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Production of Profibrotic Cytokines by Invariant NKT Cells Characterizes Cirrhosis Progression in Chronic Viral Hepatitis

Claudia de Lalla,* Grazia Galli,† Luca Aldrighetti,‡ Raffaella Romeo,§ Margherita Mariani,¶ Antonella Monno,* Sandra Nuti,† Massimo Colombo,§ Francesco Callea,† Steven A. Porcelli,* Paola Panina-Bordignon,¶ Sergio Abrignani,2‡ Giulia Casorati,2* and Paolo Dellabona2*

Invariant (inv)NKT cells are a subset of autoreactive lymphocytes that recognize endogenous lipid ligands presented by CD1d, and are suspected to regulate the host response to cell stress and tissue damage via the prompt production of cytokines. We investigated invNKT cell response during the progression of chronic viral hepatitis caused by hepatitis B or C virus infection, a major human disease characterized by a diffused hepatic necroinflammation with scarring fibrotic reaction, which can progress toward cirrhosis and cancer. Ex vivo frequency and cytokine production were determined in circulating and intrahepatic invNKT cells from controls (healthy subjects or patients with nonviral benign or malignant focal liver damage and minimal inflammatory response) or chronic viral hepatitis patients without cirrhosis, with cirrhosis, or with cirrhosis and hepatocellular carcinoma. invNKT cells increase in chronically infected livers and undergo a substantial modification in their effector functions, consisting in the production of the type 2 profibrotic IL-4 and IL-13 cytokines, which characterizes the progression of hepatic fibrosis to cirrhosis. CD1d, nearly undetectable in noncirrhotic and control livers, is strongly expressed by APCs in cirrhotic ones. Furthermore, in vitro CD1d-dependent activation of invNKT cells from healthy donors elicits IL-4 and IL-13. Together, these findings show that invNKT cells respond to the progressive liver damage caused by chronic hepatitis virus infection, and suggest that these cells, possibly triggered by the recognition of CD1d associated with viral- or stress-induced lipid ligands, contribute to the pathogenesis of cirrhosis by expressing a set of cytokines involved in the progression of fibrosis.


Approximately 6% of the world population is chronically infected by hepatitis B (HBV) or C (HCV) viruses, the most common causes of chronic liver diseases, thus posing a serious public health problem (1, 2). A major difference between the two viruses is that a relevant fraction (60–80%) of immunocompetent individuals infected by HCV (2) and only 1–2% of those infected by HBV (1) progress into chronic infection, characterized by the persistent necroinflammation of the liver. The morphologic modifications of the liver induced by the chronic infection with either HCV or HBV are difficult to distinguish: in both cases, the chronic necroinflammatory damage evolves into a fibrotic reaction, as the result of the host attempt to repair the damaged parenchyma with a scar (3). In ~10–20% and in 25–40% of patients with chronic HCV and HBV infection, respectively (1, 4), liver fibrosis progresses into cirrhosis, characterized by the disruption of liver architecture with fibrous septa and parenchymal nodules (5). Cirrhosis may evolve into severe complications such as liver failure or primary hepatocellular carcinoma (HCC) in a significant fraction of patients (6). Stellate cells, a heterogeneous group of cells of neural-crest origin resident in the subendothelial space of Disse, have been identified as the principal fibrogenic cells of the liver (3). Following liver injury of any etiology, hepatic stellate cells undergo activation and differentiation into proliferative, fibrogenic, and contractile myofibroblasts (3). Stimuli inducing stellate cell activation, such as reactive oxygen intermediates or cytokines, can derive from injured hepatocytes, endothelial cells, Kupffer cells, and infiltrating leukocytes (3). Among the cytokines implicated in the fibrotic response of the liver, TGF-β1, IL-4, and IL-13 appear to be the most potent ones (3, 7, 8). Factors concurring to (dis)regulate the fibrogenic response are still poorly understood, and cells responsible for the production of fibrogenic cytokines have not been univocally identified.

In mice, a peculiar T cell subset, defined as invariant (inv)NKT lymphocytes, is selectively enriched in liver, where it accounts for up to 25–30% of total infiltrating lymphocytes (9). In NK cells...
are remarkably conserved, and express a semi-invariant TCR, constituted by the homologous invariant Vα24-Jα18 and Vα14-Jα18 rearrangements in humans and mice, respectively, paired with junctionally diverse Vβ11 and Vβ8.2, Vβ7, and Vβ2 TCR chain in humans and mice, respectively (10, 11). invNKT cells are restricted for the non-MHC-encoded class I-like CD1d Ag-presenting molecule (12), and can be activated selectively by the xenogeneic glycosphingolipid α-galactosylceramide (α-GalCer), which binds CD1d (13). Nevertheless, invNKT display a remarkable autoreactivity for some endogenous ligands, possibly polar lipids, presented by the CD1d expressed on APCs, and can be activated to produce cytokines even in the absence of α-GalCer (14). Owing to a number of phenotypic and functional features, comprising their homogenous clonal distribution, innate memory phenotype, and immediately triggered effector functions, invNKT cells are considered to be at the interface of innate and adaptive immunity (9). A variety of animal and human studies has implicated invNKT cells in the regulation of infectious (15), tumor (16), and autoimmune conditions (17) associated with inflammation, tissue damage, and repair. Furthermore, activation and production of IL-4 and IFN-γ by intrahepatic invNKT cells have been correlated to the hepatic damage caused either by Con A administration (18) or by the genetic ablation of the suppressor of cytokine signaling 1 gene, also known as STAT-induced STAT inhibitor-1, a negative feedback molecule for cytokine signaling (19).

