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Cutting Edge: Compartmentalization of Th1-Like Noninvariant CD1d-Reactive T Cells in Hepatitis C Virus-Infected Liver

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Murine intrahepatic lymphocytes (IHL) are dominated by invariant TCR α-chain expressing CD1d-reactive NKT cells, which can cause model hepatitis. Invariant NKT (CD56\(^{+/−}\)/CD161\(^{+}\)) and recently identified noninvariant CD1d-reactive T cells rapidly produce large amounts of IL-4 and/or IFN-γ and can regulate Th1/Th2 responses. Human liver contains large numbers of CD56\(^{+}\) NKT cells but few invariant NKT. Compared with matched peripheral blood T cell lines, primary IHL lines from patients with chronic hepatitis C had high levels of CD161 and CD1d reactivity, but the invariant TCR was rare. CD1d-reactive IHL were strikingly Th1 biased. IHL also demonstrated CD1d-specific cytotoxic activity. Hepatocytes and other liver cells express CD1d. These results identify a novel population of human T cells that could contribute to destructive as well as protective immune responses in the liver. CD1d-reactive T cells may have distinct roles in different tissues. The Journal of Immunology, 2002, 168: 1519–1523.

Natural killer T cells share features of both classical T cells and NK cells. NKT are heterogeneous populations and can express αβ or γδ TCR, NK cell markers such as CD56, CD69, CD94, and CD161, and even killer inhibitory receptor (1, 2). While a fraction of these cells in the human react against the MHC-related nonpolymorphic proteins CD1a–c, molecules missing in the mouse (1, 2), a subset of NKT recognize glycolipids associated with CD1d (1–5). The classical CD1d-reactive T cell population expresses a highly restricted TCR repertoire (invariant α-chain rearrangement and limited Vβ chain usage: invariant NKT (1–3); in the human these are Va24/βαQ and predominantly Vβ11 (4). Most CD1d-reactive invariant NKT express CD161 and represent the majority of murine but not human NKT (1–4).

Murine CD161\(^{+}\) and CD161\(^{−}\) populations also include CD1d-reactive T cells using diverse TCR (6–10). In the mouse, invariant NKT preferentially accumulate in the liver and thymus, whereas noninvariant CD1d-reactive NKT are common in the spleen and bone marrow (9, 10). In contrast, a larger fraction of human peripheral blood T cells are NKT, but only a minor subset of these NKT are CD1d-reactive and express the invariant TCR and not killer inhibitory receptor (4). Although less is known of the diversity and functions of human NKT in general, noninvariant CD161\(^{−}\) CD1d-reactive T cells have recently been identified in the human bone marrow, where they are strongly Th2 biased (11).

Invariant NKT represent up to 50% of rodent intrahepatic lymphocytes (IHL) (5); Refs. 8, 9, and 12–14, consistent with expression of CD1d within rodent and human liver (15–17). Invariant NKT cause the liver damage in certain models of hepatitis (18), both directly through perforin and/or Fas ligand-mediated CD1d-specific cytotoxic activity (19–21) and by activation of NK and other immune cells via Th1 cytokines such as IFN-γ as well as Th2-like IL-4, etc. (1, 4, 21). As a consequence of their potential to kill infected cells and/or produce cytokines, NKT may also play a protective role in clearance of pathogens such as Salmonella and hepatitis B virus (22, 23) and contribute to defense against tumors (19, 24). CD1d-reactive T cells are essential for optimal response to an acute viral infection (25).

NKT are substantially enriched in human liver relative to peripheral blood (26–29). There is also preferential expression of Va24 used by invariant as well as other T cells (26, 28). However, invariant NKT are rare in human liver (30), unlike the corresponding cells in murine liver (8, 9, 12–14). Thus CD1d-reactive T cells in human peripheral blood (4, 31, 32) and bone marrow (11) and in rodents (8, 9, 12–14) display diverse phenotypes. Using functional assays, we report high levels of human hepatic Th1-biased CD1d reactivity in the absence of invariant NKT enrichment. Human T cells from the liver of patients with chronic hepatitis C virus (HCV) infection thus contained a large uniquely Th1-like population of noninvariant CD1d-reactive T cells with potentially protective and pathologic roles in hepatitis.

*Address correspondence and reprint requests to Dr. Mark A. Exley, Cancer Biology Program, Hematology/Oncology, Beth Israel-Deaconess Medical Center, Harvard Medical School, 2001 Brookline Avenue, Boston, MA 02215. E-mail address: mexley@bidmc.harvard.edu

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Materials and Methods

**Assays and reagents**

Invariant NKT cells and transfectants were used as described (4, 32). Released cytokines and chemokines from T cells assayed with transfectants and PMA were determined by ELISA. SDs are shown in Figs. 2–5. Anti-CD3 12F6 and bifunctional CD3/8 were kindly provided by Dr. J. Wong (Massachusetts General Hospital, Boston, MA).

