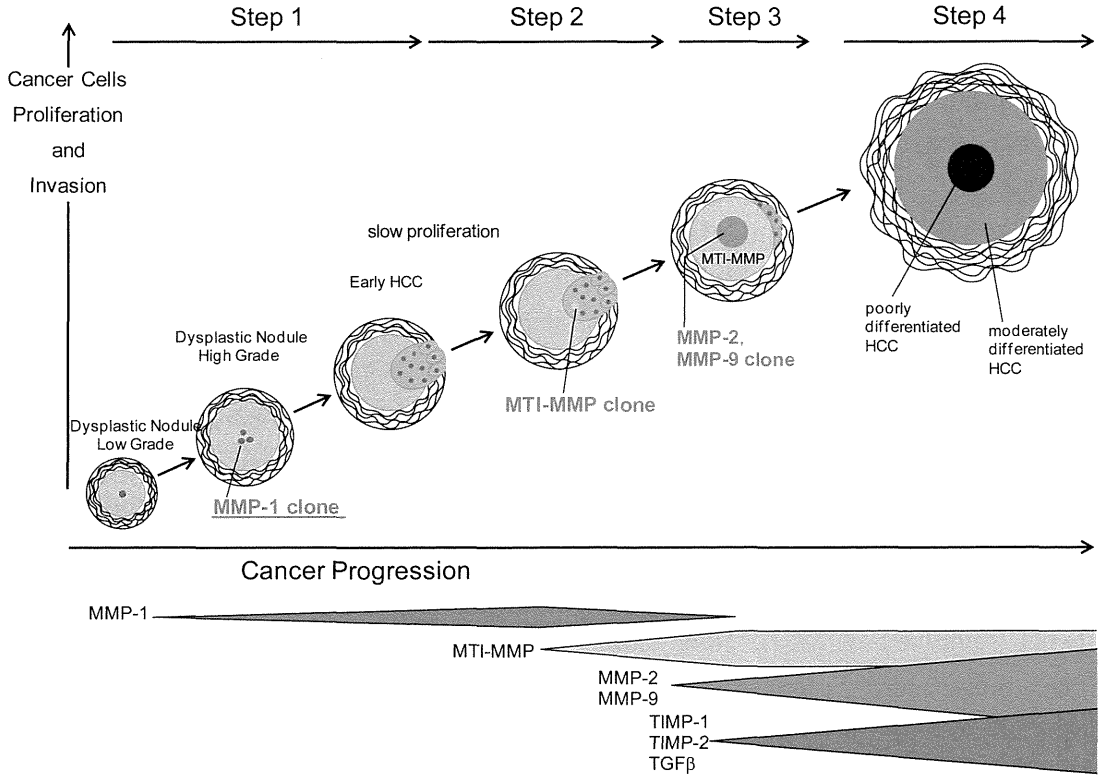
only well-differentiated HCC expressed MMP-1, two cases with both well-differentiated and moderately differentiated HCC showed positive staining, and one case with only moderately differentiated cells showed negative staining. No case of advanced HCC showed MMP-1 mRNA. The positive cells were well-differentiated cancer cells located at the invading front of the cancer. An interesting finding was that positive cells were scattered in a ratio of approximately 1% to 2% in cancer. This was very different from the findings of MMP-9 expression reported in other studies described above. Hepatocytes of non-cancerous liver did not express transcripts of MMP-1. Positive staining of MMP-1 protein was seen in early HCC with well-differentiated HCC cells and invaded portal tract. In another case with early HCC, well-differentiated HCC cells positive for MMP-1 protein were compressed by moderately differentiated cancer cells which were negative for MMP-1 staining. MMP-1 mRNA was seen in HCC cells infiltrated into the small portal tract left in tumor nodule as well as in the fibrous bands surrounding the cancer cell nodule. MMP-1 expression was associated with growth of small HCC in which well-differentiated cancer cells invade the portal tract and fibrous bands, and these fibrous tissues disappear upon participation of MMP-1 [32].

Nakatsukasa *et al.* [156] reported that TIMP-1 mRNA and TIMP-2 mRNA in cancerous tissue were homogeneously stained more intensively than in non-tumorous tissue of HCC by *in situ* hybridization. All HCC tissues contained transcripts of both TIMP-1 and TIMP-2 with stronger expression in HCC cells than in the surrounding tissue. The expression and distribution of the transcripts for TIMP-1 and TIMP-2 did not differ among the various cancer differentiation stages. The intensity of TIMPs mRNA expression varied from nodule to nodule. Stromal cells in and surrounding the HCC expressed both TIMP-1 and TIMP-2 mRNA, and their expression in stromal cells present in the capsule was especially strong. On the other hand, Musso *et al.* [145] did not observe either mRNA of MMP-2 or TIMP-2 in HCC cells, but observed them in αSMA-positive cells at the invasive front. The MMP-2(+)/TIMP-2(+)/αSMA(+) cells in the perisinusoidal space adjacent to liver tumors are considered to be HSC. This discrepancy is probably due to the histological differences in HCC used in the study above. TIMPs act to modulate the matrix/tumor interaction [138–140,142]. Furthermore, TIMPs may play an important role in cell growth, and pro-MMP-2 may be activated by MT1-MMP and TIMP-2 on the cell surface of HCC cells, stromal cells and stellate cells resulting in stromal invasion. TIMP-1 and TIMP-2 expression by stromal cells was associated with a poorer prognosis of HCC as revealed by an immunohistochemical study using tissue microarrays [153,154].

The above observations on MMP-1, -2, -9, MT-1 MMP and TIMP-2 lead to the following hypothesis, as shown in Figure 2 [35]. The conversion from low-grade dysplastic nodule to high-grade dysplastic nodule within liver cirrhosis results in a phenotype that expresses MMP-1, resulting in the formation of a new clone. Well-differentiated HCC cells proliferate slowly with the ability of stromal invasion ([32], reviewed in [35]). New clones can proliferate and invade portal tracts destroying fibrous tissue. Subsequently other clones arise to degrade effectively not only fibrous tissue but also basement membrane. These next generation clones express MT1-MMP in the process of cancer development. Several well-differentiated HCC cells express MT1-MMP and gradually small amounts of MMP-2 and MMP-9, probably stimulated by inflammatory cytokines or TGF-β, which may participate in the stromal invasion or the formation of the thick capsule of cancer nodules. Pro-MMP-9 is activated by MMP-2, or well- to moderately differentiated HCC cells to obtain a phenotype expressing MMP-9. TIMP-1 and TIMP-2 gene transcripts in HCC cells appeared with increased

expression of MMP-2 and/or MMP-9. MMP-1-positive clones (well-differentiated cancer cells) are compressed by new clones (moderately differentiated cancer cells) and subsequently disappear.

Figure 2. MMPs and TIMPs expression related with HCC progression based on the reported results in HCV-derived HCC [35]. The terminology of nodular hepatocellular lesions in this figure is modified by the report of the International Working Party [157]. Step 1: A conversion from low-grade dysplastic nodule to high-grade dysplastic nodule within liver cirrhosis obtains a phenotype to express MMP-1, resulting in the formation of a new clone. Early HCC cells proliferate slowly with the ability of stromal invasion. Step 2: New clones can proliferate and invade portal tracts and fibrous tissue. Subsequently other clones arise to degrade effectively not only fibrous tissue but also basement membrane. These clones of the next generation express MT1-MMP in the process of cancer development. Step 3: Several early HCC cells express MT1-MMP and gradually small amounts of MMP-2 and MMP-9, probably stimulated by inflammatory cytokines or TGF-β, which may participate in the stromal invasion or the formation of the thick capsule of cancer nodules. Pro-MMP-9 is activated by MMP-2, or early HCC to moderately differentiated HCC cells obtain a phenotype expressing MMP-9. TIMP-1 and TIMP-2 gene transcripts in HCC cells increased with increased expression of MMP-2 and/or MMP-9. Step 4: MMP-1 positive clones (early HCC cells) are compressed by new clones (moderately differentiated hepatoma cells) and subsequently disappear [35].



There is no direct evidence for this hypothesis. However, we have previously reported that stem cells derived from bone marrow changed their phenotypes of MMP-13 (a major interstitial collagenase in rodents with homology to human MMP-1) to MMP-9 expression in the recovery phase from experimental liver fibrosis and cirrhosis [24]. A similar switch in MMP expression may occur in the dedifferentiation process of cancer stem cells (Figure 2). On the other hand, positive staining for both MMP-2 and MMP-9 proteins was observed in inflammatory cells, fibroblasts and endothelial cells. MMP-9 mRNA was detected in mesenchymal cells inside and outside cancer nodules, and in fibrous capsules around the necrosis of cancer nodules [128]. Mesenchymal cells and inflammatory cells positive for MMP-2 and MMP-9 were numerous and stained strongly for both mRNA and protein compared with cells positive for MMP-1 [32,145,147]. These positive cells may participate in the degradation of the fibrous tissue to allow HCC cells to invade easily or to form the thick capsule around the nodule.

Cancer-associated mesenchymal stem cells (MSC) could cross-talk with tumor cells and secreted many growth factors and chemokines, which play crucial roles in tumor progression [158,159]. A recent report by Yan *et al.* [160] clearly demonstrated the presence of liver cancer-associated MSCs (LC-MSC) which promote the proliferation and metastasis of HCC cells. They indicated that S100A4 secreted from LC-MSCs can promote HCC cell proliferation and invasion, and the invasion-promoting effect of S100A4 was attenuated by a miR-155 inhibitor. MiR-155 induced by S100A4 increased MMP-9 expression; S100A4 facilitated the process by up-regulating MMP-9. S100A4 and miR-155 expression and down-regulating SOCS1 gene expression, which regulates p-STAT3, one of the mediators involved in regulating MMP-9 expression, stimulate HCC invasion. Moreover, Roderfeld *et al.*, demonstrared that MMP-9 expressing macrophages invade at the tumor front of HCC [161].

