The Journal of Immunology

The Human Liver Contains Multiple Populations of NK Cells, T Cells, and CD3$^+$ CD56$^+$ Natural T Cells with Distinct Cytotoxic Activities and Th1, Th2, and Th0 Cytokine Secretion Patterns

Derek G. Doherty, Suzanne Norris, Laura Madrigal-Estebas, Gerry McEntee, Oscar Traynor, John E. Hegarty and Cliona O'Farrelly

J Immunol 1999; 163:2314-2321; ;
http://www.jimmunol.org/content/163/4/2314

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Human Liver Contains Multiple Populations of NK Cells, T Cells, and CD3$^+$CD56$^+$ Natural T Cells with Distinct Cytotoxic Activities and Th1, Th2, and Th0 Cytokine Secretion Patterns[1]

Derek G. Doherty,[2*‡] Suzanne Norris,[3*†] Laura Madrigal-Estebas,[4*] Gerry McEntee,[†] Oscar Traynor,[†] John E. Hegarty,[5†] and Cliona O'Farrelly[5*‡]

The human liver contains significant numbers of T cells, NK cells, and lymphocytes that coexpress T and NK cell receptors. To evaluate their functional activities, we have compared the cytotoxic activities and cytokines produced by normal adult hepatic CD3$^+$CD56$^-$ (T) cells, CD3$^-$CD56$^+$ (NK) cells, and CD3$^+$CD56$^+$ (natural T (NT)) cells. In cytotoxicity assays using immunomagnetic bead-purified NK cell, T cell, and NT cell subpopulations as effectors, fresh hepatic NK cells lysed K562 targets, while NT cells could be induced to do so by culturing with IL-2. Both NT and T cells were capable of redirected cytolysis of P815 cells using Abs to CD3. Flow cytometric analysis of cytokine production by fresh hepatic lymphocyte subsets activated by CD3 cross-linking or PMA and ionomycin stimulation indicated that NT cells and T cells could produce IFN-γ, TNF-α, IL-2, and/or IL-4, but little or no IL-5, while NK cells produced IFN-γ and/or TNF-α only. The majority of NT cells produced inflammatory (Th1) cytokines only; however, ~6% of all hepatic T cells, which included 5% of Vα24 TCR-bearing NT cells and 2% of $\gamma\delta$TCR$^+$ cells, simultaneously produced IFN-γ and IL-4. The existence of such large numbers of cytotoxic lymphocytes with multiple effector functions suggests that the liver is an important site of innate immune responses, early regulation of adaptive immunity, and possibly peripheral deletion of autologous cells. ***The Journal of Immunology*****, 1999, 163: 2314–2321.**

The repertoire of lymphocytes present in the liver differs dramatically from that in peripheral blood and other organs, suggesting that the liver may have a specialized role in the immune system. The liver contains significantly higher numbers of NK cells and CD8$^+$ cytotoxic T cells compared with blood (1, 2), and up to one-half of the hepatic T cell compartment consists of an unusual subset of lymphocytes that coexpress TCR and NK stimulatory and inhibitory receptors, denoted NKT cells. In mice, these cells are either CD4$^+$ or CD4$^-$CD8$^-$, and they express an ~3-fold lower surface density of the $\alpha\beta$ TCR and the NKR-P1 receptor NK1.1 (3, 4). NK1.1$^+$ T cells are characterized by their potent effector function, as evidenced by their ability to lyse various tumor cells in the absence of prior antigenic stimulation, and to rapidly produce high levels of Th1 and Th2 cytokines, including IFN-γ and IL-4, upon activation through their TCR or NK1.1 molecules (5–7). A feature of these cells is that they frequently express a highly restricted TCR repertoire consisting of an invariant Vα14Jα281 α-chain in association with a limited number of Vβ-chains (8), which, unlike conventional MHC-restricted T cells, are positively selected by and recognize nonpeptide Ags in the context of CD1 (9, 10).

Studies in humans have also identified lymphocyte populations that coexpress $\alpha\beta$ or $\gamma\delta$ TCRs and various NK receptors, including CD16, CD56, CD69, CD161 (NKR-P1A), and/or killer cell inhibitory receptors (KIR)[6] for MHC class I (11–14). These include a human NKT cell population that coexpresses CD161 and a TCR that is structurally homologous to the murine Vα14Jα281 TCR chain, Vα24-JαQ (8), which, like their murine Vα14Jα281 T cell counterparts, can recognize nonpeptide Ags presented by CD1d (15–17) and can be induced to produce both IFN-γ and IL-4 (15, 18). T cells expressing NK cell receptors are particularly abundant in the normal human liver (2), and we have recently demonstrated that a significant proportion of hepatic, but not peripheral, CD3$^+$ cells can be induced to lyse NK-sensitive target cells (19). In these experiments, the effectors resided in a CD3$^+$CD56$^+$ cell population that makes up approximately one-third of all hepatic CD3$^+$ cells, but only ~2% of peripheral blood T cells. Human hepatic CD56$^+$ T cells are a heterogeneous population of lymphocytes that include CD4$^+$, CD8$^+$ and double-negative CD4$^-$CD8$^-$ cells expressing either $\alpha\beta$ or $\gamma\delta$ TCRs and Ag-experienced CD45RO and CD56^{dim} phenotypes. They include CD3$^+$CD161$^+$ NKT cells, some of which have invariant Vα24-JαQ TCR chains, as well as CD161-negative T cells expressing other NK receptors. Since the expression of CD56 serves better than CD161-positivity to define

*Education and Research Centre and †Liver Unit, St. Vincent's Hospital, Dublin, Ireland; and ‡Department of Medicine and Therapeutics, University College, Dublin, Ireland

Received for publication March 16, 1999. Accepted for publication June 8, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants awarded by the Irish National Liver Transplant Centre (to D.G.D. as a Newman Scholar), The Irish Health Research Board (Hepatitis C Unit Grant), Forbairt Basic Research Grants Scheme, and Baxter Healthcare (to L.M.E as a Newman Scholar).

