Osteopontin affects the persistence of β-glucan-induced hepatic granuloma formation and tissue injury through two distinct mechanisms

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Abstract

Osteopontin (OPN) plays a pivotal role in various immune responses and inflammatory diseases. OPN is expressed in various granulomatous diseases; however, the cellular and molecular role of OPN in these diseases is not well known. We analyzed the role of OPN in a β-glucan-induced hepatic granuloma model. First, we found that neither OPN deficiency nor overexpression of OPN affected the number and the size of hepatic granulomas at day 7, indicating that OPN is not involved in the formation of hepatic granulomas at the early stages. Importantly, OPN did not influence the liver tissue damage as defined by alanine aminotransferase and aspartate aminotransferase levels at early stages. Second, OPN deficiency resulted in the reduction of IL-12 and IFN-γ production at early stages. Third, at late stages, OPN deficiency resulted in a decrease in the number and size of hepatic granulomas, and a reduction of liver tissue injury. This was due to the reduction of the cellular recruitment including macrophages, CD4 T cells and dendritic cells into the liver, and the reduction of tumor necrosis factor (TNF)-α production in the liver. In contrast, overexpression of OPN resulted in the persistence of granuloma formation. These data suggest that OPN affects the persistence of hepatic granuloma formation. Our results indicate that OPN up-regulates the production of IL-12 and IFN-γ within the granulomas at early stages, and OPN has an additional role in the regulation of cellular recruitment and TNF-α production at late stages that determine the severity of liver tissue injury.

Introduction

Granulomas are localized inflammatory reactions, which are often elicited against persistent antigenic irritants that are not easily cleared by phagocytic cells. They can be a part of both immune protection and disease pathology during the course of various infectious and autoimmune diseases (1). Recent studies showed that granulomas contained not only macrophages and T cells, but also functionally mature dendritic cells (DC) which possess potent antigen-presenting function and can activate T cells (2–5). When T cell-dependent antigens enter the circulation and are captured by Kupffer cells, the traffic of DC precursors in the liver is accelerated. DC precursors capture antigen and mature in the liver, then migrate to the T cell area of the hepatic lymph nodes (LN). Here, they stimulate activation and expansion of naive CD4 T cells. Finally, activated CD4 T cells migrate back to the liver through the circulation and participate in the formation of granulomas (2,3,6). These observations indicate that granuloma formation is an ongoing immune response.

β-glucan is a component of the cell wall fraction of yeast and is a T cell-independent antigen. It has been used to induce hepatic granulomas upon i.v. injection. It was shown that Kupffer cells play a critical role in β-glucan-induced...
OPN affects the persistence of granuloma formation

granuloma formation by producing chemokines such as CCL2/MCP-1 which recruit inflammatory cells into the liver (7). Indeed, in CCL2-deficient mice, pulmonary granuloma formation in response to Schistosoma mansoni eggs was blunted (8) and in CCR2-deficient mice, which lack a prominent receptor for CCL2, hepatic granuloma formation induced by β-glucan was significantly decreased (9).

Osteopontin (OPN) is an extracellular matrix protein containing an Arg-Gly-Asp (RGD) sequence. The protein has diverse functions including mediating cell adhesion and migration by interacting with CD44 and integrins such as αvβ3 (10–14). OPN is also known as early T lymphocyte activation gene-1 (Eta-1) because its expression is found in T cells early in the course of activation (15). Recently, it was activation gene-1 (Eta-1) because its expression is found in T cells early in the course of activation (15). Recently, it was suggested that OPN was a key cytokine, contributing to the role of OPN in granuloma formation is indicated by the observed impairment of granuloma formation induced by polyvinylpyrrolidone in OPN-deficient (OPN−/−) mice (16), the cellular and molecular role of OPN during the course of liver injury and hepatic granuloma formation remains poorly defined. Therefore, we investigated the role of OPN in hepatic granulomatous disease using the β-glucan-induced hepatic granuloma model. We demonstrate in this study that OPN is significantly induced within the hepatic granulomas after β-glucan treatment and affects the persistence of hepatic granuloma formation through two distinct mechanisms. First, OPN up-regulates the production of IL-12 and IFN-γ within the granulomas at early stages. Second, at late stages, OPN has additional roles in the regulation of cellular recruitment and up-regulation of tumor necrosis factor (TNF-α) production, which results in the liver tissue damage.

