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The Portal Inflammatory Infiltrate and Ductular Reaction in Human Nonalcoholic Fatty Liver Disease

Victoria L. Gadd,[1] Richard Skoien,[1] Elizabeth E. Powell,[1,2] Kevin J. Fagan,[1] Clay Winterford,[3] Leigh Horsfall,[1] Katharine Irvine,[1*] and Andrew D. Clouston[1*]

Although nonalcoholic fatty liver disease (NAFLD) is conventionally assessed histologically for lobular features of inflammation, development of portal fibrosis appears to be associated with disease progression. We investigated the composition of the portal inflammatory infiltrate and its relationship to the ductular reaction (DR), a second portal phenomenon implicated in fibrogenesis. The portal inflammatory infiltrate may contribute directly to fibrogenesis as well as influence the fate of the DR hepatic progenitor cells (HPCs), regulating the balance between liver repair and fibrosis. The presence of portal inflammation in NAFLD was strongly correlated with disease severity (fibrosis stage) and the DR. The portal infiltrate was characterized by immunostaining NAFLD liver biopsy sections (n = 33) for broad leukocyte subset markers (CD68, CD3, CD8, CD4, CD20, and neutrophil elastase) and selected inflammatory markers (matrix metalloproteinase 9 and interleukin [IL]-17). Cells expressing all markers examined were identified throughout the liver lobules and in portal tracts, although portal tracts were more densely populated ($P < 0.01$), and dominated by $CD68^+$ macrophages and $CD8^+$ lymphocytes, at all stages of disease. An increase in portal macrophages in NAFLD patients with steatosis alone ($P < 0.01$) was the earliest change detected, even before elevated expression of the proinflammatory cytokines, *IL1B* and *TNF*, in patients with early NASH ($P < 0.05$). Portal and periductal accumulation of all other cell types examined occurred in progressed NASH (all $P < 0.05$). *Conclusion:* Knowledge of the complex cellular composition of the portal inflammatory infiltrate and HPC/DR niche in NAFLD will shape future functional studies to elucidate the contribution of portal inflammation to HPC differentiation and NAFLD pathogenesis. (HEPATOLOGY 2014;59:1393-1405)

Nonalcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver injury, yet only a proportion of patients progress to cirrhosis, liver failure, and hepatocellular carcinoma. NAFLD is conventionally assessed histologically for the presence of *lobular* features of injury, including hepatocyte ballooning, Mallory bodies, zone 3 inflammation, and perisinusoidal fibrosis. However, recent studies have identified the importance of *portal* fibrosis in predicting the subgroup of NAFLD patients that develop progressive liver disease and liver-related mortality.[1,2] Clearly, identifying the mechanisms responsible for development and progression of portal fibrosis in fatty liver disease is critically important. In

Abbreviations: ALD, alcoholic liver disease; ANOVA, analysis of variance; ASH, alcoholic steatohepatitis; BMI, body mass index; CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C chemokine receptor 2; CLD, chronic liver disease; DR, ductular reaction; HPC, hepatic progenitor cell; HCV, hepatitis C virus; IHC, immunohistochemistry; IL, interleukin; K7, keratin 7; MCP1, monocyte chemotactic protein 1; MMP, matrix metalloproteinase; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NE, neutrophil elastase; NK, natural killer; qPCR, quantitative polymerase chain reaction; RT-PCR, real-time PCR; TGF-β, transforming growth factor beta; Th, T helper; TNF, tumor necrosis factor; TWEAK, TNF-like weak inducer of apoptosis.

From the [1]Center for Liver Disease Research, School of Medicine, The University of Queensland, Translational Research Institute, Brisbane, Australia; [2]Department of Gastroenterology and Hepatology, Princess Alexandra Hospital, Brisbane, Australia; [3]HistoTechnology Facility, Queensland Institute of Medical Research, Brisbane, Australia.

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this regard, portal inflammation is observed in the majority of subjects with nonalcoholic steatohepatitis (NASH) and was acknowledged as a component of the inflammatory score in the original NAFLD grading and staging system of Brunt et al.[3] Mild portal inflammation correlates with more severe liver disease[4,5] as well as with clinical features associated with risk of progressive disease, such as increased age, body mass index (BMI), and markers of insulin resistance.[4,5]

Few data are available regarding phenotype of the portal inflammatory infiltrate or the stimuli for its presence. Previous studies describe a mixed infiltrate of lymphocytes, neutrophils, monocytes, and occasional eosinophils,[4,6] but the relative numbers of different cells and their relationship to disease progression are unknown. The degree of portal inflammation does not correlate with grade of lobular inflammation,[4] but is associated instead with portal-based changes, such as a ductular reaction (DR).[7,8] The DR, a reactive lesion at the portal tract interface comprising small biliary ductules with an accompanying complex of stroma and inflammatory cells,[9] develops when hepatocyte regeneration is impaired and hepatic progenitor cell (HPC) proliferation takes over. HPCs are bipotential and capable of proliferation and differentiation into hepatocytes, to replace injured cells, or into cholangiocytes. HPC activation and a DR are common responses to chronic liver injury, including NAFLD, and are thought to precede progressive, portal fibrosis.[10] We previously demonstrated a correlation between the DR and portal inflammation in NAFLD, and both factors were independently associated with stage of fibrosis.[7]

Within portal tracts, inflammatory cells and their mediators influence the differentiation and fate choice of HPC,[11] which, in turn, may determine the balance between liver repair and fibrogenesis. The portal inflammatory infiltrate may contribute directly to fibrogenesis through release of profibrogenic cytokines or play a key role in determining HPC fate toward a fibrogenic DR.[11-13] To identify the potential cellular sources of local fibrogenic mediators, we characterized the cellular profile of the portal and periductal inflammatory infiltrate in patients with NAFLD and analyzed the data with respect to histological and clinical features as well as whole-liver cytokine and chemokine expression. A cohort of patients with alcohol-related liver disease (ALD) was also assessed for comparison.

Materials and Methods

Patients and Clinical Data. The study involved a total of 57 adult patients with NAFLD (n = 40) or ALD (n = 17) who had undergone a liver biopsy at the Princess Alexandra Hospital (Brisbane, Australia). The diagnosis of NAFLD was established by a liver biopsy specimen showing steatosis with or without features of steatohepatitis (lobular inflammation and hepatocyte ballooning, with or without Mallory-Denk bodies or fibrosis) in the setting of increased BMI or metabolic risk factors (type 2 diabetes, hypertension, and hyperlipidemia). NAFLD patients were consuming less than 20 g of alcohol per day for women and 30 g of alcohol per day for men, and other causes of steatosis or chronic liver disease (CLD) were excluded.[14] Diagnosis of ALD was established by a history of excessive habitual intake (more than 50 [females] and 60 g [males] of alcohol per day), together with physical signs and laboratory evidence of liver disease, in the absence of viral hepatitis. Ten nondiseased liver biopsy specimens (obtained from normal liver tissue distant to liver metastases at the time of resection, normal donor biopsies before grafting, or normal liver tissue obtained at the time of laparoscopic cholecystectomy) were used as controls to obtain baseline values for immunohistochemistry (IHC; n = 8) and real-time polymerase chain reaction (RT-PCR; n = 2). Weight, height, metabolic comorbidities, and average alcohol intake (g/day) were obtained from research nurse interview at the time of liver biopsy and corroborated by longitudinal review of medical records. The protocol was approved by the University of Queensland and Metro South Health, Princess Alexandra Hospital Human Research Ethics Committees, and informed consent was obtained from each patient.

