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Hepatic CD1d Expression in Hepatitis C Virus Infection and Recognition by Resident Proinflammatory CD1d-Reactive T Cells

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A subset of CD161+CD56+ NKT cells can recognize glycolipids presented by CD1d and positively or negatively regulate inflammatory responses, including those implicated in several models of hepatitis. CD1d is expressed at very low levels in the healthy liver, but there is a large fraction of CD161+CD56+ NKT cells. There are high levels of nonclassical proinflammatory hepatic CD1d-reactive T cells in hepatitis C virus (HCV) infection. Hepatic inflammatory cells and biliary cells adjacent to portal tract fibrotic areas of HCV-infected donors specifically up-regulated CD1d. A hepatocyte cell line expressing minimal CD1d was efficiently recognized by hepatic CD1d-reactive T cells, suggesting a role for these cells in disease. Hepatic CD1d-reactive T cells from HCV-positive as well as negative donors produced large amounts of IFN-γ with some IL-13, but only rarely detectable IL-4. We confirmed large numbers of hepatic CD161+ T cells, lower levels of CD56+ T cells, and small numbers of classic invariant NKT cells. However, hepatic CD1d-reactivity was not restricted to any of these populations. We suggest virally infected hepatic cells can process potent CD1d-presented liver Ag(s), for surveillance by resident Th1 hepatic CD1d-reactive T cells. This process may be beneficial in acute viral clearance, but in chronic infection could contribute to liver injury.


T he CD1d molecule is a MHC class I-like nonpolymorphic glycolipid Ag-presenting molecule originally identified as highly expressed on thymocytes (1). Other forms of CD1d are constitutively expressed by cells of the innate and adaptive immune system, such as monocytes or macrophages, dendritic cells, and B cells, as well as in the gut, but not by resting human T lymphocytes (2–6). Intracellular CD1d is expressed at very low levels by hepatocytes in the healthy liver (2–5) and has been reported to be up-regulated by hepatocytes and cholangiocytes in certain liver diseases (7).

CD1d-reactive NKT cells, which share features of both innate and acquired immune systems, can rapidly secrete very large amounts of Th1 and/or Th2 cytokines, depending on anatomical origin and donor health status, and also display CD1d-specific cytotoxic activity (8–16). Several distinct populations of CD1d-reactive cells have been identified (17–25). The functional capabilities of these cells are as diverse as the cell surface markers used to identify them. “Invariant” NKT cells, the first CD1d-reactive cells described from both rodents and humans, express an invariant TCR α-chain rearrangement, as well as restricted Vβ chains (1, 8–13) and receptors otherwise expressed by NK cells such as CD161 (26). These cells are specifically stimulated by α-galactosylceramide (αGalCer), a marine sponge-derived lipid, absent in higher species (9, 11, 12). One or more αGalCer-related glycolipid ligands are likely presented to and stimulate invariant NKT under a range of pathological conditions (1, 9, 11–13, 27, 28), but major endogenous Ags have yet to be identified.

Various roles for the CD1d-reactive subset of CD1d-reactive NKT cells have been suggested based on their ability to influence initiation and direction of ongoing immune responses via Th1 and/or Th2 cytokines (1, 8, 11–13, 29, 30), their association with resistance to certain pathogens (12, 31–36), and their potential NK-like or CD1d-specific cytotoxicity (14–16). Physiological and vaccine-based antitumor responses have also been shown to be dependent on CD1d-reactive NKT cells in multiple model systems through IL-12 induction, direct cytotoxicity, and effector cell activation (1, 11, 12, 14, 16, 28, 37). CD1d-reactive NKT cells can activate dendritic cells and other APCs, lymphocytes, and NK cells (37–43) and are essential for optimal responses to certain viral infections (31, 33–35). Furthermore, CD1d recognition by Vγ4+ T cells is associated with viral myocarditis and autoimmune sequelae of otherwise successful anti-picornaviral responses (31, 44, 45). Remarkably, CD161+CD56+ phenotypically NKT cells can represent >30% of normal murine and human liver lymphocytes (1, 7, 8, 11, 46–59). The majority of murine hepatic NKT cells are CD1d-reactive invariant NKT cells (1, 8, 11–13, 60, 61). However, in the human liver, only a small minority of these phenotypically NKT cells are CD1d-reactive invariant T cells (24, 54, 59, 62, 63). Instead, CD1d-reactive T cells, which in the mouse may be