[2] Address correspondence and reprint requests to Dr. Derek G. Doherty, Education and Research Centre, St. Vincent's Hospital, Elm Park, Dublin 4, Ireland. E-mail address: ddoherty@svherc.ucd.ie

[3] Current address: Institute of Liver Studies, King's College School of Medicine and Dentistry, Bessemer Road, London SE5 9PJ, U.K.

[4] Current address: Infection and Immunology Group, Biology Department, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland.

[5] J.E.H. and C.O. contributed equally to the direction of this work.

[6] Abbreviations used in this paper: KIR, killer inhibitory receptor; NT cell, natural T cell; MNC, mononuclear cells; HL, hepatic lymphocyte; LAK, lymphokine-activated killing; PerCP, peridin chlorophyll protein.

0022-1767/99/$02.00

an NK receptor-positive T cell subpopulation that is characteristic of the liver (19), we have defined "hepatic natural T (NT) cells" as $CD3^+CD56^+$ cells, rather than $CD3^+CD161^+$ cells.

The above observations suggest that the human liver is a major site of NT cell activity and may thus be a uniquely specialized organ of the immune system. However, the functions of hepatic NT cells are unknown. By analogy with murine NKT cells, they are likely to have innate (20), antitumor (21, 22), and regulatory (6, 7) roles, with multiple activities that are under the control of the accessory cells and soluble factors present in their local microenvironment (23–28). Therefore, we have investigated the effector functions of human hepatic NT cells immediately after isolation from normal adult human liver specimens. By comparing the cytotoxic functions and cytokine secretion profiles of hepatic $CD3^+CD56^+$ NT cells with those of hepatic NK cells ($CD3^-CD56^+$) and conventional hepatic T cells ($CD3^+CD56^-$), we show that the NT cell fraction is capable of potent TCR-mediated and NK-like cytotoxicity, and that these cells rapidly produce proinflammatory, Th1 and Th2 cytokines. We further show that multiple subpopulations of hepatic NT cells are capable of simultaneously producing Th1 and Th2 cytokines. These results suggest that the adult liver is an important site of innate immune recognition and effector function as well as the early regulation of adaptive immune responses.

Materials and Methods

Tissue specimens

Normal liver tissue (100–300 mg biopsy specimens) was obtained from 18 adult donors at the time of liver transplantation. Liver biochemistry and histology were normal in all cases. Single hepatic cell suspensions that were enriched for mononuclear cells (MNC) were prepared using a method based on mechanical dissociation and collagenase treatment, as described previously (19). PBMC were prepared from 10 healthy adult donors by Lymphoprep density gradient centrifugation (Nycomed, Oslo, Norway). Ethical approval for this study was obtained from the Ethics Committee at St. Vincent's Hospital (Dublin, Ireland).

Abs and flow cytometry

Anti-CD3 FITC, anti-CD3 peridin chlorophyll protein (PerCP), anti-CD56 PE, anti-IFN-γ FITC, anti-TNF-α FITC, anti-IL-2 FITC, anti-IL-4 PE, anti-$\gamma\delta$ TCR biotin, isotype-matched anti-IgG controls (FITC, PE, and PerCP) and streptavidin PerCP were obtained from Becton Dickinson (Oxford, U.K.). Unconjugated anti-CD3 (HIT3a), anti-CD56 biotin, anti-IL-4 FITC, and anti-IL-5 PE were obtained from PharMingen (Oxford, U.K.). Anti-Vα24 TCR biotin was obtained from Coulter-Immunotech (Marseille, France). Phenotypic analysis of hepatic lymphocyte (HL) subsets (Fig. 1, *A* and *B*) was performed by three-color mAb staining and flow cytometry using a FACScan (Becton Dickinson) and analysis using CellQuest software (Becton Dickinson).

Reagents

Human recombinant IL-2, BSA, sodium azide, PMA, ionomycin, PHA, saponin, and brefeldin A were obtained from Sigma (Poole, U.K.).

In vitro stimulation and culture of HL

MNC-enriched hepatic cell preparations (0.1–1 $\times$ 10^6 viable cells) were plated out in 96-well round-bottom or 24-well flat-bottom plates (Nunc, Kamstrup, Denmark) and cultured with equal numbers of irradiated (3000 rads γ irradiation) allogeneic PBMC and 5 μg/ml PHA in complete RPMI medium (RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% locally produced heat-inactivated human serum) (29). Human recombinant IL-2 (30 U/ml) was added on day 3, and the cells were harvested for functional analyses on day 5. Flow cytometric analysis of 5-day cultures revealed negligible expansion or phenotypic changes to the cells. Expansion of HL was achieved by restimulation as above every 7–10 days with the addition of IL-2 on days 3, 5, and 7 (29). Long-term culture of these cells resulted in the selective expansion of $CD3^+$ cells.