Methods

Mice

Specific pathogen-free male C57BL/6 mice (7–8 weeks old) were obtained from SLC Japan. C57BL/6 × 129 OPN+/− mice previously generated (26) were backcrossed to C57BL/6 in our animal facility for 10 generations. OPN-transgenic (OPN-Tg) mice were generated as described previously (27) and were backcrossed to C57BL/6 for >15 generations. The genotype of all mice was confirmed by PCR analysis as described previously (26, 27). All animals were housed individually in a specific pathogen-free facility with unlimited access to water and laboratory chow. The experiment was approved by the Animal Care Committee of our institute and was conducted in accordance with the guidelines of the institutional animal care policy.

Induction of liver granulomas and assessment of liver damage

Mice were injected via the tail vein with 1 mg/mouse of yeast β-glucan (Zymosan A; Sigma, St Louis, MO) suspended in 200 μl PBS. At the indicated times, mice were killed and liver specimens were sampled. Granuloma formation was confirmed with hematoxylin & eosin staining of formalin-fixed 5-μm thick liver sections. The number and the size of granulomas in the section were analyzed by using NIH Image software. The degree of liver fibrosis was histologically evaluated. Briefly, liver sections were stained with Azan to visualize collagen fiber. The percentage of fibrosis area per section was analyzed using NIH Image software. Alternatively, hepatocellular damage was determined by serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Real-time quantitative PCR for OPN and chemokine gene expression in the liver

Total RNA was isolated from liver using TRIzol Reagent (Life Technologies) and reverse transcribed into cDNA by using ReverTra Ace (Toyobo, Japan). The expression of OPN, inducible nitric oxide synthase (iNOS), CCL4/MIP-1β, CCL2/MCP-1, CCL5/RANTES, CCL3/MIP-1α and CXCL9/monokine induced by IFN-γ (Mig) was determined by PCR using LightCycler–FastStart DNA Master SYBR Green 1 system (Roche Diagnostics). The sense primer for OPN was 5′-ACGACCATGATTGGCAGTG-3′ and the anti-sense primer was 5′-TTACCTCAGTCCATAAGCA-3′. The sense primer for iNOS was 5′-CTTCTCAGCCACCT-3′ and the anti-sense primer was 5′-CCTCACATACTGTGGACG-3′. The sense primer for CCL4 was 5′-CTCTCTCTCTCTTGTGCTG-3′ and the antisense primer was 5′-CTCAGTACGTGCGTTATC-3′. The sense primer for CCL2 was 5′-CCCACAAGAAGGAATGGGATC-3′ and the antisense primer was 5′-GGTTGTGGAAAGTAGTG-3′. The sense primer for CCL5 was 5′-CCGCCAAGTGTTGCCAACCC-3′ and the antisense primer was 5′-GGGTTACACTTGAGTGCCATCC-3′. The sense primer for CCL3 was 5′-TCCACCAGCTGCGCTG-3′ and the antisense primer was 5′-GTATGATATCCTTGACCC-3′. The sense primer for CXCL9 was 5′-TCTTTTGGGACATCATGTCC-3′ and the antisense primer was 5′-TGGAACGACGACCTTGG-3′. The sense primer for β-actin was 5′-TGGAATCTTGTGCTGACATCC-3′ and the anti-sense primer was 5′-TAAACAGCAGCAGTAAAC-3′.

OPN and cytokine measurement

Whole liver was homogenized in PBS. The homogenates were centrifuged, and the levels of OPN, IFN-γ, IL-12, TNF-α, IL-10 and IL-4 in supernatants were measured by ELISA. Hepatic LN cells (2.5 × 10⁶/well per 200 μl) were incubated in 96-well plates for 48 h with anti-mouse CD3 antibody (5 μg/ml, 145-2C11; PharMingen). The levels of IL-2 and IFN-γ in each culture supernatant were measured by ELISA (BD PharMingen for IFN-γ, TNF-α, IL-10, IL-4 and IL-2; IBL, Fujoka, Japan for OPN; and Biosource for IL-12). The detection limits of the assay for OPN, IFN-γ, IL-12, TNF-α, IL-10, IL-4 and IL-2 were 1 ng/ml, 31.3 pg/ml, 7.8 pg/ml, 15.6 pg/ml, 31.3 pg/ml, 7.3 pg/ml and 3.1 pg/ml respectively.