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CD161+ or CD161−, and with more diverse TCR dominate certain anatomical locations such as the murine spleen and bone marrow (17–22), human bone marrow (23), and human liver (24). Interest in CD1d-reactive T cells has been stimulated by the observations of their distinctive pro- or anti-inflammatory phenotypes in these different locations and in health vs disease (1, 7, 11–14, 27–38, 50, 51).

Several lines of evidence indicate a role for the CD1d-mediated Ag presentation pathway in the regulation of intrahepatic immune responses. Murine hepatic CD1d-reactive NKT cells can cause hepatitis in several model systems (46–51). Notably, aGalCer reduces viral replication and noninvariant CD1d-reactive NKT cells are activated in hepatitis B virus (HBV) transgenic models (50, 51). Recently, we have found high levels of reactivity to CD1d transfectants among intrahepatic T lymphocytes (IHL) derived from eight mild chronic HCV-infected patients (24). This lymphocyte population was also enriched for CD3+ CD161+ lymphocytes (24). However, very low levels of truly invariant NKT cells are found in the HCV+ liver (24, 54, 55, 57, 62, 63). CD3+ CD56− cells also represent a considerable fraction of the IHL repertoire, in both health and disease (52–59). These liver NKT cells are decreased in HCV-infected subjects with severe chronic disease (58), and their depletion has been implicated in the progression of HCV-related liver disease toward hepatocellular carcinoma (57).

HCV profoundly influences conventional hepatic T cells (64–68). Whether HCV and stage of disease modulate the expression of CD1d or hepatic CD1d-reactive T cells is unknown. Nor is it known whether CD1d-reactive IHL can recognize distinct hepatic forms of CD1d. The actual relationship between CD161+ and CD56− liver NKT and CD1d-reactive IHL has not been determined, and is not known whether these cells represent functionally distinct subsets. We now report high levels of hepatic functionally CD1d-reactive NKT cells from subjects with variable degrees of HCV liver injury and uninfected donors. Furthermore, hepatic CD1d expression is markedly elevated in HCV infection and concomitantly hepatic CD1d-reactive T cells respond strongly to hepatocyte cell line CD1d. Recognition of CD1d by IHL from HCV-negative donors and in mild HCV-mediated disease was accompanied by selective production of IFN-γ as well as some IL-13, but little or no detectable IL-4. However, cirrhotic liver CD1d-reactive T cells could also make IL-4. Significantly, hepatic CD1d reactivity was not limited to either CD161+ or CD56− phenotypically NKT cells or to classic invariant NKT cells. The results suggest that Th1-biased noninvariant resident CD1d-reactive IHL respond to increased hepatic CD1d in human hepatotropic infections with potentially protective as well as pathologic consequences.

Materials and Methods

Study subjects

Liver tissue was obtained at diagnostic biopsy from HCV seropositive patients with detectable HCV RNA, a range of fibrosis, absence of other comorbidities such as HBV, HIV, iron overload or alcohol abuse, and before treatment with either IFN or ribavirin. Only noted subjects had cirrhosis. Further HCV+ and control liver samples from donors with no clinical or histologic evidence of liver disease, with colonic carcinoma metastasis, or with nonviral liver diseases (alcoholic cirrhosis, nonalcoholic fibrosis) were obtained from the National Cancer Institute-supported Cooperative Human Tissue Network. All studies were approved by the Beth Israel Deaconess Medical Center (Boston, MA) Committee on Clinical Investigations.