Histopathologic Analysis. Histological changes of ALD or NAFLD (steatosis, lobular and portal inflammation, presence of ballooning, Mallory-Denk bodies, and fibrosis) were assessed and scored according to

Address reprint requests to: Andrew Clouston, M.B.B.S., Ph.D., F.R.C.P.A., Envoi Specialist Pathologists, 1/49 Butterfield Street, Brisbane, Australia. E-mail: andrewclouston@envoi.com.au; fax: +61 7 35524266.

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accepted criteria.[15] The NAFLD activity score (NAS) was calculated as an unweighted sum of the grade of steatosis (0-3), grade of lobular inflammation (0-3), and presence of ballooning (0-2). Progressed NASH was regarded as fibrosis stages 2-4, whereas early NASH was defined as fibrosis stages 0-1.

IHC. Of the 57 subjects selected for this study, 50 had formalin-fixed, paraffin-embedded liver biopsy sections available for IHC analysis (NAFLD, n = 33; ALD, n = 17). Sections were immunostained, as previously described,[16] for keratin 7 (K7) to highlight the DR and costained for CD3, CD4, CD8, FOXP3, CD20, CD68, neutrophil elastase (NE), interleukin (IL)−17, or matrix metalloproteinase (MMP)−9 to phenotype leukocytes. Hepatocyte senescence was highlighted using cyclin kinase inhibitor $p21^{waf1}$. Primary antibody details are summarized in Supporting Table 1. Slides underwent virtual microscopy using the Aperio ScanScope System (Aperio, Vista, CA), and fluorescent microscopy was performed using the Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Pty Ltd., North Ryde, NSW, Australia).

The DR was semiquantitatively graded (0-4) by an experienced hepatopathologist (A.D.C.): grade 0 = no evidence of DR; grade 1 = focal increased DR in portal areas involving <50% of the portal circumference; grade 2 = DR in portal areas involving >50% of portal circumference; grade 3 = distinct spurs of DR extending <50% of the lobular radius; and grade 4 = distinct spurs of DR extending >50% of the lobular radius and the presence of portal-to-portal linking. Scoring was based on the most severe area of the biopsy. Specific inflammatory cells were manually quantified as a total number of cells per portal tract (10 portal tracts per biopsy), which was also normalized to cell density per unit area (10,000 μm^2). Inflammatory cells were also counted in 10 centrilobular regions per biopsy (analyzed field area: 10,000 μm^2). Inflammatory cells were considered periductular within a three-cell diameter of each ductular structure. Results were analyzed blinded with respect to etiology and fibrosis stage.

RT-PCR. Total RNA was extracted from fresh frozen liver biopsy samples (3 mm) from 26 NAFLD patients using TRI Reagent (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions, and reverse transcribed to complementary DNA by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative real-time polymerase chain reaction (qPCR) was performed as previously described.[16] Messenger RNA (mRNA) expression of genes of interest was normalized to the geometric mean of the expression of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, human acidic ribosomal protein, and 18S ribosomal RNA). Primer and probe sequences are available in Supporting Table 2.

Statistical Analysis. Statistical analyses utilized the Student *t* test, one-way analysis of variance (ANOVA), with Tukey's post-test (where appropriate), Pearson's correlation coefficient, and Spearman's rank-correlation coefficient (where appropriate). A *P* value of <0.05 was defined as the level of significance, and all graphs and analyses were performed using GraphPad Prism (version 5.02; GraphPad Software Inc., La Jolla, CA).

Results

Clinical Characteristics. Of a total of 57 patients, 50 had paraffin-embedded tissue available for IHC analysis and 26 had frozen tissue available for RT-PCR analysis. Nineteen patients were common to both cohorts. Demographic and clinical characteristics of subjects from both cohorts are summarized in Table 1.

Portal Inflammation Is Associated With Progressed NASH. The NAS includes features of active lobular injury (hepatocellular ballooning and lobular inflammation), in addition to steatosis, and has been used as a scoring system to compare histological change in NAFLD treatment trials.[17] In our cohort of NAFLD patients, the NAS did not differ between early and progressed NASH (Fig. 1A). The importance of portal features of liver injury in predicting the subgroup of patients with progressive disease has recently been recognized.[4] In support of this, portal inflammation and DR grade were significantly associated with disease progression ($r_s = 0.84$ and $r_s = 0.83$, respectively; both $P < 0.001$; Fig. 1B,C). Consistent with histological assessment of fibrosis, expression of whole-liver *COL1A1*, encoding collagen type 1A, was elevated in patients with steatosis alone and early NASH, when compared to controls, and further still in progressed NASH (Fig. 1D). In agreement with our previous observations,[7] a $K7^+$ DR preceded significant detectable portal fibrosis (Fig. 1C). Hepatocyte senescence has been implicated in activation and proliferation of HPC/DR.[18] In this cohort of patients, senescent hepatocytes (detected by nuclear p21 expression) were present throughout centrilobular and, particularly, periportal regions at all stages of NAFLD and were absent in control subjects (Fig. 1E,F).

Table 1. Clinical and Histologic Data for Subjects With NAFLD and ALD

	Immunohistochemical Cohort		RT-PCR Cohort
	NAFLD	ALD	NAFLD
No. of patients, n	33	17	26
Age (years), mean (range)	55 (29-73)	55 (44-73)	50 (24-68)
Gender, male, n (%)	14 (42.4)	14 (82.4)	13 (50)
Body mass index,* kg/m^2 (range)	31.3 (22.9-49.9)	27.9 (19.4-36.6)	32.6 (25.8-49.9)
Type 2 Diabetes,† yes (%)	15 (46.9)	3 (17.6)	10 (40)
Alcohol at time of biopsy (g/w), mean, range	30 (0-175)	386 (0-1505)	20 (0-175)
Type of fatty liver disease, n (%)			
Steatosis alone	11 (33.3)	3	9
Steatohepatitis	22 (66.7)	13	17
Alcoholic cirrhosis		1	
Grade of steatosis, n (%)			
1	12 (36.4)	1 (5.9)	10 (38.5)
2	13 (39.4)	3 (17.6)	9 (34.6)
3	8 (24.2)	13 (76.5)	7 (26.9)
Lobular inflammation grade, n (%)			
0	5 (15.2)	3 (17.6)	5 (19.2)
1	18 (54.5)	8 (47.1)	15 (57.7)
2/3	10 (30.3)	6 (35.3)	6 (23.1)
Portal inflammation grade,‡ n (%)			
0	12 (41.4)	1 (5.9)	12 (54.5)
1	13 (44.8)	16 (94.1)	10 (45.5)
2	4 (13.8)	0	0
Stage of fibrosis, n (%)			
0	11 (33.3)	3 (17.6)	10 (38.5)
1	9 (27.3)	1 (5.9)	9 (34.6)
2	4 (12.1)	4 (23.5)	3 (11.5)
3/4	9 (27.3)	9 (52.9)	4 (15.4)

*BMI unavailable for 1 NAFLD patient.
†Information regarding diabetes unavailable for 1 NAFLD patient.
‡Portal inflammation grade unavailable for 4 NAFLD patients.