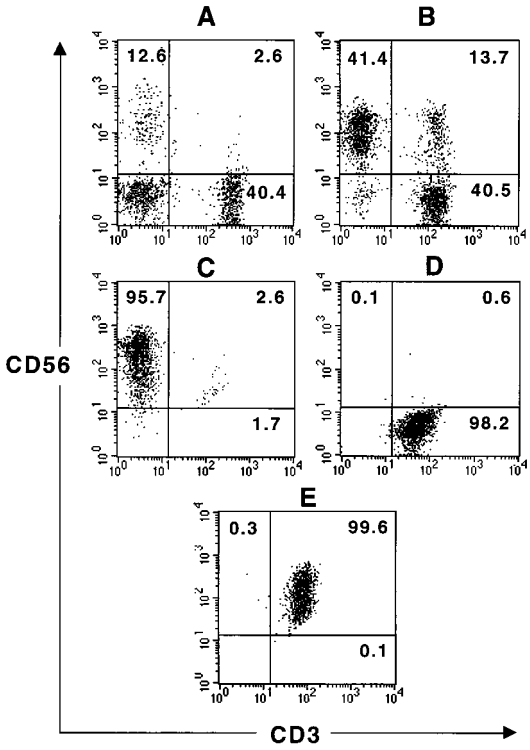

FIGURE 1. Phenotypic detection and isolation of human hepatic NT cells. *A* and *B*, Flow cytometric analysis of freshly isolated normal human peripheral blood (*A*) and HL (*B*) after staining with mAbs against CD3 and CD56. The proportions (%) of lymphocytes bearing $CD3^+CD56^-$ (T cells), $CD3^-CD56^+$ (NK cells), and $CD3^+CD56^+$ (NT cells) phenotypes are shown as numbers in the quadrants. *C–E*, Flow cytometric analysis of HL showing CD3 and CD56 expression by immunomagnetic bead-purified NK cells (*C*), T cells (*D*), and NT cells (*E*).

Isolation of HL subpopulations

Freshly isolated, in vitro-stimulated, and in vitro-stimulated and expanded hepatic NK cells, T cells, and NT cells were tested as effectors in cytotoxicity assays. Cells were separated into $CD3^-CD56^+$ (NK cells), $CD3^+CD56^-$ (T cells), and $CD3^+CD56^+$ (NT cells) fractions using mAb-coated magnetic beads (Macs Magnetic Microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany). Two approaches were employed. Double sorting of cells according to their expression of CD3 and CD56 was performed by labeling the cells with an FITC-conjugated mAb against CD3, followed by the magnetic separation of the labeled $CD3^+$ cells from the unlabeled $CD3^-$ cells using anti-FITC microbeads. The magnetic beads were then removed using the MultiSort Kit (Miltenyi Biotec). The $CD56^+$ and $CD56^-$ cells were subsequently separated from these preparations using anti-CD56 microbeads. $CD3^+CD56^+$ cells were also purified by first selectively expanding the $CD3^+$ cells in vitro, as described above, and then separating them into $CD56^+$ and $CD56^-$ populations using anti-CD56 microbeads. The purity of isolated fractions was assessed by flow cytometry (Fig. 1, *C–E*), and only preparations whose purities were >95% were used for functional studies.

Cytotoxicity assays

NK and T cell cytotoxic function was assessed in 4-h ^{51}Cr-release assays (29) using fresh, in vitro-stimulated, or stimulated and expanded effector cell populations, which were isolated by immunomagnetic bead separation, and added to the target cells at E:T ratios of 1:1 to 100:1. In NK cytotoxicity assays, the cytolytic activities of freshly prepared, freshly isolated HL subsets against the target cell line, K562, were measured. For the measurement of lymphocyte-activated killing (LAK) activity, HL were first stimulated in vitro and cultured with IL-2 for 5 days before using as effectors in cytotoxicity assays against K562 cells. The $Fc\gamma R^+$ murine mastocytoma cell line was used as the target cell for mAb-redirected cytotoxic killing using cultured HL subsets as effectors with cross-linking mAbs to the T cell or NK cell receptors CD3 or CD56 (12). In vitro expansion of the

effectors was required to obtain sufficient numbers of T cells and NT cells for use in redirected cytotoxicity assays, but since CD3-negative cells were not expanded by our method, NK cells were taken from HL cultured for 5 days only. mAbs were incubated with the effector cells for 30 min at 4°C and washed twice before adding the target cells. Percent specific lysis was expressed as: [(cpm of sample − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)].