In light of these observations, we sought to characterize both circulating and intrahepatic human invNKT cells in chronic viral hepatitis, to understand whether they play a role at different stages of liver tissue disruption. Numbers and effectors functions of invNKT cells from chronically HCV- or HBV-infected patients were directly compared with those of invNKT cells obtained from patients with a pauci-inflammatory, localized tissue damage represented by either benign or malignant intrahepatic lesions without chronic viral infection.

**Materials and Methods**

**Patients**

Forty-two patients with serologically documented chronic HBV (n = 15) or HCV (n = 27) infection were divided in three groups according to disease severity, namely: 1) without cirrhosis (n = 10); 2) with cirrhosis (n = 10); and 3) with cirrhosis and primary HCC (n = 22) (Table I). All of the patients had elevated serum levels (>40 U/L) of alanine aminotransferase, and histological diagnosis of chronic hepatitis from at least 5 years. Staging of the disease progression was performed by hepatic biopsies, and the severity of tissue damage was evaluated on the basis of infiltrates activity and fibrosis based according to the Ishak-modified histology activity index (20) (Table I). Cirrhosis was assessed by the observation of micro- and/or macroregenerative nodules in biopsy specimens.

All of the cirrhotic patients analyzed in this study were in a compensated phase of the disease (child A). The presence of HCC was documented by ultrasound and spiral triphasic computerized tomography scans and, in surgical patients, by the histological analysis of the removed mass.

As controls, we enrolled 16 patients with hepatic pathologies not related with the hepatitis virus infection, known to associate with focal liver damage and minimal intrahepatic inflammatory response, namely: 1) benign lesions (n = 3: 1 acute intrahepatic lithiasis, 1 angioma, and 1 focal nodular hyperplasia); and 2) malignant lesions (n = 13: 1 cholangiocarcinoma, 2 primary HCC, 1 metastasis from gastric carcinoma, 1 metastasis from gastrointestinal stromal tumor, 1 metastasis from pancreatic cancer, and 7 metastasis from colorectal adenocarcinoma, all confirmed by histological analysis).

In both groups of patients, invNKT cells were obtained from peripheral blood and, in case of patients undergoing hepatic surgery, from liver. Surgical specimens were required because liver biopsies did not provide enough cells for reliable intracellular flow cytometry analysis of primary invNKT cells. invNKT cells from the peripheral blood of healthy donors were used as reference control (Table I).

For reasons not related to this study, all patients with chronic viral hepatitis had never received antiviral therapy or were off therapy from at least 6 mo before being analyzed, while patients with any form of neoplastic lesion were off therapy (regional or systemic) from at least 6 mo. The study was approved by the local ethical committee, and a written informed consent was obtained from all the patients studied.

**Preparation of PBL and intrahepatic lymphocytes (IHL)**

To obtain PBL, hepatic venous blood was separated by Ficoll-Hypaque (Pharmacia, Peapack, NJ) density gradient centrifugation. To purify IHL, surgical liver specimens were washed four times with PBS to get rid of contaminating blood, and then mechanically dissociated by cutting them into small fragments with sterile scalpels, followed by manual homogenization with a syringe plunger on a cell strainer with 70-μm-pore diameter (Falcon). The filtered cell suspension was collected in RPMI 1640 (Invitrogen Life Technologies) containing 5% of normal human serum (NHS) (Euroclone, Milan, Italy) and centrifuged for 20 min at 1500 rpm. Infiltrating mononuclear cells were separated from hepatocytes by Percoll (Invitrogen Life Technologies, Carlsbad, CA) density gradient centrifugation: the cell pellet was resuspended in 40% (v/v) Percoll in RPMI 1640 and layered onto 80% (v/v) Percoll in RPMI 1640. For ~10 g of tissue, 15 ml of 40% and 80% Percoll solutions was used. The gradient was centrifuged

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<tr>
<th>Table I. Quantitative characterization of T and invNKT cells in healthy donors and in patients *</th>
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<tbody>
<tr>
<td><strong>Subject analyzed</strong></td>
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<tr>
<td><strong>Age</strong></td>
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*Triple staining with anti-CD3, anti-Vα24, and anti-Vβ11 mAbs was used to determine the frequency of T cells (CD3+ or Vα24+Vβ11+) within total T cells, respectively.