Although portal inflammation, as graded histologically, remained relatively mild at all stages of NAFLD, the fact that it can precede portal fibrosis may indicate a role for portal inflammation in disease progression. To further characterize dynamics in portal immune/inflammatory cells in NAFLD, we localized and quantified leukocytes of the major hematopoietic lineages. IHC was performed for T lymphocytes, using the pan T-cell marker, CD3, and the cytotoxic T-cell marker, CD8, as well as B lymphocytes (CD20$^+$), neutrophils (NE$^+$), and macrophages (CD68$^+$; Fig. 2). As we previously reported, in chronic hepatitis C virus (HCV) infection, all liver macrophages coexpressed CD68 and CD163 (the latter typically considered a marker of M2 macrophages) in NAFLD (Supporting Fig. 1).[16] CD4, a marker of the T-helper (Th) lymphocyte subset, does not specifically stain lymphocytes in the liver (Fig. 2); however, the strong correspondence between CD3 and CD8 cell counts suggested that CD8$^+$ T cells dominate the portal hepatic environment in this cohort (Fig. 2). The proportion of CD8$^+$CD56$^+$ natural killer (NK) T cells was unable to be determined because of concomitant expression of CD56 on other cell types in the liver, including neurons and biliary epithelial cells. Forkhead box P3–expressing regulatory T cells were rarely detected (data not shown; range, 0-19 cells across 10 portal tracts).

All leukocyte subtypes studied were identified in livers of NAFLD patients and control subjects. Lobular leukocytes were evenly distributed, and the density of cells was relatively static at all stages of disease (Fig. 2). By comparison, portal leukocytes, especially those expressing CD3, CD8, or CD68, exhibited significant, disease-stage–dependent variation (Figs. 2 and 3). Portal leukocytes were observed in control livers and at early disease stages, often at higher density than lobular leukocytes (Figs. 2 and 3). This may suggest homeostatic functions for these cells and/or low-level inflammatory activity in nominally healthy livers. Nevertheless, all types of portal leukocytes significantly increased in number in progressed NASH, where portal fibrosis was present, compared to early disease and controls (Fig. 3A-F). These data highlight portal expansion and inflammatory cell accumulation in portal tracts over time. In terms of absolute numbers, CD68$^+$ macrophages and CD8$^+$ lymphocytes dominated the inflammatory infiltrate at all stages of disease.

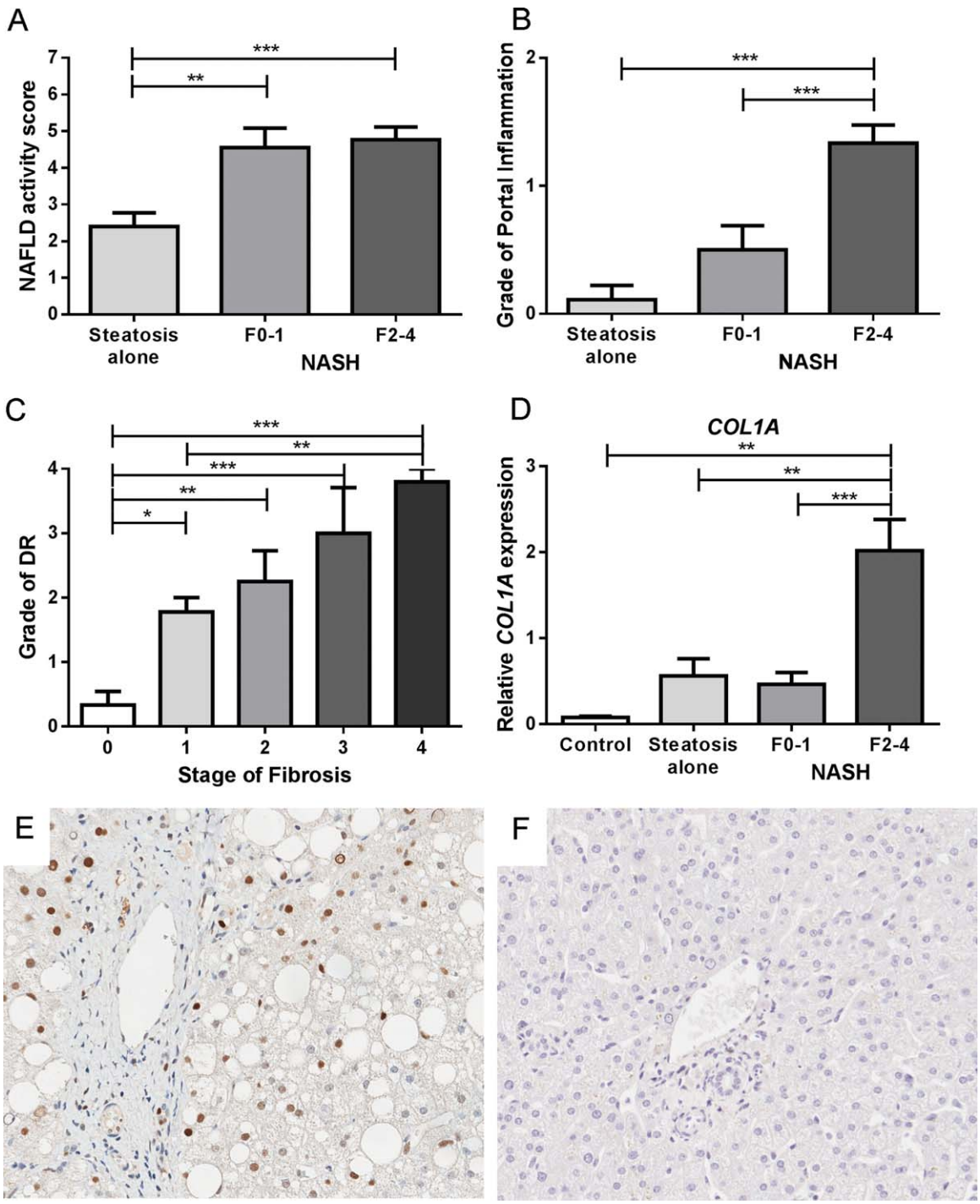

Fig. 1. Pathological features of injury in NAFLD. The NAS (A), grade of portal inflammation (B), $K7^+$ ductular reaction (C), and relative *COL1A* expression (D) in NAFLD progression. Immunostaining for p21 in a representative NAFLD biopsy section (E) and control subject (F). Data are represented as mean + standard error of the mean. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. F0-F1, early NASH; F2-F4, progressed NASH (original magnifications: 200×).