Stimulation of cells and staining for intracellular cytokines

Freshly isolated MNC-enriched hepatic or peripheral blood cells were suspended in complete RPMI medium at a density of 0.5–1 × 10^6 cells/ml and stimulated for 6 h in 24-well plates (Nunc; 0.5 ml/well) at 37°C in 5% CO_2. Cells were stimulated with either 10 ng/ml PMA plus 1 μg/ml ionomycin or with plate-bound anti-CD3 mAb (10 μg/ml HIT3a bound to plates by incubation for 6 h at 37°C in 0.1 M Na_2HCO_3) in the presence of 1 ng/ml PMA. As controls, unstimulated cells were treated similarly. Brefeldin A (10 μg/ml), an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus, was added to the cells for the last 4 h. After stimulation or incubation without stimulators, cells were washed twice with PBS containing 0.07% BSA and 0.02% sodium azide and stained for 15 min at room temperature with mAbs against cell surface CD3, CD56, Vα24 TCR, γδTCR, or isotype-matched controls. For the detection of TNF-α, IFN-γ, IL-2, and IL-4 (FITC-conjugated mAbs) production by HL subsets, anti-CD3 PerCP and anti-CD56 PE were used for cell-surface staining. For the detection of IL-5 (PE-conjugated), the cell surface was stained with anti-CD3 FITC and anti-CD56 biotin. For the simultaneous detection of IFN-γ (FITC-conjugated mAb) and IL-4 or IL-5 (PE-conjugated mAbs), cell surface staining was performed using anti-CD3 PerCP, anti-CD56 biotin, anti-Vα24 TCR biotin, or anti-γδTCR biotin. Cells were then washed twice with PBS-BSA-azide buffer and stained with detection reagents (streptavidin PerCP) where required before staining for intracellular cytokines.

For the intracytoplasmic staining of cytokines, cells were fixed with 0.5 ml 4% paraformaldehyde at room temperature for 10 min, washed with PBS-BSA-azide buffer, and permeabilized with 1 ml 0.2% saponin in PBS-BSA-azide buffer for 10 min at room temperature. Cells were pelleted and incubated with anti-cytokine mAbs (0.1 μg anti-TNF-α FITC, anti-IFN-γ FITC, anti-IL-2 FITC, anti-IL-4 FITC, anti-IL-4 PE, or anti-IL-5 PE in 50 μl 0.2% saponin) for 30–60 min. Finally, the cells were washed with PBS-BSA-azide buffer and resuspended in PBS-BSA-azide buffer for immediate analysis or in 1% paraformaldehyde for overnight storage before analysis. Coexpression of cell surface molecules and intracytoplasmic cytokines was detected by three-color flow cytometry (FACScan, Becton Dickinson) and analyzed using CellQuest (Becton Dickinson) software.

Results

Isolation and characterization of HL subsets

Preparation of cells from 100 to 300 mg liver specimens yielded 1–2.5 × 10^6 MNC. Flow cytometric analysis of CD3 and CD56 expression was used to define conventional T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), and NT cells ($CD3^+CD56^+$) (Fig. 1, *A* and *B*). Analysis of freshly isolated hepatic MNC revealed that NK cells and NT cells were significantly more frequent among HL compared with PBL (Fig. 1, *A* and *B*), as we have previously reported (2, 19). Flow cytometry of long-term HL cultures revealed that the in vitro expansion of these cells resulted in the selective expansion of $CD3^+KIR^-$ cells; therefore, functional assays using cultured NK cells were performed using cells that were cultured for 5 days only, conditions which resulted in negligible expansion or phenotypic changes to the cells.

Hepatic NT cells are capable of LAK, but not NK, cytotoxic activity

Freshly isolated or stimulated HL were separated into $CD3^-CD56^+$ (NK cells), $CD3^+CD56^-$ (T cells), and $CD3^+CD56^+$ (NT cells) fractions using immunomagnetic beads (Fig. 1, *C–E*), and each fraction was tested for its ability to lyse K562 cells in chromium release assays. When freshly isolated cells were used as effectors, only the NK cell fraction demonstrated lytic activity against this target cell line, whereas NT cells and T cells did not (Fig. 2*A*). However, after stimulation in the presence of IL-2, both the NK and NT cell fractions, but not conventional T cells, exhibited cytotoxicity against K562 targets (Fig. 2*B*), indicating that the hepatic NT cell fraction contains precursors of LAK cells.