**Isolation of intrahepatic and matched PBL T cell lines**

HCV seropositive donors with detectable HCV RNA but moderate elevations in liver enzymes and inflammation provided noncirrhotic liver biopsies under written informed consent during routine clinical evaluation. None were HIV seropositive or had been treated with IFN or ribavirin. IHL T cell lines were obtained by a single expansion as described (33). Five-millimeter sections were cultured with 100 U/ml IL-2 (Hoffman-LaRoche, Nutley, NJ), followed by CD3 or CD3/8 Abs and irradiated (30 Gy) allogeneic feeders at /H110112 days. Matched PBL lines were obtained at time of biopsy by identical stimulation. Studies were approved by the institutional committee on clinical investigations.

**Results**

**IHLs contain high levels of NKT**

Murine liver lymphoid cells contain high levels of CD1d-reactive invariant NKT (8, 9, 12). Fig. 1 shows representative results of phenotypic analysis of human IHL T cell samples expanded from HCV-infected donors in comparison to matched PBL T cell lines derived at the same time. Whereas short-term PBL T cell lines contained /H11022~5% NKT (CD3/H11001CD161/1), matched IHL lines contained 18 to >30% NKT (Fig. 1). Elevated numbers of NKT IHL did not correlate with expression of V24 TCR used by invariant NKT (4). A small V24 population was routinely detectable from PBL (1–2%), but IHL were not significantly enriched for invariant NKT-like cells (/H11349~1%; Fig. 1).

**IHLs contain high levels of Th1-polarized CD1d-reactive cells**

CD1d is the natural ligand of human invariant NKT (4), CD161 is costimulatory for these NKT (20), and NKT also include CD1a–c-reactive T cells (2). Therefore, it was determined whether high levels of NKT in IHL translated into elevated functional responses to CD1 molecules. Fig. 2, A and B, shows that whole IHL preparations, but not PBL from the same donor collected on the same day, responded with readily measurable CD1d-specific proliferation. CD1d-specific proliferative responses of both IHL (Fig. 2A)
and control invariant NKT cells (4) were specifically blocked by anti-CD1d mAb, whereas other CD1 molecules failed to elicit more than minimal proliferative responses (data not shown).

Similar to the results with proliferation, these same IHL also responded specifically to human CD1d transfectants with substantial IFN-γ secretion (Fig. 2C). Cytokine responses of IHL to CD1d were blocked by anti-CD1d mAb (Fig. 2C). Notably, the CD1d-specific IFN-γ responses of whole IHL preparations (Fig. 3, B and C) were also comparable to those of invariant NKT clones (Fig. 3A). All 12 independent IHL preparations raised with 12F6 or CD3/8 mAb from nine donors responded specifically to CD1d transfectants at levels ~10% above their response to mitogen and frequently comparably to mitogen and invariant NKT clones (Fig. 3A–C). In contrast, there were only minimal IHL responses to CD1a, -b, or -c, as with control invariant NKT (Fig. 3, A–C). Unlike in the bone marrow (11), CD1d-reactive IHL and invariant NKT responded to both B cell-derived and epithelial CD1d transfectants (Figs. 2, A and C, and 3, B and C).

In contrast to the results with IFN-γ, little IL-4 was produced by IHL in response to CD1d (Fig. 3D). However, these same IHL did produce significant levels of IL-4 in response to mitogen, indicating the presence of Th2-like conventional T cells. Somewhat higher levels of IL-4 were readily detectable in control supernatants from matched PBL treated with mitogen, indicating that they contained generally more conventional Th2 activity. Finally, as previously described (4), invariant NKT analyzed alongside made comparably high levels of IL-4 and IFN-γ in response to CD1d and mitogen (Fig. 3D).

Invariant NKT produce large amounts of other cytokines and chemokines as well as IL-4 and IFN-γ (4). These can include GM-CSF, RANTES, IL-8, Th1-like IL-2, macrophage-inflammatory protein-1α, TNF-α, Th2-like IL-5, IL-13, and regulatory (Th3) TGF-β and IL-10. In addition to the prototypical cytokines shown above (high IFN-γ, no detectable IL-4), CD1d-reactive IHL produced a markedly, though not pure, Th1-like spectrum (Fig. 4). In particular, CD1d-reactive IHL could secrete substantial amounts of macrophage-inflammatory protein-1α (one of four lines tested) and detectable TNF-α (two of four lines tested), as well as IL-13 (one of four lines tested), but only trace RANTES (one of four lines tested; 52 pg/ml) and no detectable IL-8, TGF-β, or IL-10 (<10 pg/ml; Fig. 4).

FIGURE 3. Cytokine responses of intrahepatic and peripheral blood T cell lines to CD1d. IHL lines (one round of stimulation) and established invariant NKT clone DN2.B9 (several rounds of stimulation) were incubated with C1R CD1a, -b, -c, or -d transfectants or with mitogen. Data are representative of results from five experiments. A, Invariant NKT cell clone DN2.B9 assayed for CD1d-specific IFN-γ production. B, IHL (donor B8) assayed for IFN-γ production. C, IHL from donor E8 assayed for CD1d-specific IFN-γ production. D, Comparison of CD1d-specific and mitogen-induced IL-4 responses of IHL (donor E8), matched E8 peripheral blood T cell line from the same donor, and invariant NKT control.