Although steatosis alone is generally considered benign and not associated with lobular or portal inflammation, a significant increase in number of portal macrophages was observed between control subjects and steatosis alone and increased further in patients with progressed NASH (Fig. 3A). Portal CD68 cell counts were positively associated with lobular features of liver injury, including hepatocyte ballooning ($r_s = 0.51$; $P < 0.01$) and lobular inflammation ($r_s = 0.42$; $P < 0.05$), but not steatosis or the NAS. Although other cells of the portal inflammatory infiltrate were not significantly elevated in steatosis alone,

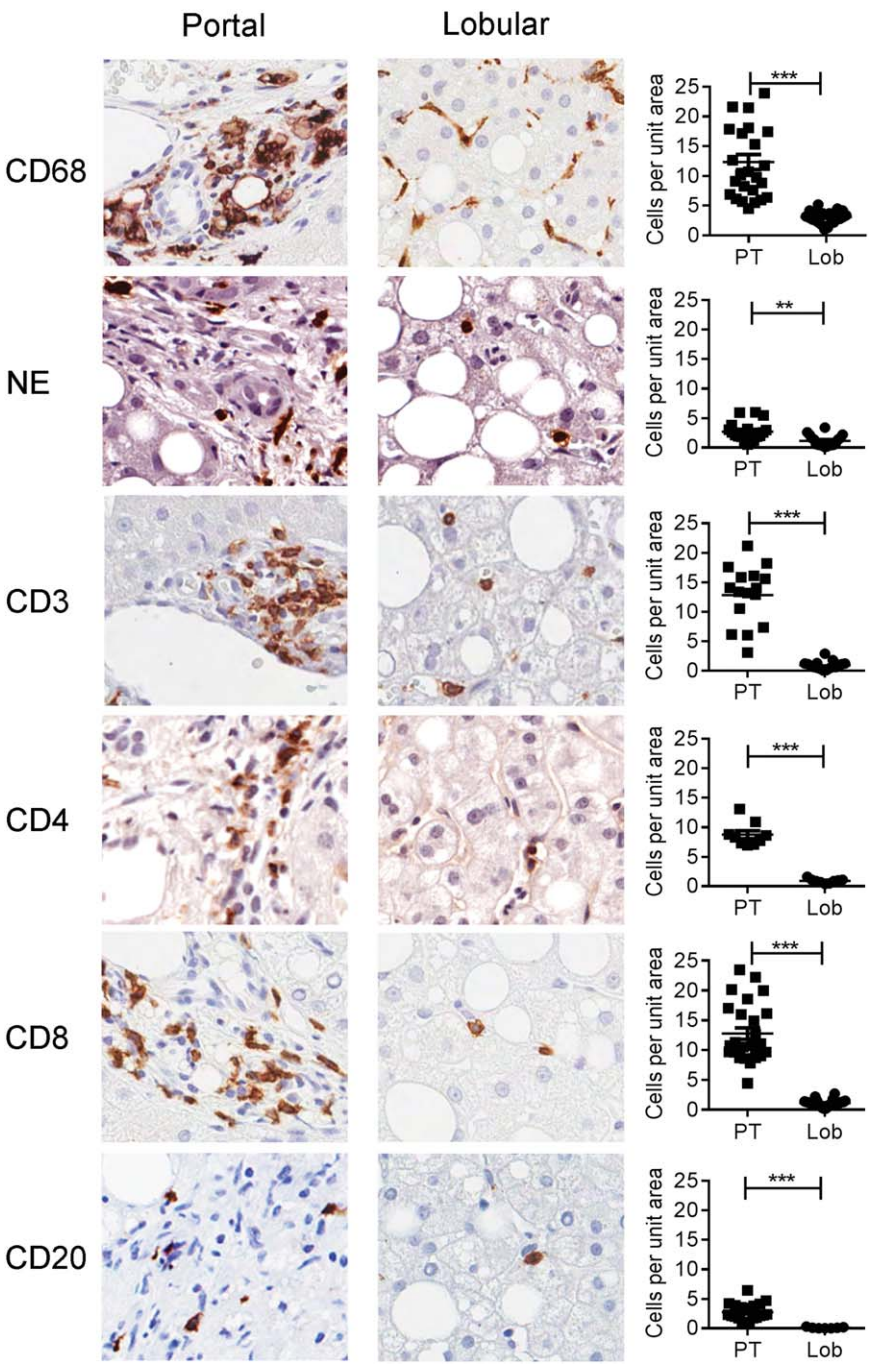

Fig. 2. Inflammatory cells are more numerous in the portal tracts than liver lobules of patients with NAFLD (original magnifications: 200×). Data are represented as mean ± standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$. PT, portal tracts; Lob, lobules.

it is certainly possible that their presence contributes to portal inflammation.

The HPC/Ductular Niche Includes a Complex Array of Nonparenchymal Cells. Inflammatory cells and their mediators have been shown to influence the behavior and differentiation of HPCs.[11,19] Cells of the portal inflammatory infiltrate were found in close proximity to the expanding DR in our cohort of patients with NAFLD (Fig. 4). Cells were not only present near the DR, but also made direct contact with strings of cholangiocytes from the canals of Hering as well as larger ductules. Excluding neutrophils and $CD4^+$ cells (because of suboptimal costaining with K7), all cell types within the periductular niche were significantly associated with increasing grade of DR (CD3 $r_s = 0.74$, CD8 $r_s = 0.81$, CD20 $r_s = 0.87$, CD68 $r_s = 0.77$; all $P < 0.05$) and stage of fibrosis (CD3 $r_s = 0.91$, CD8 $r_s = 0.86$, CD20 $r_s = 0.76$, CD68 $r_s = 0.70$; all $P < 0.001$). In contrast, the number of cells within centrilobular regions showed no correlation with portal features of disease progression, such as grade of DR or presence of portal fibrosis.