Immunohistochemistry

Wild-type C57BL/6 mice and OPN−/− mice, injected with β-glucan, were sacrificed at indicated days, and liver was obtained. Liver was embedded in Tissue-Tek OCT compound...
Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen and cut by a cryostat into 5-μm thick sections. The following anti-mouse antibodies were used: biotin-labeled MCP-1 (4E2/MCP; BD PharMingen), F4/80 (A3-1; Caltag), DEC-205 (NLDC-145; BMA Biomedicals), biotin-labeled CD4 (H129.19; BD PharMingen) and anti-mouse OPN Rabbit IgG (O-17; IBL Fujioka, Japan). After inhibition of endogenous peroxidase activity (and endogenous biotin activity for biotin-conjugated primary antibodies using Blocking Kit SP-2001; Vector), the sections were incubated with primary antibodies at 4°C overnight. They were treated with horseradish peroxidase-conjugated goat anti-rat IgG in the case of F4/80 and DEC-205, horseradish peroxidase-conjugated goat anti-rabbit IgG in the case of OPN (Jackson ImmunoResearch) or peroxidase-conjugated streptavidin; in the case of MCP-1 and CD4 (Nichirei). After visualization with 3,3′-diamino-benzidine (Nichirei), slides were counterstained with methyl green.

**Flow cytometric immunofluorescence analysis**

Freshly isolated hepatic LN cells (5 × 10^6 cells) on day 14 after β-glucan injection were incubated with the following anti-mouse mAb (all from BD PharMingen): phycoerythrin (PE)-labeled anti-mouse CD4 (L3T4), FITC-conjugated anti-mouse CD8 (Ly-2), biotin-conjugated anti-mouse CD11c (HL3) and PE-labeled I-Ab (AF6-120.1). Stained cells were analyzed by FACScan flow cytometry (Becton Dickinson, Mountain View, CA).

**Data analysis**

Statistical significance of difference was evaluated by two-way ANOVA or multiple comparison methods by Fisher. P values <0.05 were considered significant.

**Results**

**Augmentation of OPN expression in β-glucan-treated livers**

Since strong expression of OPN has been reported in a variety of granulomatous diseases such as sarcoidosis and tuberculosis in humans (20,22–24), we first investigated whether OPN was expressed in the liver after injection of β-glucan. In livers from untreated mice, a low level of OPN mRNA expression was detected. However, after β-glucan challenge, the level increased significantly, peaking at day 7 and then gradually
Fig. 2. Effect of OPN on β-glucan-induced hepatic granuloma formation. (A) Representative histology of the liver sections obtained from β-glucan-treated wild-type mice (a, c and e) and OPN−/− mice (b, d and f) at day 7 (a and b) and at day 14 (c–f). Hematoxylin & eosin staining with original magnification ×100 (a–d) or ×200 (e and f). (B) The kinetics of granuloma numbers in wild-type mice (open bars) and OPN−/− mice (closed bars) after β-glucan treatment. n = 6. (C) By using the same sections of the liver obtained at day 7 and 14, the diameters of granulomas and percentages of granuloma area per section were analyzed. Open bars and closed bars represent wild-type mice and OPN−/− mice respectively. n = 6. Data were expressed as mean ± SEM. *P < 0.05. **P < 0.005. NS, not significant.
To confirm that OPN is critically involved in the process of β-glucan-induced liver granuloma formation, OPN±/± mice were challenged with β-glucan. The number of granulomas in wild-type mice increased dramatically, peaking at day 14, then significantly decreased at day 21 and returned to almost normal levels at day 28. The kinetics of this granuloma formation is similar to OPN expression shown in Fig. 1. We found that the number of granulomas was significantly decreased in OPN−/− mice at day 14 (Fig. 2A, a and b and B). Furthermore, we analyzed the size and the area of granulomas per section. We found that the size of the granulomas in OPN−/− mice was significantly smaller than that of wild-type mice at day 14 (Fig. 2A, c and d and C). High magnification microphotography showed that there was no significant difference in histology between the two groups at day 14; granulomas consisted mainly of epithelial cells and mononuclear leukocytes in both groups (Fig. 2A, e and f). These results indicate that OPN is involved in the persistence of hepatic granuloma formation.