Expression of MMPs is controlled mainly at the transcriptional level. The promoter regions of MMPs contain several common elements where the common transcription factors such as AP-1, Ets, and/or NF-κB bind (reviewed in [35]). The present authors showed that the c-Jun NH2-terminal kinase (JNK) pathway is involved in constitutive MMP-1 expression in a well-differentiated cell line (HLE cells) among five HCC cell lines derived from various dedifferentiation stages. C-Jun is phosphorylated by JNK, one of the four distinctly regulated MAPK pathways; the other three pathways are extracellular signal-related kinases (ERK)-1/2, p38 proteins and ERK5. HLE cells constitutively expressed MMP-1 gene and protein as well as its enzymatic activity without the use of any stimulators such as phorbol ester. MMP-1 gene transcription was controlled by the activation of c-Jun through the JNK pathway [33].

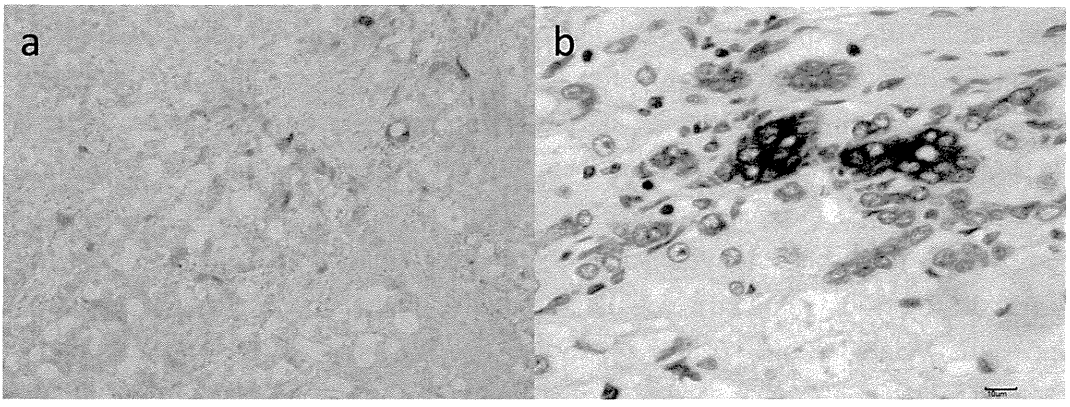
PTEN inhibits the migration and invasion of HCC by the down-regulation of MMP-2 and MMP-9 in a PI3K/Akt/MMP-dependent manner [162]. Lysophosphatidic acid (LPA) produced extracellularly by autotaxin (ATX), increases cell survival, angiogenesis, invasion and metastasis [35]. Park *et al.* [163] revealed that silencing or pharmacological inhibition of LPA1 inhibited LPA-induced MMP-9 expression and HCC cell invasion because ATX transcripts and LPA receptor type 1 (LPA1) protein levels were higher in HCC than in normal tissue. They also found that MMP-9 is downstream of LPA1. Moreover, LPA-induced MMP-9 expression with subsequent invasion was abrogated by inhibition of phosphoinositol-3 kinase (PI3K) signaling or dominant or negative mutants of protein kinase C and p38 mitogen-activated protein kinase (MAPK) [163]. Increased MMP-2, -9 and VEGF caused by

hypoxia (via ERK1/2) is suppressed with Na^+/H^+ exchanger 1 (NHE1) inhibited by 5-(*N*-ethyl-*N*-isopropyl) amiloride [164].

7.2. MMPs and TIMPs in NASH-related Carcinogenesis

There are no reports on MMPs and TIMPs in NASH-derived HCC. Figure 3a is a MMP-9 staining microscopic finding of surgically resected NASH-derived HCC. Strong staining is seen in some of the HCC cells diffused in the nodule. MMP-1 and MMP-2 staining are also positive with different distribution (not shown). The tumor size in this case is less than 2 cm in diameter, and the pathological features of HCC cells are well differentiated. According to the hypothesis shown in Figure 2, there is negative staining for both MMP-9 and MMP-2 in small cancer. This discrepancy may be based in the etiology of HCC, because the hypothesis of Figure 2 is based on HBV- or HCV-related HCC while Figure 3a is NASH-related HCC. The involvement of HPCs in NASH appears to be very different from viral chronic liver diseases as discussed above. The present authors are currently investigating the involvement of MMP-positive cells in the dedifferentiation process of early HCC cells in NASH-derived HCC.

Figure 3. Immunostaining of NASH-derived HCC: (**a**) NASH-derived HCC cells with cytoplasmic reactivity to the MMP-9 antibody are scattered sparsely throughout the nodule (100× magnification); (**b**) NASH-derived HCC cells with cytoplasmic reactivity to the CK19 antibody are also seen (400× magnification).



MMPs can be influenced by reactive oxygen species (ROS), resulting in the activation of neutrophils and macrophages at the tumor site with inflammation. These oxidants initially activate MMPs via oxidation of the pro-domain cysteine or via modification of amino acids of the catalytic domain by hydrochlorous acid in combination with myeloperoxidase (reviewed in [165]) The methylation status of cytokines in CpG dinucleotides located in the MMP promoter also plays a role in controlling gene expression of MMPs, especially in cancer cells. Recent studies on DNA methylase inhibitors, such as 5-aza-2'deoxycytidine, showed a possible induction of hypomethylation of MMPs genes at the promoter level in human HCC cells [166].

Stefanou *et al.* [167] reported that leptin up-regulated telomerase activity in HepG2 cells through the binding of STAT3 and Myc/Max/Mad network proteins to the promoter of human telomerase reversed transcriptase (hTERT). Leptin could affect the progression and invasion of HCC through its

interaction with cytokines and MMPs in the tumorigenic microenvironment. Leptin increased the levels of MMP-13 and MMP-9 in a dose- and time-dependent manner. Insulin receptor-mediated signaling promotes MMP-2 and MMP-9 expression [168]. On the other hand *Kiss*-1 gene, a putative metastasis suppressor gene, is also reported in HCC related with decreased expression of MMP-9 [169].

HPCs derived from bile ducts and the canals in the liver or bone marrow seem to play an important role in the dedifferentiation process from injured hepatocytes ([32], reviewed in [35]). Figure 3b shows the cytoplasmic reactivity to the CK19 antibody in NASH-derived HCC cancer cells. As noted above, one of characteristic pathological findings in NASH is the infiltration of HPCs. Therefore, expression of MMP-1, MMP-2 and MMP-9 seems to be expected in NASH-derived HCC, and the expression is probably different from that found in viral-derived HCC. The regulatory mechanism of TIMPs in NASH-derived HCC also remains unknown [119,170]. Further regulatory research on MMPs and TIMPs should be conducted to prevent the progress of NASH to HCC.

8. Conclusions: Future Management of NASH Focusing on MMPs and TIMPs

Distinguishing between benign NAFLD patients and advanced NASH patients is very difficult. To counter this problem the author's research group has a short-admission program for NASH patients that includes liver biopsy, metabolic examination for glucose tolerance and diet education. We follow up the courses of NASH patients focusing on the complications of HCC. Patients should undergo abdominal echography or CT/MRI and serum markers. Liver tissue obtained by needle biopsy should be checked for stem/progenitor marker-positive cells. Our group is now investigating whether MMP-1 staining or MMP-9 staining is a predictable indicator of HCC. Finally, further investigation of signal transduction pathways in MMPs and TIMPs is essential because future prevention and treatment of NASH-related HCC will be based on gene regulation of MMPs and TIMPs.

Acknowledgments

The authors thank Yoshie Muroya for her systematic search of the literature and Cecilia Hamagami for her assistance in the English revision of the manuscript.

Author Contributions

IO made a scheme of the manuscript and wrote the role of MMPs and TIMPs in fibrogenesis and carcinogenesis of NASH; TN, NT and EY wrote the mechanism of carcinogenesis in NASH from the viewpoint of surgical treatment; HK described the pathological findings of NASH cases including the immunohistochemical findings of MMPs; MN reviewed the description of HCC according to the report of the International Working Party; HY wrote the characteristics of HPC in NASH; YI reviewed the mechanism of fibrogenesis and carcinogenesis in NASH from the viewpoint of regenerating medicine.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Fierbinteanu-Braticevicp, C.; Negreanu, L.; Tarantino, G. Is fatty liver always benign and should not consequently be treated? *J. Physiol. Pharmacol.* **2013**, *64*, 3–9.
2. Puri, P.; Sanyal, A.J. Nonalcoholic fatty liver disease. In *Zakim and Boye's Hepatology*, 6th ed.; Boyer, T.D., Manns, M.P., Sanyal, A.J., Eds.; Saunders: Philadelphia, PA, USA, 2006; pp. 941–968.
3. Pinzani, M. Pathophysiology of non-alcoholic steatohepatitis and basis for treatment. *Dig. Dis. Sci.* **2011**, *29*, 243–248.
4. Starley, B.Q.; Calcagno, C.J.; Harrison, S.A. Nonalcoholic fatty liver disease and hepatocellular carcinoma: A weighty connection. *Hepatology* **2010**, *51*, 1820–1832.
5. Bhala, N.; Jouness, R.I.K.; Bugianenesi, E. Epidemiology and natural history of patients with NAFLD. *Curr. Pharm. Des.* **2013**, *19*, 5169–5176.
6. Matteoni, C.H.; Younossi, Z.M.; Gramlich, T.; Boparai, N.; Liu, Y.C.; McCullough, A.J. Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology* **1999**, *116*, 1413–1419.
7. Ludwig, J.; Viggiano, T.R.; McGill, D.B.; Ott, B.J. Nonalcoholic steatohepatitis. Mayo Clinic experience with a hitherto unnamed disease. *Mayo Clin. Proc.* **1980**, *55*, 434–438.
8. Hashimoto, E.; Tokushige, K. Prevalence, gender, ethnic variations, and prognosis of NASH. *J. Gastroenterol.* **2009**, *44*, 89–95.
9. Van der Poorten, D.; Samer, C.F.; Ramezani-Moghadam, M.; Coulter, S.; Kacevska, M.; Schrijnders, D.; Wu, L.E.; McLeod, D.; Bugianesi, E.; Komuta, M.; *et al.* Hepatic fat loss in advanced nonalcoholic steatohepatitis: Are alterations in serum adiponectin the cause? *Hepatology* **2013**, *57*, 2180–2188.
10. Kleiner, D.; Brunt, E.M.; Natta, M.V.; Behling, C.; Contos, M.J.; Cummings, O.W.; Ferrell, L.D.; Liu, Y.-C.; Torbenson, M.S.; Unalp-Arida, A.; *et al.* For the nonalcoholic steatohepatitis clinical research network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **2005**, *41*, 1313–1321.
11. Ertle, J.; Dechene, A.; Sowa, J.-A.; Penndorf, V.; Herzer, K.; Schlaak, J.F.; Gerken, G.; Syn, W.-K.; Canbay, A. Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. *Int. J. Cancer* **2011**, *128*, 2436–2443.
12. Parkin, D.M.; Bray, F.; Ferlay, J.; Pisani, P. Global cancer statistics, 2002. *CA Cancer J. Clin.* **2005**, *55*, 74–108.
13. Venook, A.P.; Papandreou, C.; Furuse, J.; de Guevara, L.L. The incidence and epidemiology of hepatocellular carcinoma: A global and regional perspective. *Oncologist* **2010**, *15*, 5–13.
14. Okazaki, I.; Maruyama, K. Collagenase activity in experimental hepatic fibrosis. *Nature* **1974**, *252*, 49–50.
15. Okazaki, I.; Brinckerhoff, C.E.; Sinclair, J.R.; Sinclair, P.R.; Bonkovsky, H.L.; Harris, E.D., Jr. Iron increases collagenase production by rabbit synovial fibroblasts. *J. Lab. Clin. Med.* **1981**, *97*, 396–402.
16. Maruyama, K.; Feinman, L.; Okazaki, I.; Lieber, C.S. Direct measurement of neutral collagenase activity in homogenates from baboon and human liver. *Biochim. Biophys. Acta* **1981**, *658*, 124–131.