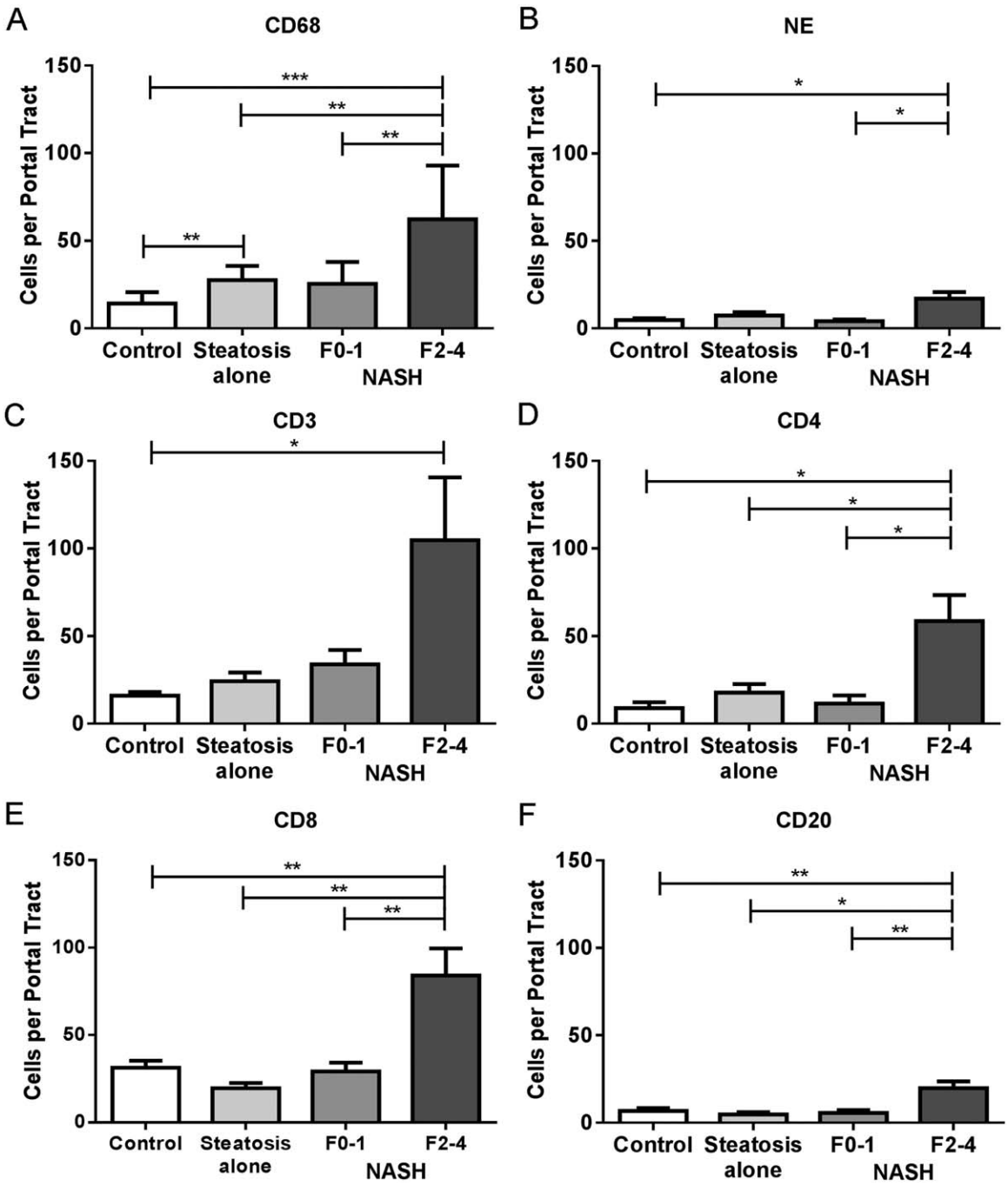

Fig. 3. Portal inflammation in NAFLD. Number of CD68 (A), NE (B), CD3 (C), CD4 (D), CD8 (E), and CD20 (F) cells per portal tract, labeled by immunostaining histologically graded NAFLD biopsy specimens. Data are represented as mean + standard error of the mean. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ F0-1, early NASH; F2-F4, progressed NASH.

A Proinflammatory Hepatic Environment Precedes Significant Portal Infiltration. To further investigate the hepatic microenvironment during NAFLD progression, we used qPCR to quantify whole-liver profibrogenic and -inflammatory cytokine gene expression. *TGFB1*, encoding the pro-fibrogenic cytokine transforming growth factor beta (TGF-β) was expressed, but did not change with disease stage (Fig. 5A). Messenger RNA (mRNA) levels of *CCL2*, encoding the macrophage chemoattractant, chemokine (C-C motif) ligand 2/monocyte chemotactic protein 1 (CCL2/MCP1), were elevated even in simple steatosis (Fig. 5B). Early inflammatory mediators that are frequently produced by activated macrophages, such as tumor necrosis factor (*TNF*) and *IL1B*, were increased in patients with early-stage NASH (as reported on previously[20,21])—preceding significant portal

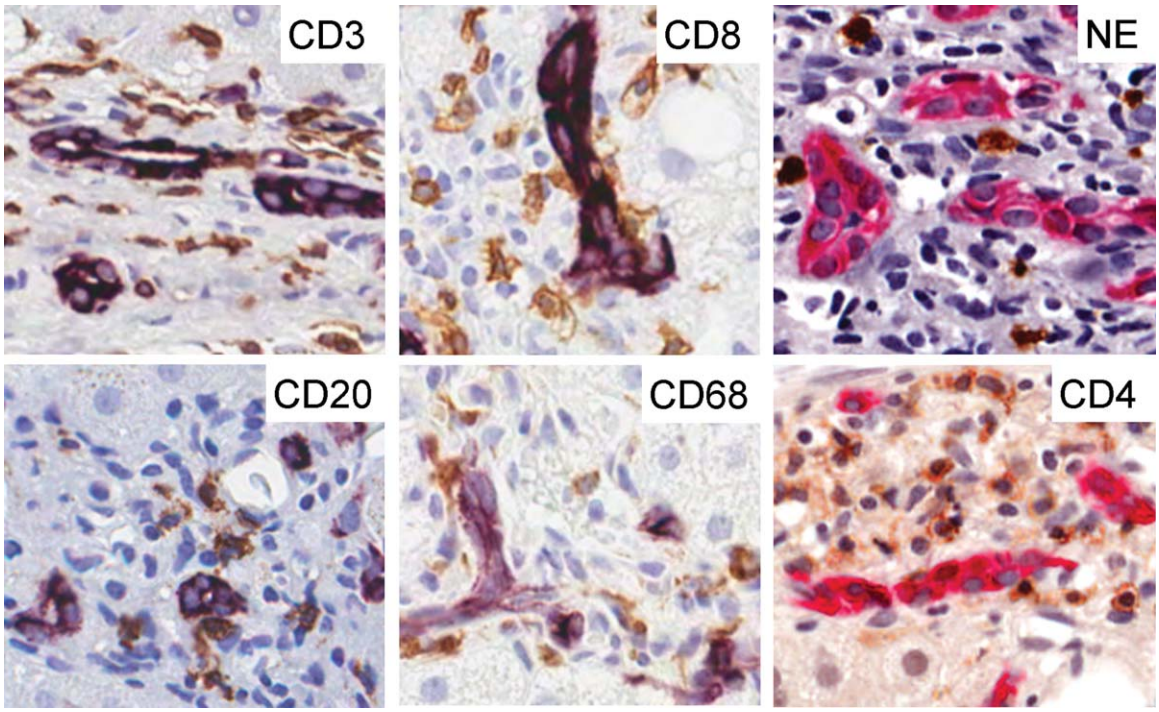

Fig. 4. The periductular niche contains an array of inflammatory cells. Localization of CD3-, CD8-, NE-, CD20-, CD68-, and CD4-labeled inflammatory cells (brown chromogen) to $K7^+$ ductular structures (purple or red chromogen) by double immunostaining histologically graded NAFLD biopsy specimens (original magnifications: 200×).

inflammatory infiltration of most types of leukocytes (Fig. 5C,D). By contrast, *IL6* and *IL8* expression increased in patients with progressed NASH (Fig. 5E,F). These changes are consistent with the hypothesis that resident and/or infiltrating macrophages respond to early triggers in NAFLD and their activation contributes to disease initiation and progression.

