that specifically recognizes the amino-terminal portion of murine OPN (Yamamoto et al., 2003), and monoclonal antibodies (mAbs) directed against β1 integrin (HMβ1) (Noto et al., 1995) and β3 integrin (HMβ3) (Yasuda et al., 1995). Anti-αv integrin (RMV-7) and anti-α4 integrin (R1-2) were purchased from PharMingen (San Diego, CA). Antibodies for FACS staining were anti-CD4-PE (L3T4), anti-CD11b-PE (M1/70), anti-Gr1-FITC (RB6-8C5), anti-NK1.1-PE(PK136), anti-TCRβ-FITC (H57–597) and streptavidin-APC (all from PharMingen, San Diego, CA), biotin-anti-F4/80 (A3-1) (Caltag Laboratories, Burlingame, CA), and biotin-anti-OPN (O-17) (IBL, Gunma, Japan). Antibodies for immunohistochemistry were biotin-anti-CD4 (L3T4), biotin-anti-Gr-1 (RB6-8C5) (all from PharMingen, San Diego, CA), and polyclonal rabbit anti-mouse OPN antibody (O-17) (IBL, Gunma, Japan).

Con A-Induced Hepatitis

Mice were injected intravenously through the tail vein with Con A (Vector Laboratories, Burlingame, CA) reconstituted in pyrogen-free PBS. In general, the dose of Con A was 15 mg/kg and 10 mg/kg for BALB/c mice and mice of the C57BL/6 background, respectively. For survival studies, mice were injected with 20 mg/kg of Con A. For the indicated experiments, 400 μg of M5 Ab or normal rabbit IgG was dissolved in 200 μl of PBS. These antibodies were administered to mice intravenously 3 hr before Con A challenge. Serum alanine aminotransferase (ALT) levels were measured by using a standard clinical automatic analyzer.

Preparation of Liver-Infiltrating Leukocytes

Liver-infiltrating leukocytes were isolated as previously described (Takahashi et al., 2001). Briefly, livers were minced, pressed through a stainless steel mesh, and suspended in PBS. After washing, the cells were resuspended in 33% Percoll solution containing heparin (100 U/ml) and centrifuged at 2000 rpm for 15 min to remove liver parenchymal cells. The pellet was treated with an RBC lysis solution, washed with PBS three times, and then resuspended in 10% FCS-DMEM.

In Vitro Migration Assay

In vitro migration assay was performed by using a 24-well Transwell tissue culture plate (Costar, Corning, NY) with polycarbonate filter (pore size, 5 μm). Recombinant murine OPN (IBL, Gunma, Japan) digested by thrombin at 10 μg of OPN per 1 U of enzyme at 37°C for 1 hr was used as a chemoattractant. In some experiments, the cells were incubated with the indicated antibodies at a concentration of 100 μg/ml at 37°C for 15 min. After incubation at 37°C for 2 hr, the migrated cell numbers were quantitated by cell counts of 100 fields by using 100 ocular grids (×100). Migrated cells were recovered and stained with Diff-Quik (International Reagents Corporation, Kobe, Japan).

Morphometric Analysis

Formalin-fixed and paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Necrotic areas were measured in each section by using NIH image 1.62 followed by calculation of the necrotic area per section.

Immunohistochemistry

The expression of OPN was assessed by using polyclonal rabbit anti-mouse OPN antibody. Sections of paraffin-embedded liver tissue were processed for immunohistochemistry as previously described (Yoneyama et al., 1998). Immunohistochemical detection of MPO was performed by using a rabbit anti-human MPO Ab (DAKO, Carpinteria, CA) that crossreacts with mouse MPO (Grone et al., 2002).

Flow Cytometry

Liver-infiltrating leukocytes were stained with anti-CD4, anti-Gr1, and anti-CD11b mAbs as previously described (Moriyama et al., 1997). For intracellular OPN staining, leukocytes obtained from the liver were cultured in the presence of 2 μM monensin for 90 min and then stained by surface markers with anti-NK1.1 and anti-TCRβ. Cells were then fixed with 4% paraformaldehyde for 10 min and permeablized with a solution of 1% FCS, 0.1% saponin, and 0.1% sodium azide in PBS. Intracellular staining was performed using biotin-anti-OPN at 3 μg/ml diluted in 0.1% saponin staining buffer and Streptavidin-APC was used as the secondary reagent. All analyses were performed on a FACSCalibur (BD, Mountain View, CA) with CellQuest software.

In Vitro and In Vivo NKT and Conventional T Cell Stimulation

NKT and conventional T cells were isolated by a combination of magnetic-activated cell sorting (MACS) and FACS as previously described (Iwabuchi et al., 2001). In brief, $CD24^{-}CD8^{-}$ cells were negatively selected by using anti-CD24 and anti-CD8 magnetic beads. The $CD24^{-}CD8^{-}$ cells were stained with FITC-anti-TCR mAb and PE-anti-NK1.1 mAb and sorted into $NK1.1^{+}TCR^{+}$ (NKT) cells and $NK1.1^{-}TCR^{+}$ (conventional T) cells with a FACS Vantage instrument (Becton Dickinson). The sorted NKT cells and conventional T cells were cultured overnight with recombinant IL-2. NKT cells were further stimulated with 100 ng/ml α-GC in the presence of 100Gy-irradiated rat basophilic leukemia (RBL) cells transfected with CD1d. T cells were stimulated with anti-CD3 (145-2C11) and anti-CD28 (37.51) mAbs. In some experiments, mice were intravenously injected with 100 μg/kg α-GC or vehicle. α-GC was kindly provided by the Institute of Pharmaceutical Research, Kirin Brewery Co. (Gunma, Japan). CD1d-transfected cells were provided by Dr. Albert Bendelac (University of Chicago, Chicago, IL).