Absence of OPN dose not alter hepatic chemokine expression induced by β-glucan injection

It has been also demonstrated that chemokines and chemokine receptors play a critical role in the recruitment of inflammatory cells and the formation of granulomas (8,9,28,29). Thus, we investigated whether the reduction of granuloma persistence in OPN±/± mice was explained by down-regulation of chemokine expression in the liver. Although the mRNA expression of CCL4, CCL2, CCL5, CXCL9 and CCL3 in the liver was markedly induced after β-glucan challenge, there was no significant difference between wild-type mice and OPN±/− mice at all time points tested. Interestingly, we found that the mRNA expression level of CCL4 and CCL2 was increased, peaking at day 7, and was decreased at day 14; in contrast, the mRNA expression levels of CCL5, CXCL9 and CCL3 remained elevated at day 14 (Fig. 3A). Next we checked protein expression of a key chemokine, CCL2, in the liver at day 14 by immunohistochemistry. Unlike OPN expression, which was expressed within the granulomas (Fig. 1C), CCL2 protein was strongly expressed in hepatocytes in both groups (Fig. 3B). Our data suggest that the reduction of the granuloma persistence in OPN±/− mice is not explained by the reduction of chemokine production.

The recruitment of inflammatory cells into the liver is decreased in OPN±/− mice

It has been demonstrated that granuloma formation involves not only macrophages and lymphocytes, but also mature DC (2–5). In addition, OPN has been shown to be a chemotactic factor for T cells and macrophages (10,20,30). Recently, it was demonstrated that OPN was involved in the migration of DC to the draining lymph nodes from the skin (31). Therefore, we hypothesized that the reduction of the granuloma persistence in OPN±/− mice was related to the reduction of the cellular recruitment. Thus, we examined the cellular components of the granulomas at day 14 by immunohistochemistry. In wild-type mice, macrophages, defined by F4/80 expression (Fig. 4A, a), were distributed throughout the granulomas and in hepatic sinusoids, and CD4+ T cells were also found in the granulomas and sinusoids (Fig. 4A, b). In contrast, DEC-205+ DC were only found within the granulomas (Fig. 4A, c). In OPN±/− mice, the distribution pattern of macrophages, CD4 T cells and DC was very similar to that detected in wild-type mice (Fig. 4A, d–f). However, the number of those cells in the liver was significantly decreased in OPN±/− mice, accompanying the reduction of granuloma size and at day 14 (Fig. 4A and B). These data indicate that OPN, which is expressed within the granulomas after β-glucan treatment, can induce the migration of not only macrophages and CD4 T cells, but also DC into the granulomatous sites and the reduction of the granuloma persistence in OPN±/− mice is due to the reduction of cellular recruitment into the liver at day 14.

Absence of OPN dose not alter regional LN reactions

We investigated whether the immune response is induced in hepatic LN in this hepatic granuloma model induced by β-glucan. We found that hepatic LN were significantly enlarged, and the number of DC, CD4 T cells and CD8 T cells in hepatic LN at day 14 increased ~5- to 7-fold as compared to untreated hepatic LN in wild-type mice (Fig. 5A). These data indicate that an immune response is induced in the hepatic LN in the β-glucan-induced hepatic granuloma model. Next, we investigated whether the reduction of the granuloma persistence in OPN±/− mice was due to an alteration of the immune response in the hepatic LN. However, there was no significant difference in total cell number of hepatic LN between wild-type mice and OPN±/− mice at day 14 (Fig. 5A: wild-type mice 28.1 × 105, OPN±/− mice 28.9 × 105). The number of CD4 T cells, CD8 T cells and DC as defined by CD11c and class II MHC double-positive cells in the hepatic LN was not different between the two groups (Fig. 5A). We further analyzed IL-2 and IFN-γ production by hepatic LN cells at day 14. Hepatic LN cells were stimulated with anti-CD3 for 48 h in vitro, and were tested for IL-2 and IFN-γ production. Neither IL-2 nor IFN-γ levels differed between wild-type mice and OPN±/− mice (Fig. 5B). These data suggest that the reduction of the granuloma persistence in OPN±/− mice is not due to the alteration of the immune response in the regional hepatic LN.