17. Maruyama, K.; Feinman, L.; Fainsilber, Z.; Nakano, M.; Okazaki, I.; Lieber, C.S. Mammalian collagenase increases in early alcoholic liver disease and decreases with cirrhosis. *Life Sci.* **1982**, *30*, 1379–1384.
18. Maruyama, K.; Okazaki, I.; Kobayashi, T.; Suzuki, H.; Kashiwazaki, K.; Tsuchiya, M. Collagenase production by rabbit liver cells in monolayer culture. *J. Lab. Clin. Med.* **1983**, *102*, 543–550.
19. Arai, M.; Niioka, M.; Maruyama, K.; Wada, N.; Fujimoto, N.; Nomiyama, T.; Tanaka, S.; Okazaki, I. Changes in serum levels of metalloproteinases and their inhibitors by treatment of chronic hepatitis C with interferon. *Dig. Dis. Sci.* **1996**, *41*, 995–1000.
20. Watanabe, T.; Niioka, M.; Hozawa, S.; Kameyama, K.; Hayashi, T.; Arai, M.; Ishikawa, A.; Okazaki, I. Gene expression of interstitial collagenase in both progressive and recovery phase of rat liver fibrosis induced by carbon tetrachloride. *J. Hepatol.* **2000**, *33*, 224–235.
21. Watanabe, T.; Niioka, M.; Ishikawa, A.; Hozawa, S.; Arai, M.; Maruyama, K.; Okada, A.; Okazaki, I. Dynamic change of cells expressing MMP-2 mRNA andMT1-MMP mRNA in the recovery from liver fibrosis in the rat. *J. Hepatol.* **2001**, *35*, 465–473.
22. Inagaki, Y.; Nemoto, T.; Kushida, M.; Sheng, Y.; Higashi, K.; Ikeda, K.; Kawada, N.; Shirasaki, F.; Takehara, K.; Sugiyama, K.; *et al.* Interferon alpha down-regulates collagen gene transcription and suppresses experimental hepatic fibrosis. *Hepatology* **2003**, *38*, 890–899.
23. Inagaki, Y.; Kushida, M.; Higashi, K.; Itoh, J.; Kawada, N.; Namikawa, K.; Kiyama, H.; Bou-Gharious, G.; Higashiyama, R.; Watanabe, T.; *et al.* Cell type-specific intervention of transforming growth factor beta/Smad signaling suppresses collagen gene expression and hepatic fibrosis in mice. *Gastroenterology* **2005**, *129*, 259–268.
24. Higashiyama, R.; Inagaki, Y.; Hong, Y.Y.; Kushida, M.; Nakao, S.; Niioka, M.; Watanabe, T.; Matsuzaki, Y.; Okano, H.; Shiota, G.; *et al.* Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. *Hepatology* **2007**, *45*, 213–222.
25. Inagaki, Y.; Kushida, M.; Shiota, G.; Kuwabara, I.; Itoh, J.; Moro, T.; Hong, Y.Y.; Nakao, S.; Higashiyama, R.; Okazaki, I.; *et al.* Hepatocyte growth factor suppresses profibrogenic signal transduction via nuclear export of Smad3 with galectin-7. *Gastroenterology* **2008**, *134*, 1180–1190.
26. Moro, T.; Shimoyama, Y.; Kushida, M.; Hong, Y.Y.; Nakao, S.; Higashiyama, R.; Sugioka, Y.; Inoue, H.; Okazaki, I.; Inagaki, Y. Glycyrrhizin and its metabolite inhibit Smad3-mediated type 1 collagen gene transcription and suppress experimental murine liver fibrosis. *Life Sci.* **2008**, *83*, 531–539.
27. Higashiyama, R.; Moro, T.; Nakao, S.; Mikami, K.; Fukumitsu, H.; Ikeda, K.; Adachi, E.; Bou-Gharios, G.; Okazaki, I.; Inagaki, Y. Negligible contribution of bone marrow-derived cells to collagen production during hepatic fibrogenesis in mice. *Gastroenterology* **2009**, *137*, 1459–1466.
28. Endo, H.; Niioka, M.; Sugioka, Y.; Itoh, J.; Kameyama, K.; Okazaki, I.; Ala-Aho, R.; Kahari, V.M.; Watanabe, T. Matrix metalloproteinase-13 promotes recovery from experimental liver cirrhosis in rats. *Pathobiology* **2011**, *78*, 239–252.

29. Watanabe, T.; Niioka, M.; Hozawa, S.; Sugioka, Y.; Arai, M.; Maruyama, K.; Okano, H.; Okazaki, I. *Extracellular Matrix and the Liver-Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 362–388.
30. Inagaki, Y.; Nemoto, T.; Nakao, A. *Extracellular Matrix and the Liver-Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 233–248.
31. Inagaki, Y.; Okazaki, I. Emerging insights into transforming growth factor-β and Smad signaling in hepatic fibrogenesis. *Gut* **2007**, *56*, 284–292.
32. Okazaki, I.; Wada, N.; Nakano, M.; Sato, A.; Takasaki, K.; Doi, M.; Kameyama, K.; Otani, Y.; Kubochi, K.; Niioka, M.; *et al.* Difference in gene expression for matrix metalloproteinase-1 between early and advanced hepatocellular carcinoma. *Hepatology* **1997**, *25*, 580–584.
33. Sugioka, Y.; Watanabe, T.; Inagaki, Y.; Kushida, M.; Niioka, M.; Endo, H.; Higashiyama, R.; Okazaki, I. C-Jun NH2-terminal kinase pathway is involved in constitutive matrix metalloproteinase-1 expression in a hepatocellular carcinoma-derived cell line. *Int. J. Cancer* **2004**, *109*, 867–874.
34. Endo, H.; Watanabe, T.; Sugioka, Y.; Niioka, M.; Inagaki, Y.; Okazaki, I. Activation of two distinct MAPK pathways governs constitutive expression of matrix metalloproteinase-1 in human pancreatic cancer cell lines. *Int. J. Oncol.* **2009**, *35*, 1237–1245.
35. Okazaki, I.; Inagaki, Y. Novel strategies for hepatocellular carcinoma based on MMPs science. *Anticancer Agents Med. Chem.* **2012**, *12*, 753–763.
36. Ong, J.P.; Elariny, H.; Collantes, R.; Younoszai, A.; Chandhoke, V.; Reines, H.D.; Goodman, Z.; Younossi, Z.M. Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. *Obes. Surg.* **2005**, *15*, 310–315.
37. Day, C.P.; James, O.F. Steatohepatitis: A tale of two "hits"? *Gastroenterology* **1998**, *114*, 842–845.
38. Van der Poorten, D.; Milner, K.L.; Hui, J.; Hodge, A.; Trenell, M.I.; Kench, J.G.; London, R.; Peduto, T.; Chisholm, D.J.; George, J. Visceral fat: A key mediator of steatohepatitis in metabolic liver disease. *Hepatology* **2008**, *48*, 449–457.
39. Adams, L.A.; Sanderson, S.; Lindor, K.D.; Angulo, P. The histological course of nonalcoholic fatty disease: A longitudinal study of 103 patients with sequential liver biopsies. *J. Hepatol.* **2005**, *42*, 132–138.
40. Rector, R.S.; Morris, E.M.; Ridenhour, S.; Meers, G.M.; Hsu, F.-F.; Turk, J.; Ibdah, J. Selective hepatic insulin resistance in a murine model heterozygous for a mitochondrial trifunctional protein defect. *Hepatology* **2013**, *57*, 2213–2223.
41. Cohen, J.C.; Horton, J.D.; Hobbs, H.H. Human fatty liver disease: Old questions and new insights. *Science* **2011**, *332*, 1519–1523.
42. Ueno, T.; Sata, M.; Tanikawa, K. Cells responsible for extracellular matrix production in the liver. In *Extracellular Matrix and the Liver Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 89–103.
43. Friedman, S.L. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N. Eng. J. Med.* **1993**, *328*, 1828–1835.
44. Li, D.; Friedman, S.L. Molecular mechanism of stellate cell activation and extracellular matrix remodeling. In *Extracellular Matrix and the Liver Approach to Gene Therapy*; Okazaki, I.,

Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 155–178.