*Median age in years with range in parentheses.

*Patients with chronic viral hepatitis were defined as noncirrhotic or cirrhotic on the basis of histological classification following Ishak-modified HAI (histology activity index) (19) as well as clinical parameters. Median grading (activity) and staging (fibrosis) scores of the hepatic disease were 7.5 (range 5–10) and 2.5 (range 1–4), respectively, for noncirrhotic patients, while they were 10 (range 5–15) and 5.5 (range 5–6), respectively, for the cirrhotic ones. The grading score is the sum of the parameters describing the activity: periportal or perisplenic interface hepatitis (piecemeal necrosis), 0–4; confluent necrosis, 0–3; focal lytic necrosis, 0–4; apoptosis and focal inflammation, portal inflammation, 0–4; with possible maximum score of 18. The staging score describes the fibrosis severity: no fibrosis, 0; fibrous expansion of some portal area, 1; fibrous expansion of some portal areas, 2; fibrous expansion of most portal areas, with occasional portal to portal bridging, 3; fibrous expansion of most portal areas, with marked bridging portal to portal as well as portal-central, 4; marked bridging with occasional cirrhotic nodules (incomplete cirrhosis), 5; cirrhosis, 6. The presence of liver cirrhosis was clinically assessed by ultrasound scan evaluation, demonstrating liver volume increase with irregular echographic pattern and spleen enlargement as well as platelet counts below 100,000, albumin below 4 g/L, and prothrombin time increase.

*PBLs were obtained from all healthy subjects and patients. IHLs were obtained in 12 of 16 patients with nonviral hepatic pathology, and in 14 of 22 patients with chronic viral hepatitis with cirrhosis and HCC.
for 10 min at 2000 rpm, and IHL were collected at the interface between 40 and 80%, washed once in RPMI 1640 with 10% NHS, and counted.

**Determination of invNKT frequency**

invNKT frequency in PBL and IHL was determined by flow cytometry using staining with cetin-conjugated anti-Vα24, FITC-conjugated anti-Vβ11 (both produced in house) (10), and allophyocyanin-conjugated anti-CD3 (BD Biosciences, San Jose, CA) mAbs, followed by CyChrome-conjugated streptavidin (BD Biosciences). Cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences), and results were analyzed using the CellQuest software (BD Biosciences). At least 2 × 10^6 gated lymphocytes were acquired for each sample.

**Characterization of intracellular cytokines expressed by invNKT cells**

Intracellular cytokine production was detected by four-color flow cytometry, as described (21), with some modifications. Briefly, IHL or PBL (1 × 10^7) were activated in RPMI 1640 with 10% NHS with 25 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml ionomycin (Sigma-Aldrich) for 1 h at 37°C in the presence of 5 U/ml human rIL-2 (Roche, Basel, Switzerland). Brefeldin A was added at 10 μg/ml (Sigma-Aldrich), and incubation continued for an additional 45 min in case of IHL, and for an additional 90 min for PBL. Cells were then stained with biotin-conjugated anti-Vα24, FITC-conjugated anti-Vβ11, and allophyocyanin-conjugated anti-CD3 mAbs for 10 min on ice, followed by CyChrome-conjugated streptavidin for 5 min on ice. Cells were then fixed in 1% paraformaldehyde (Sigma-Aldrich) for 5 min on ice, and permeabilized for 15 min at 4°C with 0.2% saponin (S-7700; Sigma-Aldrich). To overcome possible low avidity signals due to activation-dependent TCR down-regulation, mAbs specific for Vα24, Vβ11, and CD3 were also included during the intracellular staining step together with PE-conjugated mouse anti-IFN-γ, or rat anti-IL-4 or anti-IL-13 (BD Biosciences). Cells were washed with 0.2% saponin and incubated with CyChrome-conjugated streptavidin in 0.2% saponin for 5 min on ice. Cells were analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences), and results were analyzed using the CellQuest software (BD Biosciences). At least 10^6 T cells were acquired for each sample.