Abs and other reagents

Abs used, including CD1d-specific mAb and invariant Vα24-Jα18 TCR chain-specific mAb 6B11 as well as FACS analysis have been previously described (6, 10, 23, 24, 38). Blood-derived CD1d-reactive invariant NKT cell controls, and human mock and CD1d transfectants were obtained and used as described (10, 23, 24, 38). WIF-B9 cells were also used as stimulators in CD1d-reactivity assays. WIF-B9 is a highly polarized well-differentiated and fully characterized hepatocyte cell line, which stably expresses structural and functional features of normal human hepatocytes (69). WIF-B9 were transfected with human CD1d as previously described for other hepatocyte proteins, CD1d+/WIF-B9 subclones, and mock transfectants cultured as described (69).

Immunochemistry and immunoprecipitation

Frozen tissue was treated with OCT (Sakura, Torrence, CA), lightly acetone fixed and stained with anti-human CD1d mAbs CDS5 and 51.1 or isotype control mAbs as previously described for human thymus (6). Anti-mouse IgG-HRP second reagents and substrate were used to visualize Ags (Vector Laboratories, Burlingame, CA).

Liver and WIF-B9 CD1d were analyzed by immunoprecipitation and Western blot analysis essentially as previously described (6). Briefly, Triton X-100 extracts of snap-frozen liver were incubated with anti-CD1d CDS5 or control mAb covalently coupled to 1-hydroxy succinimide-activated Sepharose beads (Amersham Biosciences, Piscataway, NJ), washed, and eluted under nonreducing conditions. After reducing Ig-free precipitates, they were treated where shown with N-glycosidase F (Endo; F. Genzyme, Cambridge, MA) as recommended by the manufacturer, and analyzed by SDS-PAGE. Western blots were probed with affinity-purified rabbit anti-human CD1d (6) and secondary goat anti-rabbit IgG-HRP (Promega, Madison, WI) for chemiluminescent detection (New England Nuclear, Beverly, MA).

Isolation of intrahepatic T cell lines and T cell assays

IHL cell lines were obtained as previously described (25). Briefly, 2–5 mm samples were cultured with anti-CD3 Abs in 100 U/ml IL-2. IHLs were split and kept at 2 × 106 cells/ml until use in complete medium (RPMI 1640, 10% heat-inactivated FBS, 1-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml IL-2, obtained from National Cancer Institute-Frederick Cancer Research Foundation Biological Resources Branch (Frederick, MD) and from Hoffman-LaRoche (Nutley, NJ). CD161+ and CD56+ IHL lymphocyte fractions were obtained from the IHL by FACS (MoFlo; Cytomation, Boulder, CO) or with magnetic beads (BioSource International, Camarillo, CA) and purity was assessed by flow cytometry (FACSscan; BD Biosciences, Franklin Lakes, NJ).

T cells were assayed as described (10, 23, 24) and released cytokines were determined by ELISA (Endogen, Woburn, MA). Repeat SEs are shown.

Results

CD1d up-regulation in the HCV-infected liver

CD1d is the natural ligand of human CD1d-reactive cells (9, 10), which represent a major population in the human liver (24). Fig. 1 shows representative results of control and CD1d immunohistochemistry of healthy and HCV-infected livers. Uninfected liver from HCV-negative donors expressed very low level CD1d (Fig. 1A). Isotype control Abs gave only trace background level reactivity on HCV-infected (Fig. 1B) as well as healthy liver tissue (data not shown). However, CD1d was strongly up-regulated in HCV-infected donors at the hepatocyte-biliary border (Fig. 1, C and D) adjacent to the portal tracts. Results were confirmed using multiple CD1d-specific mAb (Fig. 1, C and D and data not shown). Marked foci of cholangiocyte/biliary hepatocyte CD1d expression were observed in moderately inflamed cases. As expected from previous study (6), CD1d was also detected on infiltrating mononuclear cells in HCV-infected liver, particularly evident in more inflamed regions (Fig. 1E), although this CD1d was at notably lower levels than on the hepatic cells (Fig. 1, C and D).

Expression of CD1d in the liver was confirmed by CD1d immunoprecipitation and Western blotting. Fig. 1F shows that fully glycosylated CD1d of similar molecular mass to the classic and thymic CD1d of ~48 kDa (6) could be readily detected in liver tissue from HCV-infected donors. However, CD1d expression was at much lower levels from control donors with other liver diseases. No CD1d-sized bands were seen with precipitates blotted with control isotype Ab (data not shown). As expected, essentially all control CD1d from high level human B cell C1R transfectants as
well as trace levels in “mock” transfected controls was reduced to ~37 kDa (Fig. 1F). Similarly, the glycosylated hepatic CD1d could be fully reduced to ~37 kDa by de-glycosylation.