Subsets of Portal and Lobular Macrophages Express MMP-9. Having previously identified MMP-9 as a marker of a subset of activated portal macrophages in HCV infection,[16] we investigated MMP-9 expression in NAFLD. In contrast to HCV,[16] a subset of *both* portal (Fig. 6A) and lobular macrophages (Fig. 6B) expressed MMP-9 in patients with steatosis alone as well as patients with progressed NASH. Furthermore, centrilobular $MMP\text{-}9^+$ macrophages frequently formed crown-like structures, surrounding large steatotic hepatocytes and lipogranulomas (Fig. 6C).

Neutrophils Express IL-17 in Progressed NASH. Because of the prominence of lymphocytes in NAFLD livers throughout disease progression, and previous reports implicating specific lymphocyte subsets in fibrogenesis,[6,22,23] we investigated the expression of the signature cytokines produced by activated and polarized Th1, Th2, and Th17 cells. No difference in *IFNG*, *IL4*, or *IL10* expression was observed between steatosis alone and NASH with mild or severe fibrosis (data not shown). Although *IL17* mRNA was expressed at very low levels (below detection in the majority of patients), we performed IHC to further investigate this proinflammatory cytokine, because it has recently been implicated in human ALD,[22] viral hepatitis,[24] and in mouse models of CLD.[25,26] Immunolabeling for IL-17 highlighted the presence of occasional positive cells in the centrilobular regions; but, these cells were significantly more abundant within portal tracts of patients with NAFLD, including the periductular niche (Fig. 7A,B). Based on morphology, the majority of these cells are likely neutrophils. Upon quantification, $IL\text{-}17^+$ cells were significantly associated with stage of fibrosis ($r_s = 0.85$; $P < 0.001$; Fig. 7C), grade of DR ($r_s = 0.84$; $P < 0.001$), and presence of portal inflammation ($r_s = 0.83$; $P < 0.001$).

NASH and Alcoholic Steatohepatitis Display Histological and Inflammatory Similarities. No significant differences in stage of fibrosis, grade of DR, or grades of portal and lobular inflammation were observed between patients with progressed NASH and patients with progressed alcoholic steatohepatitis (ASH; data not shown). Similarly, $p21^+$ senescent hepatocytes and markers of the portal inflammatory infiltrate displayed similar numbers and distribution patterns (data not shown). ALD patients with steatosis alone were excluded from the portal infiltrate analysis

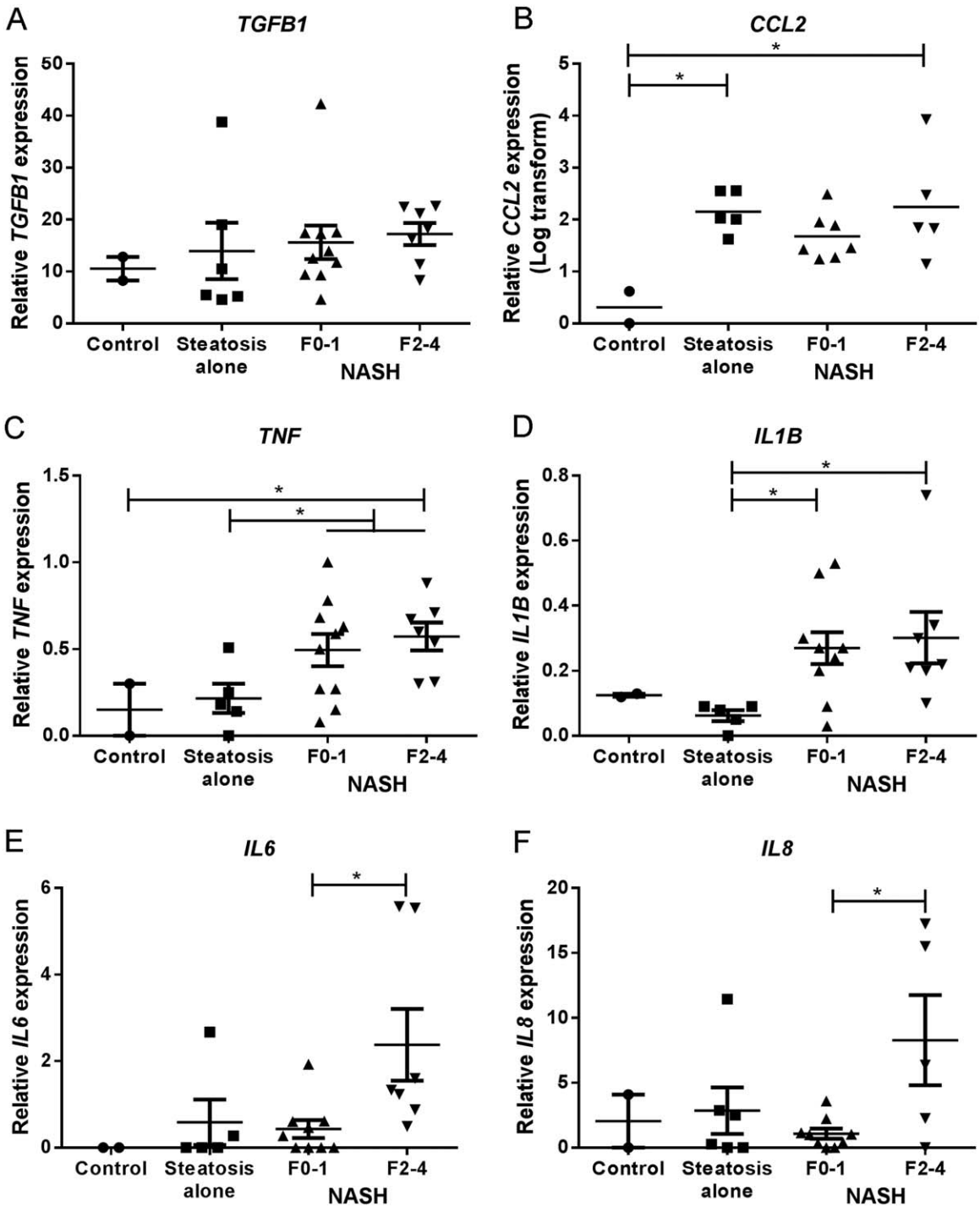

Fig. 5. A proinflammatory hepatic environment in patients with NAFLD. Whole-liver *TGFB1* (A), *CCL2* (B), *TNF* (C), *IL1B* (D), *IL6* (E), and *IL8* (F) mRNA expression was measured by qPCR in biopsy specimens from histologically graded NAFLD patients and in nondiseased liver tissue (control). Data are represented as mean ± standard error of the mean. $*P < 0.05$. F0-F1, early NASH; F2-F4, progressed NASH.

because of the small number (n = 3). Although the portal infiltrate did not significantly differ between progressed NASH and progressed ASH for any of the cell types analyzed, progressed ASH did not show a significant increase over controls for neutrophils or B lymphocytes (Supporting Fig. 2A-E).