45. Pinzani, M.; Marra, F. Profibrogenic actions of hepatic stellate cells: Major intracellular signaling pathways. In *Extracellular Matrix and the Liver Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 207–231.
46. Siegmund, S.V.; Dooley, S.; Brenner, D.A. Molecular mechanisms of alcohol-induced hepatic fibrosis. *Dig. Dis.* **2005**, *23*, 264–274.
47. Miyahara, T.; Hazra, S.; Xiong, S.; Motomura, K.; She, H.; Tsukamoto, H. Peroxisome proliferator-activated receptor γ and hepatic stellate cell activation. In *Extracellular Matrix and the Liver Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 179–188.
48. Zhu, N.-L.; Asahina, K.; Wang, J.; Ueno, A.; Lazaro, R.; Miyaoka, Y.; Miyajima, A.; Tsukamoto, H. Hepatic stellate cell-derived Delta-like Homolog 1 (DLK1) protein in liver regeneration. *J. Biol. Chem.* **2012**, *287*, 10355–10367.
49. Kisseleva, T.; Cong, M.; Paik, Y.; Scholten, D.; Jiang, C.; Benner, C.; Iwaisako, K.; Moore-Morris, T.; Scott, B.; Tsukamoto, H.; *et al.* Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 9448–9453.
50. Uyama, N.; Iimuro, Y.; Kawada, N.; Reynaert, H.; Suzumura, K.; Hirano, T.; Kuroda, N.; Fujimoto, J. Fascin, a novelmarker of human hepatic stellate cells, may regulate their proliferation, migration, and collagen gene expression through the FAK-PI3K-Akt pathway. *Lab. Invest.* **2012**, *92*, 7–71.
51. Motoyama, H.; Komiya, T.; Thuy le, T.T.; Enomoto, M.; Morikawa, H.; Iwai, S.; Uchida-Kobayashi, S.; Fujii, H.; Hagihara, A.; Kawamura, E.; *et al.* Cytoglobin is expressed in hepatic stellate cells, but not in myofibroblasts, in normal and fibrotic human liver. *Lab. Invest.* **2014**, *94*, 192–207.
52. Neubauer, K.; Krueger, M.; Quondamatteo, F.; Knittel, T.; Saile, B.; Ramadori, G. Transforming growth factor-β1 stimulates the synthesis of basement membrane proteins laminin, collagen type IV and entactin in rat liver sinusoidal endothelial cells. *J. Hepatol.* **1999**, *31*, 692–702.
53. Ramadori, G.; Neubauer, K. Role of sinusoidal endothelial cells in liver inflammation and repair. In *Extracellular Matrix and the Liver Approach to Gene Therapy*; Okazaki, I., Ninomiya, Y., Friedman, S.L., Tanikawa, K., Eds.; Academic Press: New York, NY, USA, 2003; pp. 135–151.
54. Forbes, S.J.; Russo, F.P.; Rey, V.; Burra, P.; Rugge, M.; Wright, N.A.; Alison, M.R. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* **2004**, *126*, 955–963.
55. Russo, F.P.; Alison, M.R.; Bigger, B.W.; Amofah, E.; Florou, A.; Amin, F.; Bou-Gharios, G.; Jeffery, R.; Iredale, J.P.; Forbes, S.J. The bone marrow functionally contributes to liver fibrosis. *Gastroenterology* **2006**, *130*, 1807–1821.
56. Baba, S.; Fujii, H.; Hirose, T.; Yasuchika, K.; Azuma, H.; Hoppo, T.; Naito, M.; Machimoto, T.; Ikai, I. Commitment of bone marrow cells to hepatic stellate cells in mouse. *J. Hepatol.* **2004**, *40*, 255–260.

57. Kisseleva, T.; Uchiyama, H.; Feirt, N.; Quintana-Bustamante, O.; Segovia, J.C.; Schwabe, R.F.; Brenner, D.A. Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. *J. Hepatol.* **2006**, *45*, 429–438.
58. Miyata, E.; Masuya, M.; Yoshida, S.; Nakamura, S.; Kato, K.; Sugimoto, Y.; Shibasaki, T.; Yamamura, K.; Ohishi, K.; Nishii, K.; *et al.* Hematopoietic origin of hepatic stellate cells in the adult liver. *Blood* **2008**, *111*, 2427–2435.
59. Inagaki, Y.; Higashiyama, R. Interplay between bone marrow and liver in the pathogenesis of hepatic fibrosis. *Hepatol. Res.* **2012**, *10*, 1–6.
60. Jiao, J.; Sastre, D.; Fiel, M.I.; Lee, U.E.; Ghiassi-Nejad, Z.; Ginhoux, E.; Vivier, E.; Friedman, S.L.; Merad, M.; Aloman, C. Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression. *Hepatology* **2012**, *55*, 244–255.
61. Pradere, J.-P.; Kluwe, J.; de Minicis, S.; Jiao, J.-J.; Gwak, G.-Y.; Dapito, D.H.; Jang, M.-K.; Guenther, N.D.; Mederacke, I.; Friedman, R.; *et al.* Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* **2013**, *58*, 1461–1473.
62. Xie, G.; Karaca, G.; Swiderska-Syn, M.; Michelotti, G.A.; Kruger, L.; Chen, Y.; Premont, R.T.; Choi, S.S.; Diehl, A.M. Cross-talk between notch and hedgehog regulates hepatic stellate cell fate in mice. *Hepatology* **2013**, *58*, 1801–1813.
63. Nieto, N.; Greenwel, P.; Friedman, S.L.; Zhang, F.; Dannenberg, A.J.; Cederbaum, A.I. Ethanol and arachidonic acid increase alpha 2(1) collagen expression in rat hepatic stellate cells overexpressing cytochrome P450 2E1. Role of H2O2 and cyclooxygenase-2. *J. Biol. Chem.* **2000**, *275*, 20136–20145.
64. Greenwel, P.; Dominguez-Rosales, J.A.; Mavi, G.; Rivas-Estilla, A.M.; Rojkind, M. Hydrogen peroxide: A link between acetaldehyde-elicited alpha (I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells. *Hepatology* **2000**, *31*, 109–116.
65. Alkhouri, N.; Dixon, L.J.; Feldstein, A.E. Lipotoxicity in nonalcoholic fatty liver disease: Not all lipids are created equal. *Expert Rev. Gastroenterol. Hepatol.* **2009**, *3*, 445–451.
66. Johnson, N.A.; George, J. Fitness *versus* fatness: Moving beyond weight loss in nonalcoholic fatty liver disease. *Hepatology* **2010**, *52*, 370–381.
67. Paradis, V.; Perlemuter, G.; Bonvoust, F.; Dargere, D.; Parfait, B.; Vidaud, M.; Conti, M.; Huet, S.; Ba, N.; Buffet, C.; *et al.* High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: A potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis. *Hepatology* **2001**, *34*, 738–744.
68. Ikeda, R.; Ishii, K.; Hoshikawa, Y.; Azumi, I.; Arakaki, Y.; Yasui, T.; Matsuura, S.; Matsumi, Y.; Kono, Y.; Mizuta, Y.; *et al.* Reactive oxygen species and NADPH oxidase 4 induced by transforming growth factor beta 1 are therapeutic targets of polyenylphosphatidylcholine in the suppression of human hepatic stellate cell activation. *Inflamm. Res.* **2011**, *60*, 597–604.
69. Li, J.; Fan, R.; Zhao, S.; Liu, L.; Guo, S.; Wu, N.; Zhang, W.; Chen, P. Reactive oxygen species released from hypoxic hepatocytes regulates MMP-2 expression in hepatic stellate cells. *Int. J. Mol. Sci.* **2011**, *12*, 2434–2447.

70. Asano, T.; Watanabe, K.; Kubota, N.; Gunji, T.; Omata, M.; Kadowaki, T.; Ohnishi, S. Adiponectin knockout mice on high fat diet develop fibrosing steatohepatitis. *J. Gastroenterol. Hepatol.* **2009**, *24*, 1669–1676.
71. Choi, S.S.; Syn, W.K.; Karaka, G.F.; Omenetti, A.; Moylan, C.A.; Witek, R.P.; Agboola, K.M.; Jung, Y.; Michelotti, G.A.; Diehl, A.M. Leptin promotes the myofibroblastic phenotype in hepatic stellate cells by activating the hedgehog pathway. *J. Biol. Chem.* **2010**, *285*, 36551–36560.
72. Marra, F.; Navari, N.; Vivoli, E.; Galastri, S.; Provenzano, A. Modulation of liver fibrosis by adipokines. *Dig. Dis.* **2011**, *29*, 371–376.
73. Bian, Z.; Ma, X. Liver fibrogenesis in non-alcoholic steatohepatitis. *Front. Physiol.* **2012**, *3*, 248.
74. Wree, A.; Broderick, L.; Canbay, A.; Hoffman, H.M.; Feldstein, A.E. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat. Rev. Gastroenterol. Hepatol.* **2013**, *10*, 627–636.
75. Sanyal, A.J.; Chalasani, N.; Kowdley, K.V.; McCullough, A.; Diehl, A.M.; Bass, N.M.; Neuschwander-Tetri, B.A.; Lavine, J.E.; Tonascia, J.; Unalp, A.; *et al.* Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N. Eng. J. Med.* **2010**, *362*, 1675–1685.
76. McCarty, M.F. Full-spectrum anti-oxidant therapy featuring astaxanthin coupled with lipoprivic strategies and salsalate for management of non-alcoholic fatty liver disease. *Med. Hypotheses* **2011**, *77*, 550–556.
77. Gastaldelli, A.; Harrison, S.; Belfort-Aguiar, R.; Hardies, I.; Balas, B.; Schenker, S.; Cusi, K. Pioglitazone in the treatment of NASH: The role of adiponectin. *Aliment. Pharmacol. Ther.* **2010**, *32*, 769–775.
78. Medici, V.; Ali, M.R.; Seo, S.; Aoki, C.A.; Rossaro, L.; Kim, K.; Fuller, W.D.; Vidovszky, T.J.; Smith, W.; Jiang, J.X.; *et al.* Increased soluble leptin receptor levels in morbidly obese patients with insulin resistance and nonalcoholic fatty liver disease. *Obesity (Silver Spring)* **2010**, *18*, 2268–2273.
79. Moro, T.; Nakao, S.; Sumiyoshi, H.; Ishii, T.; Miyazawa, M.; Ishii, N.; Inagaki, Y. Direct contribution of mitochondrial oxidative stress to hepatic fibrogenesis. *Hepatology* **2013**, *58*, 584A.
80. De Minicis, S.; Day, C.; Svegliati-Baroni, G. From NAFLD to NASH and HCC: Pathogenetic mechanisms and therapeutic insights. *Curr. Pharm. Des.***2013**, *19*, 5239–5249.
81. Mari, M.; Caballero, F.; Colell, A.; Morales, A.; Caballeria, J.; Fernandez, A.; Enrich, C.; Fernandez-Checa, J.C.; Garcia-Ruiz, C. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Met.* **2006**, *4*, 185–198.
82. Teratani, T.; Tomita, K.; Suzuki, T.; Oshikawa, T.; Yokoyama, H.; Shimamura, K.; Tominaga, S.; Hiroi, S.; Irie, R.; Okada, Y.; *et al.* A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells. *Gastroenterology* **2012**, *42*, 152–164.
83. Aleffi, S.; Navari, N.; Delogu, W.; Galastri, S.; Novo, E.; Rombouts, K.; Pinzani, M.; Parola, M.; Marra, F. Mammalian target of rapamycin mediates the angiogenic effects of leptin in human hepatic stellate cells. *Am. J. Physiol. Gastroenterol. Physiol.* **2011**, *301*, G210–G219.
84. Seki, E.; Brenner, D.A. Toll-like receptors and adaptor molecules in liver disease: Update. *Hepatology* **2008**, *48*, 322–335.