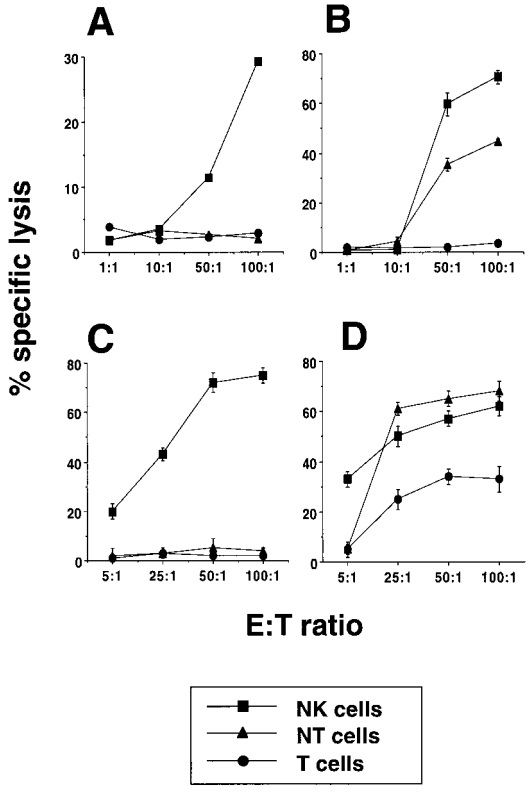

FIGURE 2. Cytotoxic activities of immunomagnetic bead-separated normal human hepatic $CD3^-CD56^+$ (NK), $CD3^+CD56^+$ (NT), and $CD3^+CD56^-$ (T) cells in chromium release assays. *A*, NK cytolytic activity of freshly isolated HL subsets against K562 target cells. Results are representative of assays of four liver samples, each set up in triplicate. *B*, LAK of K562 target cells by HL subsets that were first stimulated with mitogen and cultured for 5 days in the presence of IL-2. Results are representative of six triplicate experiments. *C*, Cytotoxic activities of cultured HL subsets against P815 target cells in the absence of cross-linking Abs. Results are representative of six triplicate assays. *D*, Redirected cytotoxicity by cultured HL subsets against P815 target cells in the presence of cross-linking mAb against CD3. Results are representative of six triplicate assays.

Cultured hepatic NT cells are capable of TCR-mediated cytotoxicity

Cultured HL were separated, using magnetic beads, into NK cells, T cells, and NT cells (Fig. 1, *C–E*) and tested for their ability to lyse murine $FcγR^+$ P815 cells in redirected cytotoxicity assays in the absence or presence of cross-linking mAbs to human CD3. Fig. 2*C* shows that purified hepatic $CD3^-CD56^+$ cells (NK cells) were capable of lysing P815 cells whether or not any Abs were present. This intrinsic cytotoxic activity was found to be the result of in vitro stimulation of NK cells (LAK activity) since little or no cytotoxicity was observed when freshly isolated $CD3^-CD56^+$ cells were used as effectors against P815 cells in the absence of cross-linking mAbs (data not shown). Hepatic T cells ($CD3^+CD56^-$) and NT cells ($CD3^+CD56^+$) did not possess LAK activity against P815 targets (Fig. 2*C*), but when cultured HL fractions were used as effectors in redirected cytotoxicity assays, CD3 cross-linking led to cytolysis of P815 targets, indicating that both hepatic NT

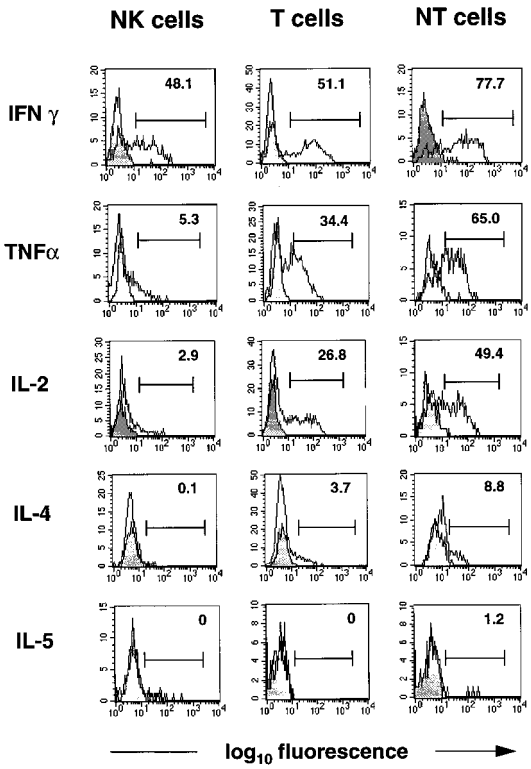

FIGURE 3. Flow cytometric detection of intracytoplasmic cytokine production by human hepatic $CD3^-CD56^+$ (NK), $CD3^+CD56^+$ (NT), and $CD3^+CD56^-$ (T) cells, which were freshly isolated from a normal liver specimen and either unstimulated or stimulated for 6 h with PMA + ionomycin. Histograms represent the log fluorescence intensities obtained upon staining for cytokines (shown on left) after gating on the HL subsets (shown above). The shaded histograms indicate cytokine production in the absence of stimulation (medium containing 1 ng/ml PMA), while the unshaded histograms show cytokine production by stimulated cells. The numbers indicate the percent of cells in each group that were positive for cytokine production, as defined by the horizontal markers, after subtraction of the values obtained for unstimulated samples.