Adoptive Transfer of NKT Cells

Intrahepatic leukocytes were stained with PE-anti-NK1.1 and FITC-anti-TCRβ mAb. Cells were also stained with α-GC-loaded CD1d-dimer (BD Biosciences PharMingen) according to the method provided by the manufacturer followed by staining with FITC-anti-TCRβ. Cells were then sorted into $NK1.1^{+}TCR^{+}$ or $CD1d\text{-}dimer^{+}TCR^{+}$ cells. These cells, in a volume of 50 μl, were injected into the liver of recipient mice (2×10^{6} cells/mouse) 1 hr before Con A challenge (10 mg/kg). Sera were obtained from individual mice at the time point indicated in the figures, and serum ALT levels were determined.

Tissue Extraction, SDS-PAGE, and Western Blot Analysis

For Western blot analysis, mouse tissues were pulverized in PBS containing protease inhibitor cocktail (Roche, Mannheim, Germany). Further homogenization was done by repeated sonication for 15 s. After calibration of protein content, 15 μg each of protein extract was electrophoresed through 10 to 20% polyacrylamide Tris HCl Ready Gels (BioRad Laboratories, Hercules, California) and probed with polyclonal anti-OPN antibody (IBL, Gunma, Japan) as described previously (Kon et al., 2002).

ELISA

OPN, MIP-2 (both from IBL, Gunma, Japan) and TNF-α and IFN-γ (both from PharMingen, San Diego, CA) concentration were measured by using ELISA kits as specified by the manufacturers. Cytokine contents in the liver extracts were expressed as amounts per 1 g of liver tissue. MPO production was measured as described (Ramsaransing et al., 2003).

Analysis of mRNA Expression

Total RNA was isolated by using Trizol (Life Technologies, Gaithersburg, MD). The specific primers used were as follows. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH): 5′-ACCACAGTCCATGCCATCAC-3′ (sense), 5′-TCCACCACCCCTGTTGCTGTA-3′ (antisense). α4 integrin: 5′-TGGAAGCTACTTAGGCTACT-3′ (sense), 5′-TCCCACGACTTCGGTAGTAT-3′ (antisense). α9 integrin, 5′-AAAGGCTGCAGCTGTCCCACATGGACGAAG-3′ (sense), 5′-TTTAGAGAGATATTCTTCACAGCCCCCAAA-3′ (antisense). MIP-2: 5′-GAACAAAGGCAAGGCTAACTGA-3′ (sense), 5′-AACATAACAACATCTGGGCAAT-3′ (antisense). β1 integrin: 5′-CAAGGAGAAGGACATTGAT

GAC-3′ (sense), 5′-TCATTTTCCCTCATACTTCGG-3′ (antisense). β3 integrin: 5′-TTCGACTACGGCCAGATGATTC-3′ (sense), 5′-TTTCT CAGTCATCAGCCCCAG-3′ (antisense). Quantitative real-time PCR analysis of mRNA expression was also carried out with LightCycler Fast Start DNA Master SYBR Green I Systems (Roche Diagnostics). The expression of mRNA was calculated by LightCycler Software, version 3. Data were standardized by G3PDH.

Construction of the GST-OPN Fusion Plasmid and Protein Purification

OPN is specifically cleaved by thrombin between R154 and S155, thus making amino-terminal and carboxy-terminal fragments of OPN. Full-length (L17–N294), amino-terminal fragments (N-half OPN) (L17–R154), and carboxy-terminal fragments (C-half OPN) (S155–N294) of murine OPN cDNA were amplified from the first strand cDNA obtained from mouse kidney by using the following primers: Full-length OPN, 5′-TAGGGATCCCTCCCGGTGAAAGTGA CTGAT-3′ (sense) and 5′-GTCTCGAGTTAGTTGACCTCAGAAGA TGA-3′ (antisense); N-half OPN, full-length OPN sense primer and 5′-AACCTCGAGTTACCTCAGTCCATAAGCCAA-3′ (antisense); C-half OPN, 5′-CAGGGATCCTCAAAGTCTAGGAGTTTCCAG-3′ (sense) and full-length OPN antisense primer. PCR products were digested with BamHI and XhoI, ligated into pGEX6P-1 (Amersham Bioscience, Piscataway, NJ), and sequenced. The recombinant GST-OPN fusion proteins were prepared in *E. coli* as described previously (Kon et al., 2002). The GST fusion proteins were purified on glutathione-Sepharose columns as described (Tanaka et al., 2004).

Statistics

Data are presented as means ± SEM and are representative of at least two independent in vitro experiments. The significance of differences between two groups was determined by using a Student's t test. *$p < 0.05$. **$p < 0.001$. NS, not significant.

Acknowledgments

We are grateful to Dr. Dean Sheppard (UCSF) for his critical comments and for reviewing the manuscript. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare of Japan.

Received: April 1, 2004
Revised: July 19, 2004
Accepted: August 18, 2004
Published: October 19, 2004

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