**CD1d-reactive invariant and hepatic NKT cells respond to CD1d expressed by WIF-B9 hepatic cells**

IHL derived from HCV-positive patients specifically respond upon encounter with lymphoid or epithelial lineage CD1d transfectants (24). Moreover, CD1d could be readily detected in liver tissue from HCV-infected donors (Fig. 1). We therefore sought to determine whether expression of CD1d in human liver could translate into functional T cell responses. Because primary human hepatocytes are difficult to culture reproducibly, we transfected CD1d into a hepatocyte cell line WIF-B9, which retains physiological polarization in vitro (69). Fig. 2A shows that upon transfection with human CD1d, a WIF-B9 cell subclone expressed detectable CD1d. Only one of the WIF-B9 transfectant clones tested (no. 7, total n = 20) had sufficient CD1d for detection by Western blotting (Fig. 2A).

We subsequently determined the ability of hepatic as well as blood-derived CD1d-reactive T cells to recognize the still very low level CD1d expressed by the WIF-B9 CD1d transfectant no. 7 (Fig. 2A, compare CD1d expression by C1R transfectants to 10 times as many WIF-B9). Peripheral blood-derived invariant CD1d-reactive NKT cells responded strongly to WIF-B9 CD1d (Fig. 2B). Remarkably, WIF-B9 CD1d could cause responses comparable to those obtained with mitogens (Fig. 2B).

Whole hepatic T cell lines from HCV donors also specifically responded to CD1d expressed by WIF-B9 transfectants with readily measurable specific IFN-γ production (Fig. 2C). Heparinic CD1d-reactive T cells from multiple donors proliferated in response to WIF-B9 CD1d (Fig. 2, D and E). Importantly, responses of both CD1d-reactive IHL (Fig. 2, C–E) and control invariant NKT cells (Fig. 2B) were specifically blocked by anti-CD1d mAb. Therefore, despite low level CD1d expression (Fig. 2A), WIF-B9 CD1d remarkably caused responses quantitatively comparable to those obtained with mitogens as well as to C1R and HeLa CD1d transfectants (24) expressing >100-fold higher levels of CD1d.
These results demonstrated that liver CD1d-reactive T cells could efficiently recognize CD1d when expressed at modest levels by a hepatocyte cell line.

**CD1d-reactivity in the HCV^+ liver is not confined to phenotypically NKT cells**

IHL from HCV^+ patients are enriched for CD3^+ CD56^+ and CD161^+ NKT lymphocytes (24, 53–59) and also show high levels of CD1d-reactivity (25). However, most of these cells are not the classical invariant NKT (24, 52, 54, 59, 62, 63). We sought to determine whether NK cell markers, such as CD161 or CD56, were a correlate of IHL CD1d-reactivity. We first determined the prevalence of CD161 or CD56 cells among hepatic T cells derived from the HCV^+ subjects following a single round expansion with anti-CD3. A mean of 99% of these cells expressed CD3 (data not shown). As shown in Fig. 3A, a substantial proportion of the hepatic T cell repertoire expressed CD161 and lesser levels of CD56 in all HCV^+ patients. Coexpression of these two NK markers was observed less frequently (Fig. 3A).

Notably, however, hepatic CD1d-reactive T cell proliferation and cytokine production were readily detected in both the CD1d-positive and negative populations from the same donors (Fig. 3, B and C). Similarly, we were able to detect significant CD1d-reactivity in CD56-negative IHL (data not shown).