OPN affects the cytokine production in the liver and subsequent liver injury

As it was demonstrated that OPN was a key cytokine contributing to the development of a Th1-type immune
response (16,17,19), we next analyzed cytokine production in the liver in OPN±/± mice after β-glucan treatment. After β-glucan challenge, both wild-type mice and OPN±/± mice showed increased production of IFN-γ in the liver as compared to untreated mice. However, OPN±/± mice showed lower levels of IFN-γ at day 7 as compared to wild-type mice (Fig. 6A). We also found that IL-12 production in the liver at day 7 was reduced in OPN±/± mice (Fig. 6A). The production of IL-4 and IL-10 in the liver was also augmented after β-glucan challenge in both wild-type mice and OPN±/± mice; however, there was no significant difference between the two groups (Fig. 6B). TNF-α has been shown to be involved in the process of hepatocellular damage (28,32). Therefore, the production of TNF-α in the liver was analyzed. After β-glucan challenge, the level of TNF-α in the liver was significantly increased in mice of both genotypes at day 7, but there was no difference between the two groups. At day 14, on the other hand, while TNF-α levels continued to rise in wild-type mice, OPN±/± mice showed a significant decrease in the levels of this cytokine (Fig. 6C, P = 0.0256). Importantly, this difference in the levels of liver TNF-α was reflected in alterations in the serum ALT (Fig. 6C, P = 0.0017) and AST levels (Fig. 6C, P = 0.0122) in OPN±/± mice.

**Fig. 3.** Effect of OPN on chemokine mRNA expression in the liver after β-glucan treatment. (A) The expression of CCL4, CCL2, CCL5, CXCL9 and CCL3 mRNA in wild-type mice liver (open bars) and OPN±/± mice liver (closed bars) after β-glucan treatment (untreated, day 7 and 14) were examined. Real-time quantitative PCR for CCL4, CCL2, CCL5, CXCL9 and CCL3 was performed as described above. These patterns are representative of the results obtained from three independent experiments. n = 6. Results are expressed as mean ± SEM. (B) Immunostaining of CCL2 in wild-type mice liver (a) and OPN±/± mice liver (b) at day 14. Original magnification ×100.
The overexpression of exogenous OPN in lymphoid tissues induces persistence of granuloma formation and subsequent fibrotic change in the liver.

To confirm that OPN is a critical molecule in the granuloma formation, we used OPN-Tg mice in which exogenous OPN is overexpressed in lymphoid tissues under the control of the Eμ promoter (27). After β-glucan challenge, the number of granulomas at day 7 was not different between wild-type mice and OPN-Tg mice (Fig. 7A). We found that the number of granulomas was significantly increased in OPN-Tg mice at day 14, but not in wild-type mice (Fig. 7A: OPN-Tg mice 2166 ± 94/cm², wild-type mice 1540 ± 88/cm², mean ± SEM, P = 0.0004). In addition, the number of granulomas returned to almost basal levels in wild-type mice at day 21, but we could still detect significant number of granulomas in OPN-Tg mice at day 21 (Fig. 7A: OPN-Tg mice 503 ± 102/cm², wild-type mice 127 ± 16/cm², mean ± SEM, P = 0.0492). Next we measured iNOS mRNA expression as an indicator of inflammatory responses. The mRNA expression of iNOS in wild-type mice peaked at day 7, then gradually declined and returned to the basal levels at day 21. In OPN-Tg mice, in contrast, high-level expression of iNOS was maintained through day.