**Immunohistochemistry**

Frozen liver serial sections (5 μm) were fixed in 4% paraformaldehyde in PBS for 10 min, washed twice in PBS with BSA (Sigma-Aldrich) for 5 min at room temperature (RT), and incubated in 0.03% H2O2 in PBS for 10 min at RT. After washing in PBS, sections were incubated in avidin/biotin-blocking reagents (Vector Laboratories, Burlingame, CA) for 15 min at RT to block non-specific staining. Normal goat serum (Sigma-Aldrich) was then added at 0.05% in PBS for 1 h at RT. Each of the following purified anti-CD1d-42, CD1d-55, CD1d-42 (22), and anti-CD3 (BD Biosciences), mAb was added separately to each serial section at a final concentration of 10 μg/ml in 40 U/ml human rIL-2 (Roche). Sections were incubated for an additional 45 min in case of IHL, and for an additional 90 min for PBL. Cells were then stained with biotin-conjugated anti-mouse IgG1 (μG1) mAb at the final concentration of 10 μg/ml. In the mouse, the same CD1d-restricted invNKT cells that show positive staining with the CD1d-ααGalCer tetramers (23). As shown in Table I and Fig. 1, the percentage of invNKT cells found in PBL of all the patients examined was largely comparable with those of healthy donors. Furthermore, unlike the situation in the mouse, human liver intrahepatic invNKT cells were also low in frequency (Table I and Fig. 1), irrespective of the pathology affecting the target organ. However, whereas in patients with hepatic metastasis the frequency of invNKT cells was at least 3.4-fold higher in invasion patients with chronic viral hepatitis and 10 cancer patients without chronic viral hepatitis, and 3–4 sections for each of the 20 patients were analyzed.

**Confocal laser microscopy**

Frozen sections of liver specimens (5 μm) were fixed in 2% paraformaldehyde for 1 h at 4°C, washed twice in wash buffer (0.1 M MOPS, pH 7.4) for 5 min at RT, and then incubated with 50 mM NH4Cl for 10 min at 4°C. After washing, the sections were incubated in 5% NHS for 20 min at RT, followed by wash in PBS, incubated with avidin/biotin-conjugated reagents (Vector Laboratories, Burlingame, CA) for 30 min at RT. After washing, sections were incubated with anti-CD1d-42 (μG1) mAb at 10 μg/ml in dilution buffer, or anti-CD3-coupled goat anti-mouse IgG1, washed once in RPMI 1640 with 10% NHS, and washed once in RPMI 1640 with 10% NHS, and counted.

**In vitro activation of invNKT cells**

PBLs from healthy donors were cultured with 50 ng/ml α-GalCer (supplied by P.D. by Kirin Brewery, Gunma, Japan) in 40 U/ml human rIL-2 (Roche). At determined time points, cells were collected and resuspended with PMA + ionomycin and brefeldin A, as described above, followed by staining with anti-CD3, anti-Vα24, and anti-Vβ11 mAbs or with anti-CD3, anti-Vα24, anti-IFN-γ, and IL-4 or IL-13 mAbs to determine the frequency and intracellular cytokine expression of invNKT cells, respectively.

**Statistical analysis**

invNKT cell frequencies were evaluated with two-tailed Mann-Whitney U test; p values ≤0.01 were considered to be significant. invNKT frequencies producing intracellular cytokines were analyzed using two-tailed Student’s t test; p values ≤0.05 were considered to be significant.

**Results**

**Intrahepatic enrichment of invNKT cells in chronic viral hepatitis**

We first determined the frequencies of invNKT cells among total T lymphocytes contained in PBL and, when available, IHL freshly isolated from: 1) patients with chronic viral hepatitis; 2) patients with benign or malignant hepatic lesions without chronic viral hepatitis; and 3) healthy donors. invNKT cells were identified as Vα24^+Vβ11^+ double-positive cells in the CD3^+ gated region. In humans, this triple staining identifies the same CD1d-restricted invNKT cells that show positive staining with the CD1d-ααGalCer tetramers (23). As shown in Table I and Fig. 1, the percentage of invNKT cells found in PBL of all the patients examined was largely comparable with those of healthy donors. Furthermore, unlike the situation in the mouse, human liver intrahepatic invNKT cells were also in frequency (Table I and Fig. 1), irrespective of the pathology affecting the target organ. However, whereas in patients with hepatic metastasis the frequency of invNKT was at most 2.30 times greater than that in PBL, a significantly higher value for this ratio (p = 0.00185) was found in chronic hepatitis patients with cirrhosis and HCC, with an invNKT frequency in the liver 10.43 times greater than that of their corresponding counterparts (Fig. 1b).

Taken together, these results indicated that, despite being in low percentage among IHL, invNKT cells are significantly enriched in chronically inflamed livers as compared with noninflamed ones. invNKT cell switch to profibrotic cytokine production characterizes progression to cirrhosis.