85. Yang, S.Q.; Lin, H.Z.; Lane, M.D.; Clemens, M.; Diehl, A.M. Obesity increases sensitivity to endotoxin liver injury: Implications for the pathogenesis of steatohepatitis. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2557–2562.
86. Seki, E.; de Meicis, S.; Osterricher, C.H.; Kluwe, J.; Osawa, Y.; Brenner, D.A.; Schwabe, R.F. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* **2007**, *13*, 1324–1332.
87. Jagavelu, K.; Routray, C.; Shergill, U.; O'Hara, S.P.; Faubion, W.; Shah, V.H. Endothelial cell toll-like receptor 4 regulates fibrosis-associated angiogenesis in the liver. *Hepatology* **2010**, *52*, 590–601.
88. Shirai, Y.; Yoshiji, H.; Noguchi, R.; Kaji, K.; Aihara, Y.; Douhara, A.; Moriya, K.; Namisaki, T.; Kawaratani, H.; Fukui, H. Cross talk between toll-like receptor-4 signaling and angiotensin-II in liver fibrosis development in the rat model of non-alcoholic steatohepatitis. *J. Gastroenterol. Hepatol.* **2013**, *28*, 723–730.
89. Henao-Mejia, J.; Elinav, E.; Jin, C.-C.; Hao, L.; Mehal, W.Z.; Strowig, T.; Thaiss, C.A.; Kau, A.L.; Eisenbarth, S.C.; Jurczak, M.J.; *et al.* Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* **2012**, *482*, 179–185.
90. Li, L.; Chen, L.; Hu, L.; Liu, Y.; Sun, H.Y.; Tang, Y.-J.; Chang, Y.-X.; Tu, Q.-Q.; Feng, G.-S.; Shen, F.; *et al.* Nuclear factor high-mobility group box1 mediating the activation of Toll-like receptor 4 signaling in hepatocytes in the early stage of nonalcoholic fatty liver disease in mice. *Hepatology* **2011**, *54*, 1620–1630.
91. Chen, A.; Tang, Y.; Davis, V.; Hsu, F.-F.; Kennedy, S.M.; Song, H.; Turk, J.; Brunt, E.M.; Newberry, E.P.; Davidson, N.O. Liver fatty acid binding protein (L-Fabp) modulates murine stellate cell activation and diet-induced nonalcoholic fatty liver disease. *Hepatology* **2013**, *57*, 2202–2212.
92. Ratziu, V.; Charlotte, F.; Bernhardt, C.; Giral, P.; Halbron, M.; Lenaour, G.; Hartmann-Heurtier, A.; Bruckert, E.; Poynard, T.; LIDO Study Group. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: Results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. *Hepatology* **2010**, *51*, 445–453.
93. Miura, K.; Yang, L.; van Rooijen, N.; Ohnishi, H.; Seki, E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2012**, *302*, G1310–G1321.
94. Tosello-Trampont, A.-C.; Landes, S.G.; Nguyen, V.; Novobrantseva, T.I.; Hahn, Y.S. Kupffer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-α production. *J. Biol. Chem.* **2012**, *287*, 40161–40172.
95. Carpino, G.; Renzi, A.; Onori, P.; Gaudio, E. Role of hepatic progenitor cells in nonalcoholic fatty liver disease development: Cellular cross-talks and molecular networks. *Int. J. Mol. Sci.* **2013**, *14*, 20112–20130.
96. Van Hul, N.K.M.; Abarca-Quinones, J.; Sempoux, C.; Horsmans, Y.; Leclercq, I.A. Relation between liver progenitor cell expansion and extracellular matrix deposition in a CDE-induced murine model of chronic liver injury. *Hepatology* **2009**, *49*, 1625–1635.
97. Boulter, L.; Govaere, O.; Bird, T.G.; Radulescu, S.; Ramachandran, P.; Pellicoro, A.; Ridgway, R.A.; Seo, S.S.; Spee, B.; Rooijen, N.V.; *et al.* Macrophage derived Wnt signaling opposes Notch

signaling in a Numb mediated manner to specify HPC fate in chronic liver disease in human and mouse. *Nat. Med.* **2012**, *18*, 572–579.

98. Nobili, V.; Carpino, G.; Alisi, A.; Franchitto, A.; Alpini, G.; de Vito, R.; Onori, P.; Alvaro, D.; Gaudio, E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* **2012**, *56*, 2142–2153.
99. Friedman, S.L. Convergent pathways that cause hepatic fibrosis in NASH. *Nat. Rev. Gastroenterol. Hepatol.* **2013**, *10*, 71–72.
100. Wagner, M.; Zollner, G.; Trauner, M. Nuclear receptors in liver disease. *Hepatology* **2011**, *53*, 1023–1034.
101. Belfort, R.; Harrison, S.A.; Brown, K.; Darland, C.; Finch, J.; Hardies, J.; Balas, B.; Gastaldeli, A.; Tio, F.; Pulcini, J.; *et al.* A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N. Eng. J. Med.* **2006**, *355*, 2297–2307.
102. Syn, W.-K.; Choi, S.S.; Liaskou, E.; Karaca, G.F.; Agboola, K.M.; Oo, Y.H.; Mi, Z.; Pereira, T.A.; Zdanowicz, M.; Malladi, P.; *et al.* Osteopontin is induced by hedgehog pathway activation and promotes fibrosis progression in nonalcoholic steatohepatitis. *Hepatology* **2011**, *53*, 106–115.
103. Okazaki, I.; Watanabe, T.; Hozawa, S.; Niioka, M.; Arai, M.; Maruyama, K. Minireview Series for the 50th Volume: Reversibility of hepatic fibrosis: From the first report of collagenase in the liver to the possibility of gene therapy for recovery. *Keio J. Med.* **2001**, *50*, 58–65.
104. Arthur, M.J.; Friedman, S.L.; Roll, F.J.; Bissell, D.M. Lipocytes from normal rat liver release a neutral metalloproteinase that degrades basement membrane (type IV collagen). *J. Clin. Invest.* **1989**, *84*, 1076–1085.
105. Takahara, T.; Furui, K.; Funaki, J.; Nakayama, Y.; Itoh, H.; Miyabayashi, C.; Sato, H.; Seiki, M.; Ooshima, A.; Watanabe, A. Increased expression of matrix metalloproteinase-II in experimental liver fibrosis in rats. *Hepatology* **1995**, *21*, 787–795.
106. Takahara, T.; Furui, K.; Yata, Y.; Jin, B.; Zhang, L.P.; Nambu, S.; Sato, H.; Seiki, M.; Watanabe, A. Dual expression of matrix metalloproteinase-2 and membrane-type-1-matrix metalloproteinase in fibrotic human livers. *Hepatology* **1997**, *26*, 1521–1529.
107. Okazaki, I.; Nabeshima, K. Introduction: MMPs, ADAMs/ADAMTSs research products to achieve big dream. *Anticancer Agents Med. Chem.* **2012**, *12*, 688–706.
108. Iredale, J.P.; Benyon, R.C.; Pickering, J.; McCullen, M.; Northrop, M.; Pawley, S.; Hovell, C.; Arthur, M.J. Mechanism of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J. Clin. Invest.* **1998**, *102*, 538–549.
109. Yoshiji, H.; Kuriyama, S.; Yoshii, J.; Ikenaga, Y.; Noguchi, R.; Nakatani, T.; Tsujinoue, H.; Yanase, K.; Namisaki, T.; Imazu, H.; *et al.* Tissue inhibitor of metalloproteinase-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* **2002**, *36*, 850–860.
110. Ljumovic, D.; Diamantis, I.; Alegakis, A.K.; Kouroumalis, E.A. Differential expression of matrix metalloproteinases in viral and non-viral chronic liver diseases. *Clin. Chim. Acta* **2004**, *349*, 203–211.
111. D'Amico, F.; Consolo, M.; Amoroso, A.; Skarmoutsou, E.; Mauceri, B.; Stivala, F.; Malaponte, G.; Bertino, G.; Neri, S.; Mazzarino, M.C. Liver immunolocalization and plasma levels of MMP-9

in non-alcoholic steatohepatitis (NASH) and hepatitis C infection. *Acta Histochem.* **2010**, *112*, 474–481.