Fig. 4. OPN regulates the recruitment of macrophages, CD4 T cells and DC into the liver. (A) Immunostaining of granulomatous cells in wild-type mice (a–c) and OPN−/− mice (d–f) at day 14 after β-glucan treatment. (a and d) Immunostaining for F4/80 (brown), (b and e) immunostaining for CD4 (brown) and (c and f) immunostaining for DEC-205 (brown). Original magnification x100. (B) Positive area of F4/80, DEC-205 and CD4 cells. The photo images of the immunostained liver sections were analyzed by using NIH Image software. n = 3. Results are expressed as mean ± SEM. *P < 0.05.
This persistence of hepatic granulomas in OPN-Tg mice was accompanied by elevated levels of serum ALT and AST (Fig. 7C). Interestingly, we found that the subsequent fibrotic changes in the liver were more severe in OPN-Tg mice at day 28 (Fig. 7D). These data indicate that OPN may be a key factor for controlling the persistence of granuloma formation and inflammatory responses, and thus the severity of liver tissue injury.

**Discussion**

OPN has been clearly characterized as a chemotactic factor for smooth muscle cells, endothelial cells, fibroblasts, lymphocytes and macrophages by interacting with integrin and CD44 (10–14,33,34). OPN is expressed in various types of cells including activated T cells and macrophages (15,22). It was recently shown that OPN protein was strongly expressed in the pathological foci of patients with various granulomatous diseases including tuberculosis and sarcoidosis (20,22,24).

The role of OPN in granulomatous diseases is thought to involve regulation of the migration of inflammatory cells from the circulation into the inflammatory sites (20,30). In fact, pulmonary granuloma formation induced by embolization of *Schistosoma mansoni* eggs was delayed and the granulomas formed contained fewer macrophages in OPN±/± mice in comparison to wild-type mice (25). Furthermore, it was reported that OPN±/± mice were defective in granuloma formation in skin following s.c. injection of polyvinyl pyrrolidone (16). More importantly, the degree of OPN protein expression in granulomas of tuberculosis correlated with the prognosis of patients (24). These data suggest that OPN is involved not only in the pathological process of granuloma formation, but also in the immunological reaction against pathogens. Thus, it is very important to define the cellular and molecular role of OPN during the course of granuloma formation.

Here, using the hepatic granuloma model induced by β-glucan, we found that the expression of OPN protein in the liver was significantly induced from day 7 to 21 after injection in wild-type mice. Furthermore, immunohistochemical analysis demonstrated that OPN protein was present at high levels within the granulomas. These results suggest that OPN is involved in this hepatic granuloma formation induced by β-glucan.

The first important finding is that the absence of OPN does not affect the number and size of hepatic granulomas induced by β-glucan at early stages (at day 7). It should be noted that overexpression of OPN in OPN-Tg mice does not affect the number of granulomas at day 7. On the other hand, we found that the persistence of granuloma formation was reduced in OPN±/± mice; the number and the size of granulomas were significantly decreased in OPN±/± mice at late stages (at day 14). Again, in sharp contrast, the number of granulomas was increased in OPN-Tg mice at late stages. Thus, OPN is involved in the persistence of granuloma formation. It has been shown that chemokines are critically involved in the process of granuloma formation (8,9,28,29). We found that the mRNA expression of CCL3, CCL4, CCL2, CCL5 and CXCL9 was significantly induced after β-glucan treatment both in wild-type and OPN±/± mice, but there was no difference between the two groups. Furthermore, we found that one of a key chemokine, CCL2 protein, was expressed in hepatocytes at day 14 in both wild-type and OPN±/± mice. Thus, the reduction of the persistence of the granuloma formation in OPN±/± mice was not explained by the reduction of chemokine expression.

In addition, we found that OPN deficiency resulted in a reduced accumulation of macrophages, CD4 T cells and DC in the liver at day 14, but not at day 7. Recent investigations showed that OPN was involved in the migration of Langerhans...
cells/DC from the skin to the draining LN (31). We demonstrated in this study that OPN can induce the migration of not only macrophages and CD4 T cells, but also DC to the granulomatous sites. Thus, the reduction of cellular recruitment into the liver in OPN−/− mice at day 14 accounts for the reduction of the persistence of granuloma formation at day 14. However, the critical question that should be raised here is why OPN deficiency results only in the reduced recruitment of inflammatory cells at day 14, but not at day 7. It is likely that chemokines other than OPN are responsible for the initial stage of hepatic granuloma formation. In this regard, we demonstrated that the mRNA expression of chemokines is significantly induced in the liver at day 7 after β-glucan treatment.