To further confirm these findings, we subjected hepatic CD1d-reactive T cell lines to limiting dilution and selected for CD1d-reactivity. Fig. 4 show representative flow cytometry and functional characterization of these cells. We readily found hepatic CD1d-reactive cells showing no expression of CD1d1 (Fig. 4, B and C), a marker stable in culture of invariant and noninvariant NKT cells (10, 23, 24, 38). We also tested a limited number of these limiting dilution lines/clones for CD56 expression and found no CD56 CD1d-reactive lines (data not shown). However, CD56 is known to be down-regulated by extended culture of invariant and noninvariant NKT cells (10). Flow cytometric analysis of these CD1d-reactive cells further established the absence of Vα24/ Vβ11 or Vα24-Joα TCR chain expression (Fig. 4, A and B; data not shown). In conclusion, CD1d-reactivity could be readily detected from hepatic T cells with no detectable CD56, CD161, Vα24/Vβ11, or Vα24-Joα TCR chain expression.

**Hepatic CD1d-reactive IHL are present independent of HCV infection**

We next tested whether the presence of high levels of Th1-like hepatic CD1d-reactive T cells was dependent upon HCV infection or stage of HCV-mediated disease. IHL were expanded from a spectrum of HBV and HCV-negative donors with no hepatitis (transplant donors, nonviral fibrosis, cirrhosis, colonic metastases, etc.). Although there were relatively few inflammatory cells obtained from healthy control donors and cirrhotics, IHL were readily obtained using available larger samples. Such IHL also contained high level CD1d-reactivity (Fig. 5A), as seen with...
HCV donors (Figs. 2–5). Overall, hepatic CD1d-reactive T cells from 7 of 10 HCV-negative donors could produce significant levels of IFN-γ, greater in some cases on a per cell basis than certain HCV+ IHL (Fig. 5A) and at ~10% of mitogen responses (Fig. 5A). Consistent with our previous results (24), 20 of 20 HCV+ noncirrhotic donors made CD1d-specific IFN-γ.

Production of Th2 cytokines by CD1d-reactive IHL in advanced grade liver disease

Invariant NKT cells can produce high levels of IL-4 and IL-13 upon recognition of CD1d (8–13). Although traditionally thought of as Th2 cytokines, recent evidence suggests that IL-4 and IL-13 can be profibrotic and production of Th2 cytokines by hepatic T cells may be associated with progression of liver fibrosis (70–72). Thus, we further characterized functionally the in vitro response of liver CD1d-reactive T cells to WIF-B9 and HeLa transfectants by CD161+ IHL from HCV+ donor. CD1d-specific and mitogen responses by CD161-negative IHL from the same donor as in B.

CD1d-reactive IHL from several fibrotic or cirrhotic donors secreted modest levels of IFN-γ and significant quantities of both IL-13 and also IL-4 (Fig. 6A), suggesting a broadening of the hepatic CD1d-reactive T cell response during disease progression to include potentially profibrotic Th2 as well as inflammatory Th1 cytokines. As with IL-13 (Fig. 6B) and IFN-γ (Figs. 2, 3, and 5), mitogen produced detectable IL-4 responses from all IHLs tested (Fig. 6B).

Cytokine responses from hepatic CD1d-reactive T cells are summarized in Fig. 7. Although IHL produced less IFN-γ than...
peripheral blood-derived invariant T cell clones and highly enriched cell line controls (Fig. 7A), this likely represents the fact that only a fraction of the total IHL used were CD1d-reactive. Remarkably, therefore, the rarely detectable levels of IL-4 produced by noncirrhotic hepatic CD1d-reactive T cells in the presence or absence of HCV infection (Fig. 7B) suggested strong compartmentalization of these responses, as previously proposed (24). Cirrhotic as well as fibrotic IHL, independently of HCV infection, produced high levels of CD1d-reactive T cell IL-4, even relative to pure invariant NKT cells (Fig. 7B). Levels of hepatic CD1d-dependent IL-13 were also minimal in noncirrhotic IHL, but were comparable to IL-13 produced by the invariant NKT controls (Fig. 7C). We therefore conclude that although conventional T cell responses in advanced grade liver disease may broaden toward Th2 (Fig. 6B) as well as the Th1 cytokine production dominant in healthy and relatively mildly inflamed liver (Figs. 2–5; Ref. 24), the greatest increase in IL-4 cytokine production occurs in the CD1d-specific hepatic T cell population (Figs. 6A and 7, B and C).