Despite previous reports suggesting IL-17$^+$ cells are associated with ALD, we found that the average number of portal IL-17$^+$ cells did not increase between control subjects and patients with progressed ASH. Furthermore, patients with progressed NASH displayed greater numbers of portal IL-17-expressing

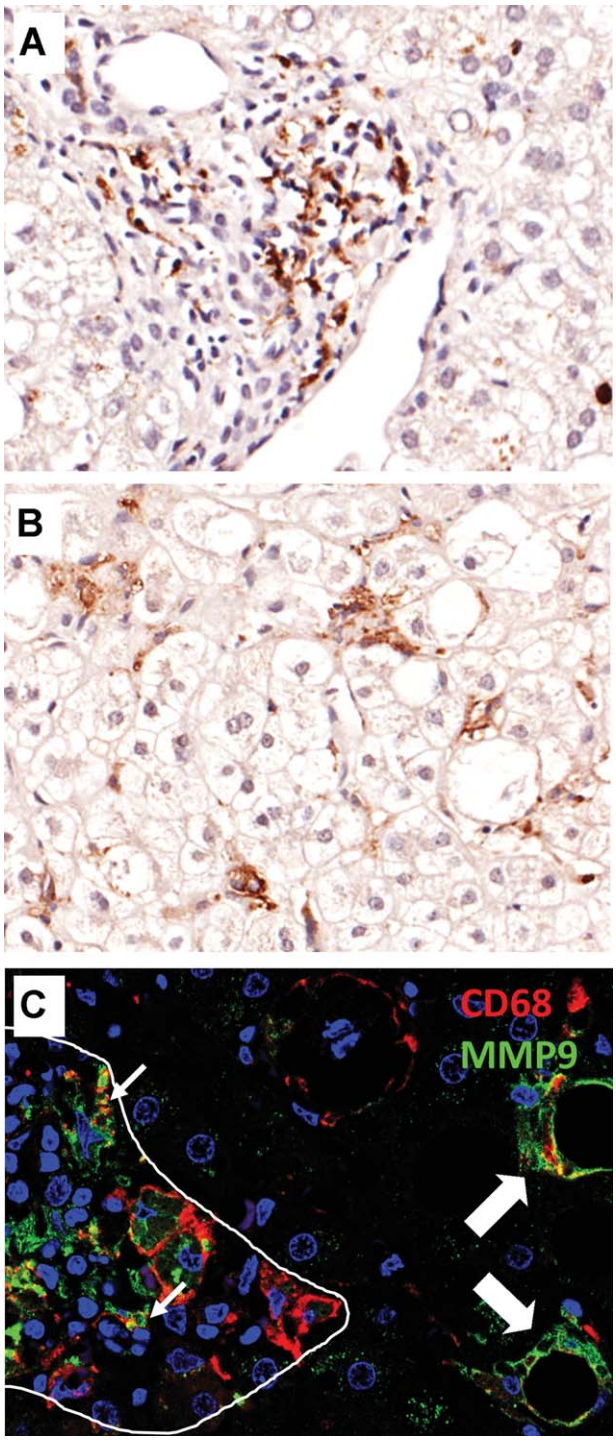

Fig. 6. Portal and lobular localization of MMP-9$^+$ macrophages in NAFLD. Immunolabeling for MMP-9 in portal (A) and centrilobular (B) regions in a representative NASH biopsy specimen. CD68 and MMP-9 double immunofluorescence confirming MMP-9 expression by a subset of portal (thin arrows) and lobular macrophages (C). Lobular MMP-9$^+$ macrophages are associated with large steatotic hepatocytes, forming crown-like structures (block arrows). (Original magnifications: A and B, 200×; C, 630×).

cells than patients with progressed ASH (Supporting Fig. 2F).

Discussion

Although portal inflammation is neither a diagnostic criterion for NAFLD nor a component of the NAS, it is reported to be associated with clinical and histological features of advanced disease. The current study was undertaken to profile the cells comprising the portal inflammatory infiltrate and their association with the DR and portal fibrosis. The findings demonstrate that a mix of inflammatory cells, including T cells, B cells, macrophages, and neutrophils, accumulate within portal tracts of NASH patients with portal fibrosis, supporting a role for portal inflammation in progressive disease. Despite differences in the underlying etiology of disease, the portal inflammatory infiltrate was similar in NASH and ASH patients, suggesting that these diseases may share inflammatory and fibrogenic mechanisms.

An important new finding of this study is the significant increase in portal macrophages in subjects with steatosis alone. In contrast, other inflammatory cell numbers did not show a significant increase over control subjects until portal fibrosis was present. The early accumulation of macrophages, and elevated expression of the proinflammatory cytokine mRNAs, *TNF* and *IL1B*, supports an initiating role for the innate immune system in NAFLD, but the specific stimulus for its activation is unclear. Portal macrophage accumulation was further amplified in steatohepatitis and was positively associated with hepatocyte ballooning and lobular inflammation, but not with the extent of steatosis or senescent hepatocyte burden. So, whereas lobular injury could plausibly trigger macrophage activation, the factors that recruit or retain macrophages, and subsequently other immune cells, specifically in portal tracts are unknown. Candidate chemoattractants include CCL2/MCP-1, chemokine (C-X3-C motif) ligand 1, and chemokine (C-X-C motif) ligand 12, which have been shown to be produced by inflamed biliary epithelia or proliferating ductules in inflammatory liver diseases.[27-29] We observed elevated *CCL2* expression in early-stage NASH, consistent with the early accumulation of portal macrophages. The CCL2/CCR2 (C-C chemokine receptor 2) axis has been implicated in the pathogenesis of CLD in some mouse models, but seems to be irrelevant in others.[30,31] Elevated hepatic and systemic CCL2 have been reported in human CLD, including NASH.[32] However, as we previously demonstrated in chronic HCV infection,[16]