invNKT cells are implicated in the regulation of the response to cell stress and tissue damage through the production of diverse cytokines. The above data thus raised the question as to whether invNKT cells could be involved in some way in the progressive
damage of liver tissue in the course of the chronic viral hepatitis. To characterize peripheral and intrahepatic invNKT cells, lymphocytes were isolated from peripheral blood of all categories of patients and from healthy donors (Table I). Total lymphocytes were immediately stimulated with PMA/ionomycin, and analyzed by flow cytometry for the intracellular expression of either IFN-γ, or IL-4 or IL-13 within the invNKT or T cell subsets. The major fraction of peripheral blood-derived invNKT cells produced IFN-γ, irrespective of whether they were obtained from healthy donors or patients, suggesting that this is the dominant cytokine made by this subset of NKT cells (Fig. 2). Nevertheless, there was some variation from one category of patients to the other, with patients with chronic viral hepatitis and cirrhosis notably exhibiting the highest frequencies of IFN-γ-producing invNKT cells, and those with hepatic metastasis the lowest. Unlike IFN-γ, however, IL-4 and IL-13 displayed a markedly different distribution among circulating invNKT cells in different patient groups. Both cytokines were in fact nearly undetectable in invNKT cells from healthy donors, from patients with hepatic lesions without chronic viral hepatitis, and 14 patients affected by chronic viral hepatitis with cirrhosis and HCC (n = 8 HCV; n = 6 HBV).

Because the effector functions of T lymphocytes are displayed in the target organs, we also compared the pattern of cytokines produced by primary intrahepatic invNKT cells obtained from patients with hepatic malignant lesions without chronic viral hepatitis, or patients with chronic viral hepatitis, cirrhosis, and HCC. As shown in Fig. 3, the majority of intrahepatic invNKT cells from both groups of patients produced IFN-γ, but only those from cirrhotic patients with HCC exhibited significant expression of IL-4 and IL-13.

Interestingly, circulating and intrahepatic conventional αβ T cells also showed a significantly increased frequency of IL-4-producing cells in cirrhotic patients (data not shown), confirming that progression toward cirrhosis is accompanied by the up-regulation of type 2 cytokines in conventional T cells (25, 26).

These data therefore show that invNKT cells modify substantially their effector potential with the progression to cirrhosis of chronic viral hepatitis, although independently of the presence of an intrahepatic neoplasm.

**CD1d is strongly expressed in cirrhotic liver**

To assess whether invNKT could undergo a TCR-specific activation in the liver, we determined the level of expression and cellular distribution of CD1d in liver sections, obtained from patients with chronic viral hepatitis at different stages of activity and fibrosis, as well as from patients with hepatic metastasis from gastrointestinal neoplasia, displaying little or no liver inflammation and fibrosis. Serial sections from liver tissues were analyzed for the presence of infiltrating T cells, identified with anti-CD3 mAb, as a direct measure of the degree of chronic inflammation, and for CD1d expression (Fig. 4). In noncirrhotic livers with benign lesions or metastasis from colorectal cancer, there was nearly any inflammatory T cell infiltrate within the parenchyma. CD1d expression was extremely rare too, and apparently confined within the infrequent lymphoid aggregates found in the intact parenchyma (Fig. 4, a and b). In patient with hepatic metastasis, however, some CD1d expression was present in the proximity of the fibrotic tissue surrounding the neoplastic lesion, in which some infiltrating T lymphocytes were also present, suggesting some degree of reactivity around the lesion (data not shown). By contrast, in chronically inflamed livers, the expression of CD1d was strong, and it increased in parallel with the progression of the inflammatory infiltrate and fibrosis, as shown by the representative immunohistochemistry analysis of hepatic tissue obtained from patients with chronic viral hepatitis and associated fibrosis with or without cirrhosis (Fig. 4, c–h). CD1d expression was localized in portal inflammatory infiltrates (c–f), as depicted by the presence of infiltrating T lymphocytes (d and j), as well as within the fibrous septa surrounding the cirrhotic lobules (g). The pattern of CD1d expression was quite comparable in chronic HBV- or HCV-infected livers (data not shown).

Collectively, these data show that CD1d is highly expressed in the liver of chronic viral hepatitis patients with progressing fibrosis. CD1d is not only distributed within the portal inflammatory infiltrates, but also in fibrotic tissue, suggesting that it might be a molecular signature of the tissue damage progression.

**CD1d is expressed by APCs infiltrating liver parenchyma and on cells juxtaposed to hepatocellular stellate cells**

To obtain a more precise definition of CD1d-expressing cells in the cirrhotic liver, confocal laser microscopy was performed on sections by costaining with CD1d and one of the following markers: CD3 for T cells, CD20 for B cells, CD68 for macrophages, DC-LAMP for DC, and α-SMA for stellate cells (3). The costaining...
experiments showed that CD1d and CD3 were expressed by different cells within the portal inflammatory infiltrate (Fig. 5, a–c). By contrast, a conspicuous fraction of B cells, either in the periportal lymphoid aggregates (Fig. 5, d–f) or scattered in the fibrotic areas (data not shown), coexpressed CD1d. In the portal infiltrate, the majority of CD68+ macrophages coexpressed CD1d (Fig. 5, g–i), as well as a sizeable fraction of CD1d molecules colocalized with DC-LAMP on DCs, mainly in portal lymphoid aggregates (Fig. 5, j–l). However, most of CD68+ macrophages contained within either the fibrous septa or the hepatic parenchyma did not express CD1d, while DC-LAMP+ DCs were substantially absent from these two locations (data not shown). We also determined whether stellate cells, the principal fibrogenic cell of the liver, expressed CD1d. Cooexpression of cirrhotic liver sections with CD1d and α-SMA did not reveal coexpression of the two molecules within the same cells (Fig. 5, m–o). Interestingly, though, cells expressing either molecule were found in close proximity and specifically situated within the fibrous septa juxtaposed to the regenerating lobule, suggesting a possible indirect effect of CD1d recognition by invNKT cells in the activation of bystander stellate cells.