The second important finding in this paper is that the production of the Th1-type cytokine, IFN-γ, is significantly decreased in the liver in OPN−/− mice at day 7. Furthermore, IL-12 production in the liver was significantly reduced in OPN−/− mice at day 7, suggesting that OPN modulates the cytokine expression within the granulomas toward higher amounts of IFN-γ production by up-regulating the IL-12 expression from macrophages. Importantly, the production of Th2-type cyto-

Fig. 6. Effect of OPN on cytokine production in the liver and tissue damage. (A) IFN-γ levels in the liver homogenates in wild-type mice (open bars) and OPN−/− mice (closed bars) after β-glucan treatment (untreated, day 7 and 14) were measured by ELISA. IL-12 levels in the liver homogenates in wild-type mice and OPN−/− mice at day 14 were measured by ELISA. (B) IL-4 and IL-10 levels in the liver homogenates in wild-type mice (open bars) and OPN−/− mice (closed bars) after β-glucan treatment (untreated, day 7 and 14) were measured by ELISA. (C) TNF-α levels in the liver homogenates in wild-type mice (open bars) and OPN−/− mice (closed bars) after β-glucan treatment (untreated, day 7 and 14) were measured by ELISA. Representative data from three independent experiments. n = 6. Serum ALT and AST levels in wild-type mice (open square) and OPN−/− mice (closed square) after β-glucan treatment (untreated, day 7, 14, 21 and 28). n = 6. Data were expressed as mean ± SEM. *P < 0.05. **P < 0.005.
kines, IL-4 and IL-10, was not different between the two groups. Therefore, OPN may not be a regulator of classical T<sub>1</sub>/T<sub>2</sub> paradigm (16,17,21) in this model. It has been reported that IFN-γ mediates the pathogenesis of liver injury by regulating macrophage infiltration and activation. IFN-γ also stimulates TNF-α production from macrophages (35-37). In accordance with those reports, we found that TNF-α production in the liver was decreased in OPN<sup>−/−</sup> mice at day

Fig. 7. Augmentation of granuloma formation in OPN-Tg mice. (A) Granuloma numbers in wild-type mice (open bars) and OPN-Tg mice (dotted bars) after β-glucan treatment. n = 6. (B) The expression of iNOS mRNA in wild-type mice liver (open bars) and OPN<sup>−/−</sup> mice liver (dotted bars) after β-glucan treatment. These patterns are representative of the results obtained from three independent experiments. n = 3. (C) Serum ALT and AST levels in wild-type mice liver (open square) and OPN-Tg mice liver (closed square) after β-glucan treatment. n = 6. (D) Sections of wild-type mice liver (a) and OPN-Tg mice liver (b) at day 28 after β-glucan treatment were stained with Azan to visualize collagen fiber (blue). Original magnification ×100. Fibrosis area per section in wild-type mice and OPN-Tg mice at day 28 after β-glucan treatment. Wild-type mice n = 9, OPN-Tg mice n = 11. Results are expressed as mean ± SEM. *P < 0.05. **P < 0.005. NS, not significant.
this hepatic granuloma model, OPN within granulomas induced by β-glucan treatment regulates the persistence of hepatic granuloma formation and liver tissue injury through two distinct mechanisms. First, at early stages of hepatic granuloma formation, OPN up-regulates the production of IL-12 and IFN-γ within the granulomas. Second, at late stages of hepatic granuloma formation, OPN has an additional role in the regulation of cellular recruitment into the liver or into the granulomas and up-regulation of TNF-α production that determines the severity of liver tissue injury.

**Abbreviations**

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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<td>AST</td>
<td>Aspartate aminotransferase</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>LN</td>
<td>Lymph node</td>
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<td>OPN</td>
<td>Osteopontin</td>
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<td>PE</td>
<td>Phycocyanin</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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**References**

OPN affects the persistence of granuloma formation