Discussion

A subset of NKT cells responding to the nonpolymorphic ligand CD1d is highly conserved across mammalian evolution and responds to certain glycolipids bound to the hydrophobic CD1d pocket, stimulating maturation of APCs and IL-12 release (1, 9, 11–13, 39, 40, 73, 74).

Different cell types can express different CD1d isoforms (1, 6) and may present distinct glycolipids to diverse CD1d-reactive T cell populations (1, 11–25, 74). Therefore, CD1d-reactive T cells likely have distinct roles in different anatomical locations. To date, based on expression restricted primarily to APC, CD1d presentation has been thought of as purely MHC class 2-like (1, 8). In support of a model for a class 1-like CD1d tissue presentation (Fig. 8), we have found that CD1d was strongly up-regulated in HCV-infected donors at the hepatocyte-biliary border adjacent to the portal tracts as well as by mononuclear infiltrating cells during HCV infection. These results are consistent with the findings of biliary CD1d up-regulation and increased numbers of hepatic invariant NKT cells in primary biliary cirrhosis (7, 63). HCV-infected human liver across a range of disease stages contained large numbers of CD1d-reactive nonvariant T cells that could recognize hepatocyte cell line-expressed CD1d. Therefore, at least for HCV-infected liver as well as for coxsackievirus-infected myocytes (44, 45), CD1d can be substantially up-regulated during these diverse viral infections. CD1d could presumably alert resident CD1d-reactive T cells of potential tissue infection, because such reactivity was found in both cases and CD1d is critical for picornavirus as well as HSV resistance (31, 33, 35). CD1d-reactive T cells have also been anecdotally linked to viral resistance in the human (36). Interestingly, although our proposed model for hepatic CD1d presentation is functionally like MHC class 1 in being tissue rather than APC-based, CD1d expression is apparently up-regulated in response to infection. In this sense, hepatic CD1d presentation could be thought of as class 2-like, whereas classic class I presentation, as well as APC CD1d presentation, are constitutive functions. Therefore, MHC and CD1d presentation represent complementary but analogous systems (1, 8, 11–13, 73, 74). Given that viral infections do not provide potential novel glycolipid Ags in the way that bacteria can, this may provide an important and complementary immunological system for the early detection of viral infection in certain tissues to rapidly initiate innate and adaptive responses.

Very low level CD1d expressed by a hepatocyte cell line was able to stimulate hepatic CD1d-reactive NKT cell proliferation and IFN-γ Th1 cytokine production within an order of magnitude of very high level CD1d expressed on transfectants. Peripheral blood
invariant NKT cells use CD161 as a costimulatory molecule analogous to CD28 on classical T cells (15). Costimulatory requirements for CD1d-reactive IHL are unknown, and indeed our new data show that neither CD56 nor CD161 were essential for CD1d-reactivity. However, the current results are consistent with the possibility that ligands for other CD1d-reactive IHL costimulatory molecules are present and could be preferentially expressed on hepatocytes. A not mutually exclusive alternative is that endogenous glycolipid Ags may be preferentially expressed on infected hepatocytes.

Unique phenotypic features of the various CD1d-reactive T cell subsets have yet to be established. Further studies are also needed to determine their role in defense against various hepatotropic and other viruses. Significantly, high levels of hepatic CD1d-reactive T cells are not limited to either HCV-mediated disease or stage. The functional requirements of these cells and their TCR usage are currently being further evaluated. The increased expression of CD1d by the HCV-infected liver implicates the CD1d-mediated Ag presentation pathway in the modulation of intrahepatic immune responses in chronic hepatitis C. It remains to be established whether CD1d up-regulation is 1) an activation marker of liver inflammation, 2) part of general intrahepatic antiviral mechanisms, whether CD1d up-regulation is 1) an activation marker of liver inflammation, 2) part of general intrahepatic antiviral mechanisms, or 3) part of a specific anti-HCV strategy. Such a strategy may be more successful in acute hepatotropic infections, which may consequently be more efficiently eliminated, but prove deleterious in chronic infection.

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