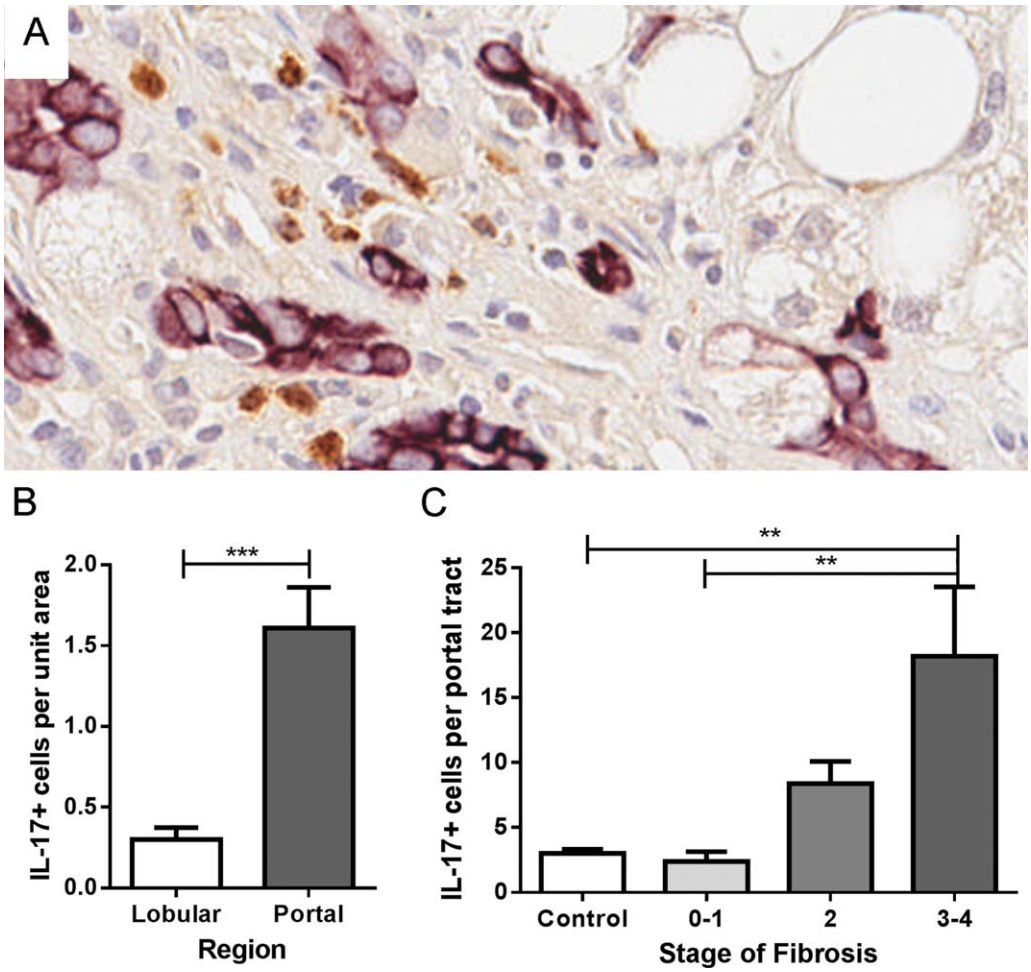

Fig. 7. IL-17$^+$ cells are a feature of the portal inflammatory infiltrate in NAFLD. Identification of IL-17$^+$ cells (brown) in portal and periductular (K7$^+$ ductular structures; purple) regions (A). Number of IL-17$^+$ cells per unit area (10,000 μm^2) in lobular and portal regions (B) and per portal tract in biopsy specimens from histologically graded NAFLD patients (C). Data are represented as mean + standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$ (original magnifications: A, 200×).

lobular, but not portal, macrophages expressed CCR2 in NASH (data not shown). This may suggest that CCR2 is not important for portal macrophage homing in human CLD or simply that the receptor is dynamically regulated in the inflammatory environment.

Although the most significant portal accumulation of inflammatory cells occurred in progressed NASH, the activated hepatic environment that arises early in disease—as evidenced by proinflammatory cytokine expression—likely influences the phenotype and function of resident cells. Having identified MMP-9 as a marker of a subpopulation of portal macrophages associated with fibrosis in chronic HCV infection,[16] we investigated its expression in NASH. MMP-9$^+$ macrophages were present in portal tracts of NASH patients, but, in contrast to HCV, also formed crown-like structures around large steatotic hepatocytes in centrilobular regions. MMP-9 functions in multiple pathways have been implicated in fibrogenesis and its resolution, and may play different roles at different stages of disease.[33,34] The role of MMP-9 in NASH progression is not known. However, the different localization of MMP-9$^+$ macrophages in NASH and HCV may correspond to different patterns of fibrosis in the two diseases, supporting the conclusion that MMP-9 acts locally to regulate tissue remodeling.

Portal inflammation can persist after surgical or drug treatment for NAFLD, even as steatohepatitis and fibrosis improve.[35-37] After rosiglitazone therapy, the ratio of portal to lobular inflammation increased, but actual portal inflammation severity was not specified.[35] Others found that semiquantitative portal inflammation scores did not change significantly.[36,37] Currently, it remains unclear how treatment of NASH affects the extent and composition of portal inflammation or whether inflammation associated with the DR changes. This warrants further study. Additionally, although the current study suggests that portal, rather

than lobular, inflammatory populations correlated more closely with fibrosis, until changes in portal inflammation post-therapy are better understood, it cannot yet be used as an endpoint in clinical trials of NAFLD treatment.

Portal, but not lobular, inflammatory cell populations were significantly associated with extent of periportal DR. Inflammatory cells form an important component of the DR—a complex of matrix and cells, including cholangiocytes, stem cells, and myofibroblasts. Inflammatory cells were not only present in the periductular niche, but also made direct contact with strings of K7-positive, immature HPCs. Cellular crosstalk between leukocytes, matrix, and other cells in the ductular niche may have a pivotal role in regulating both fibrogenesis and liver repair. Although HPCs are at least bipotential, capable of differentiating into hepato- and cholangiocytes, their capacity for epithelial to mesenchymal transition is controversial.[38] The context of the inflammatory niche may determine the outcome of their activation. In mouse liver injury models, for example, the capacity of activated HPCs to replace injured hepatocytes and restore liver function was dependent upon the type of liver injury and resulting DR.[39] The factors underpinning this heterogeneity are not known, but several candidates have been identified, some of which are produced by macrophages, including TWEAK (TNF-like weak inducer of apoptosis)[13,40] and WNT proteins.[11] Other inflammatory cell populations colocalizing with expanding and migrating HPCs are also likely to contribute regulatory and effector cytokines, including IL-17$^+$ cells, NK cells, and T lymphocytes.[22,25,26,41]

Whether HPC activation and proliferation directly drives portal fibrosis as well as liver regeneration is controversial.[10] In a recent study of liver regeneration in mice with carbon-tetrachloride–induced fibrosis, HPC expansion was accompanied by increased profibrogenic gene expression and *de novo* collagen deposition.[42] Blocking the HPC mitogen, TWEAK, inhibited HPC proliferation, prevented fibrogenic response, and increased hepatocyte replication.[42] However, it is unclear whether HPC and stellate cell activation and proliferation are interdependent or whether they occur in parallel as a result of a common stimulus (e.g., TWEAK) or to secretion of profibrogenic cytokines and growth factors from the DR or surrounding inflammatory cells.

In summary, we show that early macrophage infiltration and subsequent portal inflammation are key features of NAFLD progression. Portal, but not lobular, inflammatory cell populations were significantly associated with stage of fibrosis, suggesting that recruitment and consequences of portal and lobular inflammation should be considered separately, because different immunopathogenic processes may be involved. Understanding the cellular composition of the periductular niche and the cross-talk occurring within this niche will help shape functional studies to elucidate the cellular and molecular mediators of HPC differentiation, as well as the role of HPCs in liver fibrosis and repair.

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