112. Cao, Q.; Mak, K.; Lieber, C.S. Leptin represses matrix metalloproteinase-1 gene expression in LX2 human hepatic stellate cells. *J. Hepatol.* **2007**, *46*, 124–133.
113. Wanninger, J.; Walter, R.; Bauer, S.; Eisinger, K.; Schaffler, A.; Dorn, C.; Weiss, T.S.; Hellerbrand, C.; Buechler, C. MMP-9 activity is increased by adiponectin in primary human hepatocytes but even negatively correlates with serum adiponectin in a rodent model of non-alcoholic steatohepatitis. *Exp. Mol. Pathol.* **2011**, *91*, 603–607.
114. Tarrats, N.; Moles, A.; Garcia-Ruiz, C.; Fernandez-Checa, J.C.; Mari, M. Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. *Hepatology* **2011**, *54*, 319–327.
115. Adams, L.A.; Lymp, J.F.; St. Sauver, J.; Sanderson, S.O.; Lindor, K.D.; Feldstein, A.; Angulo, P. The natural history of nonalcoholic fatty liver disease: A population-based cohort study. *Gastroenterology* **2005**, *129*, 113–121.
116. Ascha, M.S.; Hanouneh, I.A.; Lopez, R.; Tamimi, T.A.-R.; Feldstein, A.F.; Zein, N.N. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* **2010**, *51*, 1972–1978.
117. Fu, J.; Chen, Y.; Cao, J.; Luo, T.; Qian, Y.; Yang, W.; Ren, Y.; Su, B.; Cao, G.; Yang, Y.; *et al.* P28 GANK overexpression accelerates hepatocellular carcinoma invasiveness and metastasis via phosphoinositol 3-kinae/AKT/hypoxia-inducible factor-1α pathways. *Hepatology* **2011**, *53*, 181–192.
118. Hayashi, Y.; Osanai, M.; Lee, G.H. Fascin-1 expression correlates with repression of E-cadherin expression in hepatocellular carcinoma cells and augments their invasiveness in combination with matrix metalloproteinases. *Cancer Sci.* **2011**, *102*, 1226–1235.
119. Wang, B.; Hsu, S.; Majumder, S.; Kutay, H.; Huang, W.; Jacob, S.T.; Ghoshal, K. TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. *Oncogene* **2010**, *29*, 1787–1797.
120. Man, K.; Ng, K.T.P.; Xu, A.; Cheng, Q.; Lo, C.M.; Xiao, J.W.; Sun, B.S.; Lim, Z.X.H.; Cheung, J.S.; Wu, E.X.; *et al.* Suppression of tumor growth and metastasis by adiponectin in nude mice through inhibition of tumor angiogenesis and downregulation of Rho kinase/IFN-inducible protein 10/matrix metalloproteinase 9 signaling. *Clin. Cancer Res.* **2010**, *16*, 967–977.
121. Gentilini, A.; Rombouts, K.; Galastri, S.; Caligiuri, A.; Mingarelli, E.; Mello, T.; Marra, F.; Mantero, S.; Roncalli, M.; Invernizzi, P.; *et al.* Role of the stromal-derived factor-1 (SDF-1)-CXCR4 axis in the interaction between hepatic stellate cells and cholangiocarcinoma. *J. Hepatol.* **2012**, *57*, 813–820.
122. Marrero, J.A.; Fontana, R.J.; Su, G.L.; Conjeevaram, H.S.; Emick, D.M.; Lok, A.S. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology* **2002**, *36*, 1349–1354.
123. Ratziu, V.; Bonyhay, L.; di Charlotte, F.; Cavallaro, L.; Sayegh-Tainturier, M.H.; Giral, P.; Grimaldi, A.; Opolon, P.; Poynard, T. Survival, liver failure and hepatocellular carcinoma in obesity-related cryptogenic cirrhosis. *Hepatology* **2002**, *35*, 1485–1493.

124. Paradis, V.; Zalinski, S.; Chelbi, E.; Guedj, N.; Degos, F.; Vilgrain, V.; Bedossa, P.; Belghiti, J. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: A pathological analysis. *Hepatology* **2009**, *49*, 851–859.
125. Zhang, D.Y.; Friedman, S.L. Fibrosis-dependent mechanism of hepatocarcinogenesis. *Hepatology* **2012**, *56*, 769–775.
126. Yin, C.; Evason, K.J.; Asahina, K.; Stainer, D.Y.R. Hepatic stellate cells in liver development, regeneration, and cancer. *J. Clin. Invest.* **2013**, *123*, 1902–1910.
127. Muir, K.; Hazim, A.; He, Y.; Peyressatre, M.; Kim, D.-Y.; Song, X.; Beretta, L. Proteomic and lipidomic signatures of lipid metabolism in NASH-associated hepatocellular carcinoma. *Cancer Res.* **2013**, *73*, 4722–4731.
128. Page, J.M.; Harrison, S.A. NASH and HCC. *Clin. Liver Dis.* **2009**, *13*, 631–647.
129. Bohinc, B.N.; Diehl, A.M. Mechanism of disease progression in NASH: New paradigms. *Clin. Liver Dis.* **2012**, *16*, 549–565.
130. Weiskopf, K.; Ring, A.M.; Ho, C.C.M.; Volkmer, J.-P.; Levin, A.M.; Volkmer, A.K.; Özkan, E.; Fernhoff, N.B.; van de Rijn, M.; Weissman, I.L.; *et al.* Engineered SIRPα variants as immunotherapeutic adjuvants to anti-cancer antibodies. *Science* **2013**, *341*, 88–91.
131. Yoshimoto, S.; Loo, T.M.; Atarashi, K.; Kanda, H.; Sato, S.; Oyadomari, S.; Iwakura, Y.; Oshima, K.; Morita, H.; Hattori, M.; *et al.* Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* **2013**, *499*, 97–101.
132. Lin, H.; Yan, J.; Wang, Z.; Hua, F.; Yu, J.; Sun, W.; Li, K.; Liu, H.; Yang, H.; Lv, Q.; *et al.* Loss of immunity-supported senescence enhances susceptibility to hepatocellular carcinogenesis and progression in Toll-like receptor 2-deficient mice. *Hepatology* **2013**, *57*, 171–182.
133. Wang, Z.; Yan, J.; Lin, H.; Hua, F.; Wang, X.; Liu, H.; Lv, X.; Yu, J.; Mi, S.; Wang, J.; *et al.* Toll-like receptor 4 activity protects against hepatocellular tumorigenesis and progression by regulating expression of DNA repair protein Ku70 in mice. *Hepatology* **2013**, *57*, 1869–1881.
134. Murphy, S.K.; Yang, H.; Moylan, C.A.; Pang, H.; Dellinger, A.; Abdelmalek, M.F.; Garrett, M.; Ashley-Koch, A.; Suzuki, A.; Tillmann, H.L.; *et al.* Relationship between methylome and transcriptome in patients with nonalcoholic fatty liver disease. *Gastroenterology* **2013**, *145*, 1076–1087.
135. Arii, S.; Mise, M.; Harada, T.; Furutani, M.; Ishigami, S.; Niwano, M.; Mizumoto, M.; Fukumoto, M.; Imamura, M. Overexpression of matrix metalloproteinase 9 gene in hepatocellular carcinoma with invasive potential. *Hepatology* **1996**, *24*, 316–322.
136. Ashida, K.; Nakatsukasa, H.; Higashi, T.; Ohguchi, S.; Hino, N.; Nouso, K.; Urabe, Y.; Yoshida, K.; Kinugasa, N.; Tsuji, T. Cellular distribution of 92-kd type IV collagenase B in human hepatocellular carcinoma. *Am. J. Pathol.* **1996**, *149*, 1803–1811.
137. Ogata, R.; Torimura, T.; Kin, M.; Ueno, T.; Tateishi, Y.; Kuromatsu, R.; Shimauchi, Y.; Sakamoto, M.; Tamaki, S.; Sata, M.; *et al.* Increased expression of membrane type 1 matrix metalloproteinases and matrix metalloproteinase-2 with tumor differentiation in hepatocellular carcinomas. *Hum. Pathol.* **1999**, *30*, 443–450.
138. Sakamoto, Y.; Mafune, K.; Mori, M.; Shiraishi, T.; Imamura, H.; Mori, M.; Takayama, T.; Makuuchi, M. Overexpression of MMP-9 correlates with growth of small hepatocellular carcinoma. *Int. J. Oncol.* **2000**, *17*, 237–243.

139. Maatta, M.; Soini, Y.; Liakka, A.; Autio-Harmainen, H. Differential expression of matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1-MMP in hepatocellular and pancreatic adenocarcinoma: Implications for tumor progression and clinical prognosis. *Clin. Cancer Res.* **2000**, *6*, 2726–2734.
140. Syed, I.; Rathod, J.; Parmar, M.; Parmar, M.; Corcoran, G.B.; Ray, S.D. Matrix metalloproteinase-9, -10, and -12, MDM2 and p53 expression in mouse liver during diethylnitrosamine-induced oxidative stress and genomic injury. *Mol. Cell Biochem.* **2012**, *365*, 351–361.
141. Fang, J.-H.; Zhou, H.-C.; Zeng, C.; Yang, J.; Liu, Y.; Huang, X.; Zhang, J.-P.; Guan, X.-Y.; Zhuang, S.-M. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. *Hepatology* **2011**, *54*, 1729–1740.
142. Sato, H.; Takino, T.; Okada, Y.; Cao, J.; Shinagawa, A.; Yamamoto, E.; Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature* **1994**, *370*, 61–65.
143. Strongin, A.Y.; Collier, I.; Bannikov, G.; Marmer, B.L.; Grant, G.A.; Goldberg, G.L. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. *J. Biol. Chem.* **1995**, *270*, 5331–5338.
144. Yamamoto, H.; Itoh, F.; Adachi, Y.; Sakamoto, H.; Adachi, M.; Hinoda, Y.; Imai, K. Relation of enhanced secretion of active matrix metalloproteinases with tumor spread in human hepatocellular carcinoma. *Gastroenterology* **1997**, *112*, 1290–1296.
145. Musso, O.; Theret, N.; Campion, J.P.; Turlin, B.; Milani, S.; Grappone, C.; Clement, B. *In situ* detection of matrix metalloproteinase-2 (MMP2) and the metalloproteinase inhibitor TIMP2 transcripts in human primary hepatocellular carcinoma and in liver metastasis. *J. Hepatol.* **1997**, *26*, 593–605.
146. Harada, T.; Arii, S.; Imamura, T.; Higashitsuji, H.; Furutani, M.; Niwano, M.; Ishigami, S.; Fukumoto, M.; Seiki, M.; Sato, H.; *et al.* Membrane-type matrix metalloproteinase-1 (MT1-MMP) gene is overexpressed in highly invasive hepatocellular carcinoma. *J. Hepatol.* **1998**, *28*, 231–239.
147. Theret, N.; Musso, O.; L'Helgoualc'h, A.; Campion, J.-P.; Clement, B. Differential expression and origin of membrane-type 1 and 2 matrix metalloproteinases (MT-MMPs) in association with MMP2 activation in injured human livers. *Am. J. Pathol.* **1998**, *153*, 945–954.
148. Yamamoto, H.; Itoh, F.; Adachi, Y.; Fukushima, H.; Itoh, H.; Sasaki, S.; Hinoda, Y.; Imai, K. Messenger RNA expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human hepatocellular carcinoma. *Jpn. J. Clin. Oncol.* **1999**, *29*, 58–62.
149. McKenna, G.J.; Chen, Y.; Smith, R.M.; Meneghetti, A.; Ong, C.; McMaster, R.; Scudamore, C.H.; Chung, S.W. A role for matrix metalloproteinases and tumor host interaction in hepatocellular carcinomas. *Am. J. Surg.* **2002**, *183*, 588–594.
150. Giannelli, G.; Bergamini, C.; Marinosci, F.; Fransvea, E.; Quaranta, M.; Lupo, L.; Schiraldi, O.; Antonaci, S. Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma. *Int. J. Cancer* **2002**, *97*, 425–431.
151. Ishii, Y.; Nakasato, Y.; Kobayashi, S.; Yamazaki, Y.; Aoki, T. A study on angiogenesis-related marix metalloproteinase networks in primary hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.* **2003**, *22*, 461–470.