In any of the examined tissue sections, however, we were unable to find CD1d expression on hepatocytes, at a variance with what has been reported in the mouse (28, 29).

Together, these findings document that APCs express high levels of CD1d in parallel with the progression of inflammation and tissue damage.

**In vitro activated invNKT cells acquire the effector functions found in cirrhosis**

The increasing expression of CD1d in the liver during chronic viral hepatitis could be responsible for the sustained Ag-dependent stimulation of invNKT cells, accounting for the cytokine switch documented in cirrhotic patients. We therefore tested whether invNKT cells could be induced to switch toward the production of fibrogenic cytokines, upon activation in vitro with the specific CD1d ligand α-GalCer and low doses of IL-2. As shown in Fig. 6a, primary invNKT cells from the peripheral blood of healthy donors modified progressively their cytokine pattern, going from the expected high IFN-γ, low IL-4, and no IL-13 production found immediately after their purification, to the high IFN-γ, high IL-4, and high IL-13 production found as early as 1 wk of culture, and reminiscent of the one exhibited by primary invNKT cells from cirrhotic patients. Interestingly, in vitro activated invNKT cells expressed either IFN-γ alone, or both IFN-γ and IL-4 or IL-13, indicating that these cells assume either a type 1 or a type 0 cytokine profile, but only rarely a pure type 2 one (Fig. 6b). The cytokine pattern found in primary invNKT cells from cirrhotic patients could thus reflect a previous Ag-driven activation event in vivo.

**Discussion**

We have found that invNKT cells respond to the progressive liver damage caused by chronic hepatitis virus infection. invNKT cells are in fact significantly enriched, relative to peripheral blood, in the chronically inflamed organs as compared with the noninflamed ones. Moreover, invNKT cells undergo a substantial modification in their effector potential, which characterizes the cirrhotic state, consisting in the capacity to produce IL-4 and IL-13 in addition to IFN-γ. These functional changes in invNKT cells are present in both chronic HBV and HCV infections, suggesting that these cells respond to factors released as a consequence of tissue damage rather than to viral Ags.
Our findings confirm that the human liver harbors significantly less invNKT cells than the mouse one (23, 24, 30–32), suggesting the possibility that the two species differ in some major molecular pathways involved in the migration and/or the accumulation of invNKT cells in this organ. Furthermore, the data presented in this study underscore the migratory capacity of invNKT cells toward inflammatory stimuli, a property described for these cells in animal models of bacterial infection as well as in primary biliary cirrhosis, a human autoimmune disease characterized by chronic inflammation state of the liver (32, 33). In line with this property, invNKT cells constitutively express functional receptors for inflammatory chemokines (34), and the expression of the receptors for the inflammatory chemokines IFN-γ-inducible protein-10 and liver and activation-regulated chemokine is maintained in the cells isolated from the cirrhotic livers (C.d.L., P.D., and G.C., unpublished data).

Migration and accumulation of invNKT cells into inflammatory sites do not require the presence of CD1d molecules (33); however, CD1d expression at the inflammation site may be crucial for the display of effector functions of these cells locally. invNKT cells have been defined “autoreactive by design” (35), as they recognize endogenous ligands, presumably polar lipids, bound to CD1d. These endogenous ligands could be unleashed in target organs or by signals triggered by the inflammatory stimuli and/or cell death, two conditions fulfilled in chronic viral hepatitis. Accordingly, we have found a strong expression of CD1d in chronically inflamed livers, and documented its increase with the progression of both fibrosis and parenchyma damage. The colocalization studies show that CD1d is indeed found mainly on B cells and macrophages infiltrating the portal space or along the fibrous septa, or on DC in the portal space. Because several studies show that invNKT cells recognize in vitro DC and B cells in a self-reactive, CD1d-specific manner (21, 36–38), it is conceivable that this interaction may very well occur in vivo. In light of these considerations, we propose that the cytokine switch in invNKT cells observed in cirrhosis would reflect the sustained activation of these cells consequent to the described high CD1d expression, possibly in association with inflammation-induced CD1d ligands. Moreover, a reciprocal influence between invNKT cells and APCs has been described and may also take place in chronically inflamed liver; for instance, invNKT cell-dependent activation of B cells might take part in the pathogenesis of both cryoglobulinemia and B cell lymphoma, which occurs in some chronic HCV infections. The observed switch of invNKT cells infiltrating the cirrhotic liver to produce IL-4 and IL-13, two important growth factors implicated in the invNKT-dependent help of B cells (21), would further...