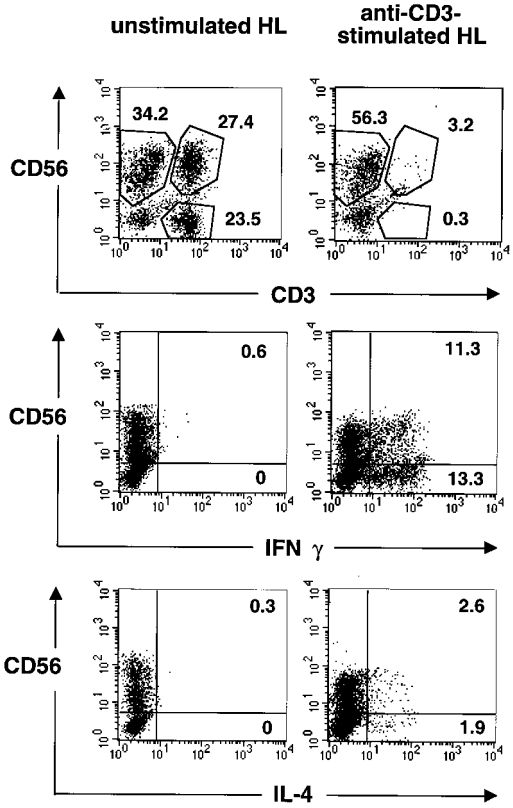

FIGURE 4. Flow cytometric detection of CD3 and CD56 expression and intracytoplasmic cytokine production by human $CD3^+CD56^+$ (NT) and $CD3^+CD56^-$ (T) cells, which were freshly isolated from a normal liver specimen and either unstimulated or stimulated for 6 h with plate-bound anti-CD3 mAb. The profiles of unstimulated HL are shown to the left, and the corresponding profiles obtained upon stimulation are shown to the right. The top profiles show that stimulation of cells by CD3 cross-linking resulted in the blocking of CD3, thus interfering with its subsequent detection by flow cytometry. The numbers indicate the percent of the cells that expressed NK, T, and NT cell phenotypes as detected by mAb staining before and after stimulation. The middle and bottom profiles show the expression of CD56 and the production of IFN-γ (*middle*) and IL-4 (*bottom*) by unstimulated (*left*) and anti-CD3-stimulated (*right*) cells. The figures show the percentages of events detected in each quadrant. Since only cells that express surface CD3 would be stimulated as a result of CD3 cross-linking, the frequencies of NT cells and T cells producing cytokines were calculated from the proportion of $CD56^+$ and $CD56^-$ that produced cytokines as follows: % of NT cells producing cytokines upon anti-CD3 stimulation = [(% $CD56^+$ $cytokine^+$ cells after stimulation) − (% $CD56^+$ $cytokine^+$ cells before stimulation)]/[% $CD3^+CD56^+$ cells before stimulation]; % of T cells producing cytokines upon anti-CD3 stimulation = [(% $CD56^-$ $cytokine^+$ cells after stimulation) − (% $CD56^-$ $cytokine^+$ cells before stimulation)]/[% $CD3^+CD56^-$ cells before stimulation]. Thus, in the example shown: % of NT cells producing IFN-γ = [11.3 − 0.6]/27.4 = 39.1%; % of NT cells producing IL-4 = [2.6 − 0.3]/27.4 = 8.4%; % of T cells producing IFN-γ = [13.3 − 0]/23.5 = 56.6%; and % of T cells producing IL-4 = [1.9 − 0]/23.5 = 8.1%.

cells and conventional T cells are capable of TCR-mediated cytolysis (Fig. 2*D*). No cytolysis of P815 cells was observed after cross-linking with anti-CD56 or isotype-matched control mAbs (data not shown). Since in vitro expansion of HL was required to obtain sufficient numbers of T cells and NT cells for use as effectors in these assays, we have not determined whether freshly isolated HL subsets could also lyse P815 cells in redirected assays. These results indicate that, like conventional T cells, cultured hepatic NT cells are capable of TCR-mediated cytolysis, but, in contrast to T cells, these cells can also be activated through receptors that mediate LAK activity.

Cytokine production by hepatic and peripheral T cells, NT cells, and NK cells

Freshly isolated hepatic MNC were stimulated for 6 h with PMA and ionomycin or plate-bound anti-CD3 mAb, or incubated in the absence of stimulator, and the frequencies of cytokine-producing HL subsets were determined by flow cytometry after mAb staining for surface CD3 and CD56 and intracellular IFN-γ, TNF-α, IL-2, IL-4, and IL-5. Following stimulation with PMA and ionomycin, cytokine production by NK cells, T cells, and NT cells was determined by three-color flow cytometry of gated $CD3^-CD56^+$, $CD3^+CD56^-$, and $CD3^+CD56^+$ cells, respectively, as shown in Fig. 3. The numbers of cells expressing CD3 and CD56 were not changed by these 6-h stimulations. Stimulation of the cells by CD3 cross-linking resulted in the blocking of CD3, thus interfering with the subsequent detection of this molecule by flow cytometry (Fig. 4). Since only the cells that express surface CD3 would produce cytokines as a result of activation by anti-CD3 mAb, the frequencies of NT cells that produced cytokines were calculated as the percentages of HL that expressed CD56 and produced cytokines upon stimulation through CD3, divided by the percentages of unstimulated HL that expressed the $CD3^+CD56^+$ phenotype (Fig. 4).

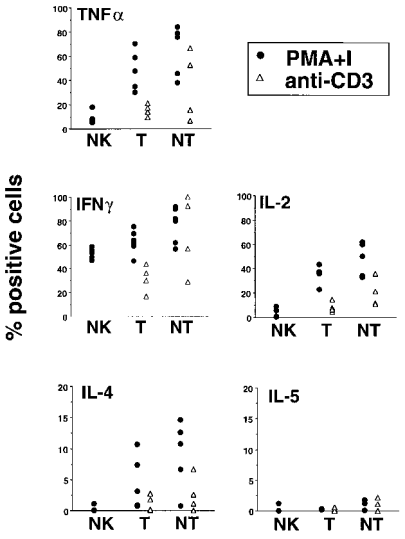

FIGURE 5. Production of proinflammatory cytokine TNF-α, Th1 cytokines IFN-γ and IL-2, and Th2 cytokines IL-4 and IL-5 by freshly isolated normal human hepatic $CD3^-CD56^+$ (NK), $CD3^+CD56^-$ (T), and $CD3^+CD56^+$ (NT) cells from normal liver specimens stimulated for 6 h with PMA + ionomycin (n = 6 liver samples) or plate-bound anti-CD3 mAb (n = 4 liver samples). The percent of positive cells was determined as described in the legends to Figs. 3 and 4.