152. Gao, Z.H.; Tretiakova, M.S.; Liu, W.H.; Gong, C.; Farris, P.D.; Hart, J. Association of E-cadherin, matrix metalloproteinases, and tissue inhibitors of metalloproteinases with the progression and metastasis of hepatocellular carcinoma. *Mod. Pathol.* **2006**, *19*, 533–540.
153. Altadill, A.; Rodriguez, M.; Gonzalez, L.O.; Junquera, S.; Corte, M.D.; Gonzalez-Dieguez, M.L.; Linares, A.; Barbon, E.; Fresno-Forcelledo, M.; Rodrigo, L.; *et al.* Liver expression of matrix metalloproteinases and their inhibitors in hepatocellular carcinoma. *Dig. Liver Dis.* **2009**, *41*, 740–748.
154. Tretiakova, M.S.; Hart, J.; Shabani-Rad, M.T.; Zhang, J.; Gao, Z. Distinction of Hepatocellular adenoma from hepatocellular carcinoma with and without cirrhosis using E-cadherin and matrix metalloproteinase. *Mod. Pathol.* **2009**, *22*, 1113–1120.
155. Gorrin Rivas, M.J.G.; Arii, S.; Furutani, M.; Harada, T.; Mizumoto, M.; Nishiyama, H.; Fujita, J.; Imamura, M. Expression of human macrophage metalloelastase gene in hepatocellular carcinoma: Correlation with angiostatin generation and its clinical significance. *Hepatology* **1998**, *28*, 986–993.
156. Nakatsukasa, H.; Ashida, K.; Higashi, T.; Ohguchi, S.; Tsuboi, S.; Hino, N.; Nouso, K.; Urabe, Y.; Kinugasa, N.; Yoshida, K.; *et al.* Cellular distribution of transcripts for tissue inhibitor of metalloproteinases 1 and 2 in human hepatocellular carcinomas. *Hepatology* **1996**, *24*, 82–88.
157. International Working Party. Terminology of nodular hepatocellular lesions. *Hepatology* **1995**, *22*, 983–993.
158. Korkaya, H.; Liu, S.; Wicha, M.S. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. *J. Clin. Invest.* **2011**, *121*, 3804–3809.
159. McLean, K.; Gong, Y.; Choi, Y.; Deng, N.; Yang, K.; Bai, S.; Cabrera, L.; Keller, E.; McCauley, L.; Cho, K.R.; *et al.* Human ovarian carcinoma-associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. *J. Clin. Invest.* **2011**, *121*, 3206–3219.
160. Yan, X.-L.; Jia, Y.-L.; Chen, L.; Zeng, Q.; Zhou, J.-N.; Fu, C.-J.; Chen, H.-X.; Yuan, H.-F.; Li, Z.-W.; Shi, L.; *et al.* Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: Role of the S100A4-mIR155-SOCS1-MMP-9 axis. *Hepatology* **2013**, *57*, 2274–2286.
161. Roderfeld, M.; Rath, T.; Lammert, F.; Dierkes, C.; Graf, J.; Roeb, E. Innovative immunohistochemistry identifies MMP-9 expressing macrophages at the invasive front of murine HCC. *World J. Hepatol.* **2010**, *2*, 175–179.
162. Tian, T.; Nan, K.; Guo, H.; Wang, W.; Ruan, Z.; Wang, S.; Liang, X.; Lu, C. PTEN inhibits the migration and invasion of HepG2 cells by coordinately decreasing MMP expression via the PI3K/Akt pathway. *Oncol. Rep.* **2010**, *23*, 1593–1600.
163. Park, S.Y.; Jeong, K.J.; Panupinthu, N.; Yu, S.; Lee, J.; Han, J.V.V.; Kim, J.M.; Lee, J.S.; Kang, J.; Park, C.G.; *et al.* Lysophospatidic-acid augments human hepatocellular carcinoma cell invasion through LPA1 receptor and MMP-9 expression. *Oncogene* **2011**, *30*, 1351–1359.
164. Yang, X.; Wang, D.; Dong, W.; Song, Z.; Dou, K. Inhibition of Na(+)/H(+) exchanger 1 by 5-(*N*-ethyl-*N*-isopropyl) amiloride reduces hypoxia-induced hepatocellular carcinoma invasion and motility. *Cancer Lett.* **2010**, *295*, 198–204
165. Kessenbrock, K.; Plaks, V.; Werb, Z. Matrix metalloproteinases: Regulators of the tumor microenvironment. *Cell* **2010**, *141*, 52–67.

166. Murugan, R.S.; Vinothini, G.; Hara, Y.; Nagini, S. Black tea polyphenols target matrix metalloproteinases, RECK, proangiogenic molecules and histone deacetylase in a rat hepatocarcinogenesis model. *Anticancer Res.* **2009**, *29*, 2301–2305.
167. Stefanou, N.; Papanikolaou, V.; Fukukawa, Y.; Nakamura, Y.; Tsezou, A. Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase. *BMC Cancer* **2010**, *10*, 442.
168. Chen, Y.; Boyartchuk, V.; Lewis, B. Differential roles of insulin-like growth factor receptor- and insulin receptor-mediated signaling in the phenotypes of hepatocellular carcinoma cells. *Neoplasia* **2009**, *11*, 835–845.
169. Shengbing, Z.; Feng, L.J.; Bin, W.; Lingyun, G.; Aimin, H. Expression of KISS-1 gene and its role in invasion and metastasis of human hepatocellular carcinoma. *Anat. Rec. (Hoboken)* **2009**, *292*, 1128–1134.
170. Martin, D.C.; Sanchez-Swatman, O.H.; Ho, A.T.; Inderdeo, D.S.; Tsao, M.; Khokha, R. Transgenic TIMP-1 inhibits simian virus 40 T antigen-induced hepatocarcinogenesis by impairment of hepatocellular proliferation and tumor angiogenesis. *Lab. Invest.* **1999**, *79*, 225–234.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).

Lymph Node Stromal Cells Negatively Regulate Antigen-Specific CD4$^+$ T Cell Responses

Jun Abe,[*,†,‡] Shigeyuki Shichino,[*,†] Satoshi Ueha,[*,†] Shin-ichi Hashimoto,[*,†,§] Michio Tomura,[¶] Yutaka Inagaki,[†,‖] Jens V. Stein,[‡] and Kouji Matsushima[*,†]

Lymph node (LN) stromal cells (LNSCs) form the functional structure of LNs and play an important role in lymphocyte survival and the maintenance of immune tolerance. Despite their broad spectrum of function, little is known about LNSC responses during microbial infection. In this study, we demonstrate that LNSC subsets display distinct kinetics following vaccinia virus infection. In particular, compared with the expansion of other LNSC subsets and the total LN cell population, the expansion of fibroblastic reticular cells (FRCs) was delayed and sustained by noncirculating progenitor cells. Notably, newly generated FRCs were preferentially located in perivascular areas. Viral clearance in reactive LNs preceded the onset of FRC expansion, raising the possibility that viral infection in LNs may have a negative impact on the differentiation of FRCs. We also found that MHC class II expression was upregulated in all LNSC subsets until day 10 postinfection. Genetic ablation of radioresistant stromal cell–mediated Ag presentation resulted in slower contraction of Ag-specific CD4$^+$ T cells. We propose that activated LNSCs acquire enhanced Ag-presentation capacity, serving as an extrinsic brake system for CD4$^+$ T cell responses. Disrupted function and homeostasis of LNSCs may contribute to immune deregulation in the context of chronic viral infection, autoimmunity, and graft-versus-host disease. *The Journal of Immunology*, **2014, 193: 1636–1644.**

Lymph nodes (LNs) are the primary site of Ag encounter by lymphocytes. Circulating lymphocytes reside there for up to 24 h (1) and undergo multiple rounds of cell-to-cell contact with Ag-presenting dendritic cells (DCs) (2). The LN structure and microenvironment are designed to concentrate Ags transported from the periphery and to promote the migration and survival of lymphocytes and DCs (3–5).

LN stromal cells (LNSCs) play a critical role in the functional organization of the LNs (6). LNSCs consist of multiple types of fibroblastic cells, lymphatic endothelial cells (LECs), and blood vessel endothelial cells (BECs). One subset of fibroblastic LNSCs, fibroblastic reticular cells (FRCs) in the T cell region, produce IL-7 and exist in close contact with T cells and DCs (7, 8). FRCs also express the homeostatic chemokines CCL19/CCL21 (9–11) and ICAM-1, which together act as the guiding cue and driving force for the intranodal migration of lymphocytes and DCs (4, 12). Moreover, FRCs regulate vascular growth in reactive LNs during inflammation (13), thereby ultimately controlling lymphocyte trafficking. Therefore, FRCs represent one of the key cellular components providing structural and microenvironmental support for adaptive immune responses in LNs.

Recently, a newly described role for LNSCs as APCs in adaptive immunity has attracted much attention. In the steady-state, LNSCs differentially express peripheral tissue–specific Ags and present them to CD8$^+$ T cells for the maintenance of self-tolerance (14–16). In contrast, LNSCs stimulated with LPS for 12 h in vivo upregulate MHC class II (17). Furthermore, *Pdpn*-Cre–driven expression of a model Ag in LECs and FRCs leads to the expansion of adoptively transferred Ag-specific CD4$^+$ T cells, even under noninflammatory conditions (16). These observations suggest that the outcome of Ag presentation to CD4$^+$ T cells by LNSCs could be either immunogenic or tolerogenic, depending on the inflammatory environment. However, because previous studies used sterile models of inflammation, it remains unclear how FRCs and other LNSCs adapt functionally during replicative cytopathic virus infections.