**FIGURE 3.** Increased production of profibrotic cytokines by intrahepatic invNKT from cirrhotic patients. Intracellular IFN-γ, IL-4, and IL-13 accumulation in intrahepatic invNKT cells obtained from patients with hepatic metastasis from colorectal cancer without chronic viral infection (n = 5), or patients affected by chronic viral infection with cirrhosis and HCC (n = 5). Total IHL were stimulated and stained, as described in Fig. 2. a. Representative dot plots from invNKT cells from individual patients are shown. Numbers indicate the percentage of cells expressing the given cytokine. b. Overall percentage of intrahepatic invNKT cells from the two groups of patients expressing the given intracellular cytokine. Statistically significant difference between the percentage of invNKT cells expressing a given cytokine in chronic viral hepatitis vs nonchronic viral hepatitis patients is indicated by asterisks, with the following values: *, p = 0.01; **, p = 0.001; and ***, p = 0.0001.

**FIGURE 4.** CD1d expression in livers with or without chronic viral hepatitis. Frozen liver serial sections were stained with anti-CD1d (a, c, e, and g) or anti-CD3 (b, d, f, and h)-specific mAbs. A representative immunohistochemistry analysis of hepatic tissues from patients with: a and b, hepatic metastasis from colon adenocarcinoma without chronic viral hepatitis. Only rare CD1d-expressing cells are observed in the liver parenchyma differentiated by design “autoreactive” (a, arrow) and inset, localized in a modest lymphoid aggregate related to intrahepatic blood flow stasis (b); c and d, chronic HBV hepatitis with fibrosis and HCC, without cirrhosis (27). Shown is a portal inflammatory infiltrate (PI). Both CD1d- and CD3-expressing cells are in the portal infiltrate, but not in the liver parenchyma; e–h, chronic HBV hepatitis with active cirrhosis and HCC (27). Shown are a portal inflammatory infiltrate (PI) with surrounding fibrosis (e and f), or a portal fibrosis (PF) with fibrous septa (FS) and cirrhotic nodules (CN) (g and h). CD1d is expressed by cells within the portal infiltrate, as well as by cells within the fibrous areas. At variance with T cells, no CD1d-expressing cells are present in the liver parenchyma. (Original magnification ×10.)
support a possible pathogenetic role of invNKT cells in these HCV-related immunopathologies.

We cannot discount also the possibility that environmental factors present in the chronically inflamed liver may facilitate the cytokine change occurring in these cells. An interesting candidate could be IL-18, which can induce type 2 cytokines in murine invNKT cells, concomitant to the maintenance of sustained IFN-γ production, in the presence of invTCR engagement and of limiting amount of IL-12 (39). IL-18 is indeed found abundantly expressed during chronic viral hepatitis and cirrhosis (40), supporting its possible role as type 2 cytokine inducers in human invNKT cells.

In line with our results, primary human invNKT cells from healthy donors have been found biased as a whole toward Th1 cytokine profile; however, the CD4+ and CD4- invNKT subsets exhibit a Th0 and Th1 cytokine profile, respectively (41, 42). We could not find significant variation in the CD4+ vs CD4- subset distribution in cirrhotic patients compared with healthy donors (C. de Lalla, unpublished results). Thus, the increased frequency of Th0 invNKT cells found in cirrhotic patients cannot be simply related to an expansion of the CD4+ subset.

Reversible qualitative changes in invNKT cells have been described in patients with advanced prostate cancer (43), or in association with the clinical progression of multiple myeloma (44). In both tumors, however, the change consisted in the loss of IFN-γ, with the acquisition of IL-4 in the prostate cancer only, indicating that the modification of the invNKT cell effector functions in these pathologies is substantially different from those found in chronically infected liver with cirrhosis. This would indicate certain plasticity in invNKT cell responses, which might not univocally differentiate toward an invariable effector phenotype.

What are the possible effects of type 2 cytokines produced by invNKT cells on the pathogenesis of cirrhosis? The pathologic hallmark of cirrhotic progression is the sustained inflammatory liver damage, with necrotic tissue replaced by the host wound healing response accompanied by the disordered attempt to regenerate