FIGURE 4. Cytokine and chemokine production by CD1-reactive IHL. Net CD1d-specific (response to CD1d − level with mock transfectant) production of a range of Th1, Th2, and Th3 cytokines and chemokines by IHL lines (one round of stimulation) and established invariant NKT cell clone DN2.B9 (several rounds stimulation). IL-8, IL-10, and TGF-β were not detectable.

FIGURE 5. CD1d-specific cytotoxicity of IHL. Net CD1d-specific (response to CD1d − level with mock transfectant) cytotoxicity of activated CD161⁺ IHL ~2 wk after first biopsy stimulation and activated invariant NKT clones.
CD1d-specific cytotoxic activity of CD161+ IHL T cells

Activated human invariant NKT have potent perforin-mediated CD1d-specific cytotoxic activity (20). Fig. 5 shows that after subtraction of reactivity against mock transfectants, whole IHL retained significant CD1d-specific cytotoxic activity. Higher backgrounds of IHL presumably reflect activated alloreactive CTL and/or lymphokine-activated killer-like cells in these preparations. Therefore, as for proliferative and Th1-like cytokine responses, IHL contained measurable CD1d-reactive cytotoxic activity. Combined with high levels of NKT and trace amounts of invariant-like T cells, these results indicate that human liver lymphocyte lines contained high levels of noninvariant Th1-like CD1d-reactive T cells.

Discussion

Our phenotypic results with single-round expanded IHL lines from HCV+ patients are consistent with published ex vivo data showing similar levels of CD56- and CD161+ NKT in healthy human liver (27, 28), which may increase and later fall in HCV infection (26, 27, 29). This suggests most are CD56"CD161", as bone marrow (11). Also consistent with our results, Vα24+ cells are reported to make up to ~1% of IHL, irrespective of HCV infection (26, 28). Recent reports using staining with α-galactosylceramide-loaded soluble CD1d show that most NKT are invariant in rodent liver (13, 14), but very few within human liver are invariant NKT (0.03–0.34%), comparable to the low numbers in human blood (30), therefore providing a lowest possible estimate for the number of CD1d-reactive NKT.

Approximately 50% of human bone marrow CD161+ T cells are noninvariant CD1d-reactive NKT (11). This report demonstrates a comparably high level of CD1d-reactive T cells in IHL lines from HCV+ donors. Levels of functionally CD1d-reactive T cells in healthy human liver and in other diseases remain to be determined. Although most human blood NKT are not CD1d reactive, high levels of CD1d-reactive IHL suggest that, as in bone marrow, these were NKT. A substantial fraction of human IHL (26) and murine hepatic NKT are apoptotic, although the latter have high regenerative potential (14, 34). This study used single round expansion after brief incubation to rest active cells. Cell lines analyzed could not be apoptotic. However, this would indicate that we underestimated CD1d reactivity by use of cell lines. CD1d-reactive T cells have potentially distinct immunoregulatory functions in different anatomical locations.

The major CD1d-reactive population within IHL had a pronounced Th1 bias, whereas conventional T cells in the same IHL made IL-4, albeit less than matched blood T cells, consistent with previous phenotypic reports of IHL NKT and conventional T cells (28, 33–36). This contrasts with healthy human bone marrow, where noninvariant CD161+ CD1d-reactive T cells are Th2 biased (11). Phenotypic and functional compartmentalization of conventional T cells also occurs in chronic HCV infection, where intrahepatic HCV-specific T cells are more numerous, recognize distinct epitopes, produce different cytokines, and use distinct TCR (33–37). Irrespective of whether our findings result from normal physiology or are a result of pathology, we demonstrate both phenotypic and functional compartmentalization of human CD1d-reactive T cells.

IFN-γ from CD1d-reactive IHL and CD8+ HCV-specific CTL fails to prevent HCV replication (33–37). Instead, these cells may cause tissue damage. Invariant NKT mediate Con A- and Salmonella-induced hepatitis (18, 22). Activation of invariant NKT results in their apoptosis and concomitant liver damage (34). In addition to potential cytotoxicity against hepatic cell surface CD1d induced by infection or inflammation, up-regulation of Fas ligand on activated CD1d-reactive NKT may induce apoptosis of Fas-expressing hepatocytes (18). NKT also could directly and perhaps indirectly increase TNF-α levels.

In summary, we have found that human liver, like bone marrow, but unlike blood, contains large numbers of CD1d-reactive non-invariant NKT. However, IHL CD1d-reactive T cells were Th1 polarized, representing a novel lineage from the previously defined invariant and bone marrow NKT. Such cells have the potential for distinct functions uniquely expressed within the liver microenvironment.

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