Similarly, the frequencies of cytokine-producing T cells were calculated as the percentages of HL that were $CD56^-$ and produced cytokines upon CD3 cross-linking divided by the percentages of unstimulated HL expressing $CD3^+CD56^-$ phenotypes (Fig. 4). In all experiments, background cytokine production by similarly gated unstimulated cells or cells incubated with 1 ng/ml PMA was negligible, but values obtained were nevertheless subtracted from those obtained with stimulated cells to avoid subjectivity when defining positivity and negativity for cytokine production (Figs. 4 and 5).

Hepatic MNC from six normal donors were stimulated in vitro with PMA and ionomycin, and hepatic MNC from four donors were stimulated with anti-CD3 mAb. In addition, PBMC from six and two healthy donors were stimulated with PMA + ionomycin and anti-CD3 mAb, respectively. The proportions of the hepatic NK cells, T cells, and NT cells from these individuals that produced TNF-α, IFN-γ, IL-2, IL-4, and IL-5 are summarized in Fig. 5, and the mean percentages positivity for these cytokines are compared with those obtained with peripheral blood NK and T cells in Table I. Significant and comparable proportions of hepatic NT cells and T cells were capable of producing TNF-α, IFN-γ, IL-2, and IL-4, but not IL-5, upon PMA and ionomycin stimulation or CD3 cross-linking (Fig. 5). The percentages of cells expressing these cytokines were higher after stimulation with PMA and ionomycin than after CD3 cross-linking. In all donors examined, the majority (57–92%) of hepatic NT cells produced IFN-γ, while smaller proportions produced TNF-α (38–84%), IL-2 (32–61%), IL-4 (1–15%), and IL-5 (0–2%) (Fig. 5). Cytokine production by PMA and ionomycin-stimulated hepatic NK cells was limited to TNF-α (8.7%) and IFN-γ (53%) (Fig. 5). The proportions of hepatic NK cells and T cells that produced TNF-α, IFN-γ, IL-2, IL-4, and IL-5 upon PMA + ionomycin or anti-CD3 mAb stimulation were comparable to those of peripheral blood NK and T cells (Table I).

Th0 cytokine profiles of HL subpopulations

To examine coexpression of Th1 and Th2 cytokines by individual HL subsets, fresh MNC-enriched hepatic cells were stimulated with PMA and ionomycin and stained for surface expression of CD3, CD56, Vα24 TCR, or $\gamma\delta$TCR and intracytoplasmic production of IFN-γ and IL-4 or IL-5. Fig. 6*A* shows that the proportions of hepatic $CD3^+$, $CD56^+$, Vα24 TCR^+, or $\gamma\delta TCR^+$ cells that produce either or both of these cytokines could be determined using three-color flow cytometry with gating on the cells expressing the surface markers. The proportions of these HL subsets from five normal liver specimens that were capable of simultaneously producing IFN-γ and IL-4 or IFN-γ and IL-5 are shown in Fig. 6*B*. While the numbers of cells producing IL-5 were very low, up to 12% (mean 6%) of $CD3^+$ HL simultaneously produced IFN-γ and IL-4. This Th0 cell population included up to 11% (mean 5.1%) of Vα24 TCR^+ HL, which accounted for a mean of 2.7% of the total hepatic $CD3^+$ T cell compartment. The mean proportions of $CD56^+$ cells and $\gamma\delta TCR^+$ cells that demonstrated Th0 cytokine profiles were lower (means 1.0% and 1.6%, respectively). The majority of IL-4-producing cells in the $CD3^+$ (82.1%), $CD56^+$ (56.8%), V$\alpha 24^+$ (87.0%), and $\gamma\delta TCR^+$ (59.8%) HL subsets also produced IFN-γ, indicating that most hepatic IL-4-secreting cells exhibit Th0 phenotypes.

Discussion

The human liver is known to be a potent site of cytotoxic activity (11, 19, 30). In the present study, we have shown that the normal adult human liver contains at least three distinct populations of cytotoxic lymphocytes: NK cells, T cells, and NT cells, which can be defined by their expression of the $CD3^-CD56^+$, $CD3^+CD56^-$,

Table I. *Cytokine production by freshly isolated human hepatic $CD3^-CD56^+$ (NK), $CD3^+CD56^-$ (T), and $CD3^+CD56^+$ (NT) cells and peripheral blood NK cells and T cells upon stimulation with PMA and ionomycin or anti-CD3 mAb*[a]

	PMA + Ionomycin Stimulation					Anti-CD3 mAb Stimulation		
	Liver (n = 6)			Blood[b] (n = 6)		Liver (n = 4)		Blood[b] (n = 2)
Cytokine	NK	T	NT	NK	T	T	NT	T
TNF-α	8.7	48.4	64.7	12.1	47.0	15.7	35.5	5.4
IFN-γ	53.0	62.4	77.5	41.7	25.2	31.8	69.4	11.8
IL-2	3.3	35.0	47.7	2.4	32.5	8.2	19.9	ND
IL-4	0.2	4.6	9.1	0	1.4	1.2	2.6	2.5
IL-5	0.4	0.2	1.0	0.1	1.0	0.2	1.1	ND

[a] Intracytoplasmic cytokine production was determined by anti-cytokine mAb staining and flow cytometry, as described in *Materials and Methods*. The numbers give the mean percentages of gated NK, T, and NT cells that stained positive for each cytokine upon stimulation, as described in the legends to Figs. 3 and 4.