**FIGURE 5.** In the cirrhotic liver, CD1d is expressed by DC, B cells, macrophages, and unidentified cells juxtaposed to stellate cells. Shown are representative cases of chronic HBV infection with active cirrhosis and HCC. Sections were stained with anti-CD1d mAb in red (a, d, g, j, and m) and one of the following markers in green: CD3 for T cells (b), CD20 for B cells (e), CD68 for macrophages (h), DC-LAMP for DC cells (k), and α-SMA for stellate cells (n). Colocalization is shown in yellow (c, f, i, l, and o): CD1d is almost completely excluded from CD3+ T cells in portal infiltrates (a–c), and very infrequent colocalization is observed (arrowhead). Note that CD1d is expressed on cells often surrounding biliary ductules (c, inset). CD1d is heavily coexpressed with CD20 by B cells in lymphoid aggregates flanking the fibrous septa (d–f). At the interface between fibrous septa and lymphoid aggregate CD1d is expressed by CD68+ macrophages (g–i). A small fraction of CD1d molecules colocalizes with DC-LAMP (j–l) on DC cells in lymphoid aggregates. CD1d+ cells are also closely juxtaposed to stellate cells within fibrous septa (m–o). (Original magnification ×40.)

**FIGURE 6.** invNKT cells from healthy donors produce IL-4 and IL-13 upon CD1d-dependent activation in vitro. invNKT cells from the peripheral blood of healthy donors were activated in vitro with the CD1d-restricted ligand α-GalCer in the presence of 40 U/ml, and their pattern of intracellular cytokine expression was determined by four-color flow cytometry at the indicated time points, as described in Materials and Methods. One representative experiment of three is reported. a, Both frequency of invNKT cells expressing either IFN-γ, or IL-4 or IL-13, and of invNKT cells among total T cells are indicated. Day 0 of culture indicates primary invNKT cells, activated ex vivo with PMA + ionomycin and brefeldin A. b, Intracellular cytokines produced by invNKT cells at day 21 of activation. Total lymphocytes were activated with PMA + ionomycin and brefeldin A, stained with anti-CD3 and anti-Vα24 mAbs, permeabilized, and stained with anti-IFN-γ and anti-IL-4 or anti-IFN-γ and anti-IL-13. The percentage of cells coexpressing each pair of cytokines is shown in the quadrants. All of the cells stained by anti-CD3 and anti-Vα24 mAbs also expressed Vβ11 (data not shown).
the hepatic lobule. We propose that the production of type 2 cytokines by invNKT cells at a peculiar stage of the chronic viral disease plays an active role in promoting this scenario. One of the most significant functions of IL-4 and, especially, of IL-13 is in fact the promotion of wound healing and fibrosis, to counteract necroinflammatory responses (7). IL-4 and/or IL-13 lead to increased deposition of collagen fibers in the extracellular matrix by inducing the expression of the enzymes arginase I and II, responsible for the synthesis of ornithine-derived proline, an essential precursor for the production of collagen, with the concomitant suppression of NO generation (7). IL-13 is a potent direct inducer of collagen production in fibroblasts (7); moreover, it exerts an indirect profibrotic role by inducing TGF-β, which is a major profibrotic cytokine (7). Blocking IL-13 action with a soluble chimeric IL-13R-Fc molecule indeed counteracts the fibrotic reaction occurring in the liver of mice infected by Schistosoma mansoni, a clear example of hepatic inflammation and fibrotic response evolving from an early type 1 into a sustained type 2 cytokine-type response (7). IL-4, in contrast, acts primarily as an indirect profibrotic signal, possibly by regulating the expression of TGF-β (8), although it may stimulate synthesis of the extracellular matrix proteins by human dermal fibroblasts in vitro (45).

As already discussed, a peculiar aspect of the hepatic fibrosis is that it entails the activation of stellate cells, which are the main profibrotic cells in the liver (3). Our confocal analysis rules out CD1d expression on stellate cells, yet it reveals the expression of CD1d in some unidentified cells situated in close contact with stellate cells. The recognition of CD1d expressed on these yet unidentified cells could trigger invNKT cells to produce type 2 profibrotic cytokines, which in turn act on the adjacent stellate cells to activate, with other factors, their fibrogenic program.

The cytokine switch in circulating invNKT cells is already present in cirrhotic patients who have not yet developed primary HCC, and is maintained when HCCs develop, suggesting that the acquisition of IL-4 and IL-13 production by invNKT cells precedes the tumor onset. Recently, IL-13 produced by CD1d-dependent NKT cells was shown to induce a TGF-β-dependent suppression of antisarcoma immune response in a mouse model (46). Thus, the invNKT cell-dependent cytokine network that we implicate in the cirrhosis progression of chronic viral hepatitis might also play an immunosuppressive role, favoring in some way the development of HCC.

In conclusion, our findings reveal a possible pathogenic role of CD1d-dependent invNKT in chronic viral hepatitis: consistent with their sentinel role in the immune system, invNKT cells may be activated by CD1-dependent stimulation to halt the tissue injury through the production of type 2 cytokines. The persistent production of type 2 cytokines, however, may trigger excessive healing and contribute to the progression of the fibrotic process toward cirrhosis and, possibly, also to HCC outgrowth. Targeting type 2 cytokines could thus be of clinical benefit in liver cirrhosis.