In the current study, we performed a detailed analysis of LNSC responses during vaccinia virus (VV) infection. BECs and LECs showed rapid expansion, peaking at day 14 postinfection (p.i.). In contrast, we observed a delayed, but sustained, expansion of terminally differentiated FRCs, which were replenished by noncirculating progenitor cells in a lymphotoxin-β–dependent manner. Of note, FRCs and other LNSCs expressed MHC class II p.i., which contributed to the contraction of Ag-specific CD4$^+$ T cells at the later time points of the immune response. Taken together, our data elucidate the dynamics of LNSC expansion and function during antimicrobial immune responses.

*Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan; †Japan Science and Technology Agency, Tokyo 102-8666, Japan; ‡Theodor Kocher Institute, University of Bern, CH-3012 Bern, Switzerland; §Division of Nephrology, Department of Laboratory Medicine, Kanazawa University, Ishikawa 920-1192, Japan; ¶Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; and ‖Center for Matrix Biology and Medicine, Graduate School of Medicine, Institute of Medical Sciences, Tokai University, Kanagawa 259-1143, Japan

Received for publication October 31, 2013. Accepted for publication June 5, 2014.

This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas 19059004 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, the Japan Science and Technology Core Research for Evolutional Science and Technology program (to K.M.), and Grant-in-Aid for Young Scientists (B) 2379052 from the Japan Society for the Promotion of Science (to J.A.).

Address correspondence and reprint requests to Dr. Kouji Matsushima, Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail address: koujim@m.u-tokyo.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; bm12, B6.C-H2-Ab1^{bm12}; Col1a2, type I collagen α2; DC, dendritic cell; DN, double negative; FRC, fibroblastic reticular cell; HEV, high endothelial venule; LN, lymph node; LNSC, LN stromal cell; LTβR, lymphotoxin-β receptor; LTβR-Ig, rIg Fc region–fused soluble recombinant lymphotoxin-β receptor protein; #639/#474 mice, FucciG$_1$-#639×FucciS/G$_2$/M-#474 double-transgenic mice; p.i., postinfection; popLN, popliteal lymph node; Tfh, T follicular helper; VV, vaccinia virus; VV-OVA, OVA expressing recombinant VV; WT, wild-type.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302946

Materials and Methods

Mice, viral infection, and immunization

C57BL/6 (B6) mice were purchased from Japan SLC. *Rag2*$^{-/-}$ $OVA_{323-339}$-specific TCR-transgenic (OT-II) mice were purchased from Taconic. B6.C-H2-Ab1^{bm12} (bm12) and B6.SJL (CD45.1) mice were purchased from The Jackson Laboratory. FucciG_1-#639×FucciS/G_2/M-#474 double-transgenic mice (#639/#474 mice) on a B6 background (18) were provided by Atsushi Miyawaki (RIKEN). *Col1a2*-GFP mice were generated as described previously (19). Mice were 6–8 wk old at the commencement of experiments. Mice were either infected s.c. with 1×10^4 PFU OVA-expressing recombinant VV (VV-OVA) (20) or were hock immunized (21) with 50 μg OVA protein emulsified in CFA. In some experiments, mice were given an i.p. injection of 100 μg rIg Fc region–fused soluble recombinant lymphotoxin-β receptor (LTβR) protein (LTβR-Ig) 1 d before immunization and on a weekly basis thereafter. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Tokyo.

Abs

mAbs against CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (6D5), β1 integrin (Ha2/5), CD31 (390), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL1), VCAM-1 (429), CD138 (281-2), CD157 (BP-3), ICOS (7E.17G9), PD-1 (RMP1-30), CXCR5 (2G8), gp38 (8.1.1), Ly-6G/C (RB6-8C5), NK1.1 (PK136), Ter-119 (TER-119), BrdU (Bu20a), MHC class I (28-14-8), I-A/I-E (M5/114.15.2), TCR Vα2 (B20.1), and TCRγδ (UC7-13D5) were purchased from BioLegend, BD Biosciences, or eBioscience and used for flow cytometry. For the immunofluorescent staining of tissue sections, purified anti–pan-endothelial Ag (MECA-32) and biotinylated anti-gp38 (8.1.1) mAbs were purchased from BioLegend, anti-type IV collagen rabbit antiserum was purchased from LSL, and Alexa Fluor–labeled secondary Abs and streptavidin were purchased from Life Technologies.

Flow cytometry

LNs were either minced by filtering through a cell strainer (BD Biosciences) or digested in 0.1% crude collagenase (Wako; cat. #32-10534), 0.96 mg/ml Dispase II (Roche), and 20 kU/ml DNase I (Calbiochem) in DMEM supplemented with 2% FCS and 10 mM HEPES for 30 min at 37°C. Throughout the digestion process, the suspension was stirred gently using a small magnetic stirrer bar. Aliquots of the resulting single-cell suspension were stained with appropriate Ab mixtures. Data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo software (TreeStar).

Measurement of viral load in tissues

Viral load in reactive LNs and infected footpads was measured by copy number assay. In brief, LNs and footpads were homogenized using a beads-based cell disruptor, and total DNA was isolated using a Tissue Genomic DNA Extraction Mini Kit (Favorgen). Copy numbers of *Ova* gene were measured by subjecting 45 ng total DNA/reaction to quantitative PCR with FastStart TaqMan Probe Master (Roche), a TaqMan Gene Expression Assay kit Gg03366808_m1 (for *Ova*), and a TaqMan Copy Number Reference Assay kit (Applied Biosystems). The limit of detection of the assay was 10 copies.

In vivo BrdU labeling

For short-term pulse labeling, mice were injected i.p. with 1 mg/mouse BrdU (Sigma-Aldrich) 16 h before sacrifice. For long-term continuous labeling, mice were provided with drinking water containing 0.8 mg/ml BrdU for the duration of the experiment, commencing immediately p.i. BrdU water was replaced every 2–3 d.

Immunofluorescent staining of LN sections

Six-micron-thick cryosections of fresh LNs were prepared and fixed for 10 min in acetone. Fixed sections were rehydrated with PBS, blocked with Blocking One reagent (Nacalai Tesque), and stained with Ab mixtures (in PBS containing 2% BSA) for 1 h at room temperature. Sections were incubated further with appropriate secondary Abs. For BrdU staining, sections were fixed for 10 min with 4% paraformaldehyde in PBS after the staining of the other Ags. Fixed sections were treated with 2 M HCl for 1 h at 37°C before incubation with anti-BrdU Ab. After staining, sections were mounted with ProLong Gold reagent (Life Technologies). Immunofluorescently stained sections were observed under a TCS SP5 confocal microscope (Leica Microsystems). The acquired images were processed and quantified using ImageJ software (National Institutes of Health).

Parabiosis

Parabiosis surgery was performed on body weight–matched partners. A longitudinal incision was made through the skin and fascia of mice deeply anesthetized with pentobarbital. The incised skins and fascia of partner mice were joined by sutures. More than 6 wk after the surgery, mice were infected s.c. with VV-OVA via the footpad distal to the conjoined side of the body.

Generation of bone marrow chimeric mice and adoptive transfer

CD45.1$^-$ CD45.2$^+$ bm12 and B6 recipient mice were exposed to 8.5 Gy irradiation before transplantation with 5×10^6 CD45.1$^+$ CD45.2$^+$ whole bone marrow (BM) cells the following day. More than 6 wk after transplantation, 2×10^4 CD45.1$^+$ CD45.2$^-$ *Rag2*$^{-/-}$ OT-II T cells were adoptively transferred into recipient mice via the tail vein 1 d before immunization. For OT-II T cell preparation, CD4$^+$ T cells were enriched from the spleen of OT-II mice by negative selection using streptavidin MicroBeads and an AutoMACS cell separator (Miltenyi Biotec). Before magnetic labeling with MicroBeads, cells were labeled with biotinylated Abs against CD8α, CD11b, CD11c, CD19, CD138, Ly-6G/C, NK1.1, Ter-119, and TCRγδ. The purity of CD4$^+$ TCR Vα2$^+$ OT-II T cells was routinely >95%.

Statistical analysis

Data were analyzed for statistical significance by Student *t* test (for comparison of two groups) or one-way ANOVA (for comparison of three of more groups; post hoc tests were performed as described in the figure legends). All statistical analyses were performed using GraphPad Prism software. The p values < 0.05 were considered statistically significant.

Results

LNSCs expand in a subset-specific manner during viral infection

In a recent study, immunization with protein Ag and CFA caused the number of FRCs and endothelial cells in the LNs to increase in parallel with the lymphocyte number (22). We confirmed this observation in a sterile OVA/CFA immunization model (Supplemental Fig. 1A–C). However, the expansion of LNSC subsets during antimicrobial immune responses remained to be investigated. Therefore, we developed a protocol for the flow cytometric analysis of stromal cells in reactive popliteal LNs (popLNs) following s.c. VV-OVA infection, using contralateral nonreactive popLNs as controls.

LNSCs were identified based on the differential expression of gp38 and CD31 among CD45$^-$ Ter-119$^-$ nonhematopoietic cells in the LNs (23) (Fig. 1A, Supplemental Fig. 1A). Using this approach, we observed a >10-fold increase in total popLN cell numbers, peaking on day 10 p.i. (Fig. 1B, 1C). LEC and BEC expansion peaked on day 14 p.i., with a 5-fold increase in their numbers. Strikingly, FRC expansion was significantly delayed (Fig. 1B, shaded area) but was sustained until day 28 p.i., whereas expansion of LECs and BECs began as early as day 4 p.i. (Fig. 1B, 1C). The numbers of LNSCs in the contralateral nonreactive popLNs were not affected by VV infection. The percentage of T cell region reticular cells (9) among total FRCs, identified by CD157 expression, remained essentially stable (data not shown). Of note, the onset of FRC expansion coincided with the decline of VV-OVA copy number in reactive popLNs (Fig. 1D). Viral gene was almost undetectable on day 10 p.i. in reactive popLNs, whereas virus persisted for longer at the infection site. Reactive popLNs harbored considerably less virus than the infection site throughout the time course of our analysis. Importantly, the delayed expansion of FRCs relative to that of lymphocytes and other LNSC subsets clearly demonstrates that reactive LN growth and increased FRC numbers are not synchronized in the VV-OVA infection model, unlike in sterile inflammation (22).