[b] Insufficient NT cells were present in peripheral blood for accurate cytokine analyses.

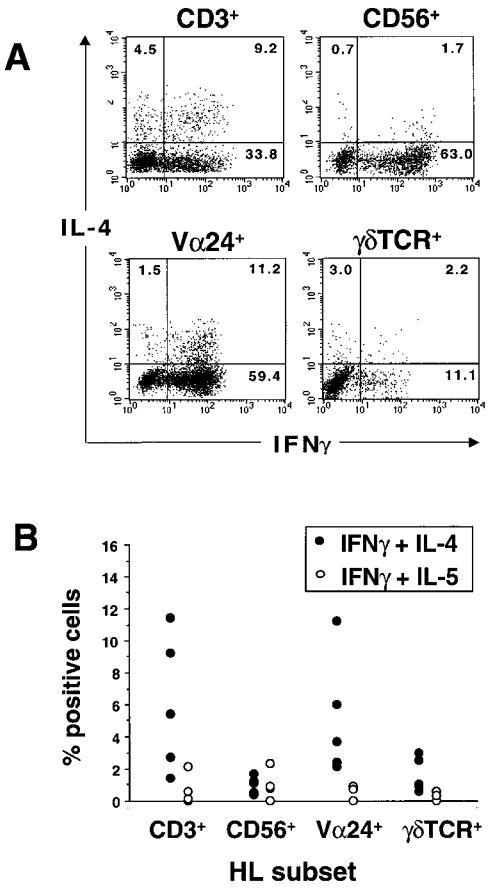

FIGURE 6. Simultaneous production of Th1 and Th2 cytokines by normal human HL subsets. *A*, Flow cytometric detection of IFN-γ and IL-4 production by HL from a normal liver specimen stimulated with PMA + ionomycin after gating on cells expressing CD3 (*top left*), CD56 (*top right*), Vα24 TCR (*bottom left*), and γδ TCR (*bottom right*). The numbers represent the proportions of the gated cells in each quadrant. *B*, Summary of the proportions of normal human hepatic $CD3^+$, $CD56^+$, Vα24 TCR^+, and γδ TCR^+ HL that simultaneously produced IFN-γ and IL-4 ($n = 5$ liver samples) or IFN-γ and IL-5 ($n = 4$ liver samples) upon stimulation with PMA + ionomycin.

and $CD3^+CD56^+$ phenotypes, respectively. Hepatic NK cells are capable of spontaneously lysing the NK-sensitive K562 target cell line and can be induced by culturing with IL-2 to lyse murine P815 cells. In contrast, conventional hepatic ($CD3^+CD56^-$) T cells do not kill NK-sensitive targets, but are capable of TCR-mediated cytolysis of P815 cells in mAb-redirected cytotoxicity assays. A duality of cytotoxic functions was observed with hepatic NT cells. Freshly isolated NT cells are not capable of spontaneously lysing K562 cells, but can be induced to do so by stimulation with PHA and culture with IL-2 (LAK activity). Unlike hepatic NK cells, this LAK activity does not extend to P815 targets, but cultured NT cells can be triggered by mAb cross-linking of CD3 to lyse P815 cells. Hepatic NT cells are thus capable of LAK and TCR-mediated cytotoxic function. These activities are similar to those of murine NKT cells expressing the NK1.1 receptor, which account for a similar proportion of normal murine HL (3, 4). $NK1.1^+$ T cells are capable of redirected lysis of Fc receptor-bearing cells in the presence of Abs to the CD3-TCR complex (20) and can be induced by exposure to cytokines, such as IL-2, IL-4, or IL-12 to lyse various NK-sensitive and NK-resistant tumor cells (23, 31). In particular, IL-12 administered systemically or produced by Kupffer cells in response to LPS, induces the proliferation and cytotoxic function of hepatic $NK1.1^+$ T cells (21, 23, 32), and these cells are thought to be the major effectors of IL-12-mediated tumor rejection (21, 22). Human cytotoxic $CD3^+CD56^+$ NT cells in peripheral blood are also selectively expanded by a combination of IL-2 and IL-12 (33), suggesting that the high numbers of NT cells present in the liver may simply reflect high concentrations of such inflammatory cytokines in this organ. Within this milieu, it is not surprising that hepatic NT cells express KIR and CD94/NKG2 receptors (19) that regulate their activities through interactions with MHC class I molecules on target cells (12, 13, 14).

The results of the present study also indicate that the human liver contains lymphocytes capable of the rapid secretion of proinflammatory, inflammatory (Th1 or Tc1), and helper (Th2 or Tc2) cytokines. Significant numbers of hepatic $CD3^+CD56^-$ T cells and $CD3^+CD56^+$ NT cells can produce TNF-α, IFN-γ, IL-2, and/or IL-4, but little or no IL-5, upon stimulation with PMA and ionomycin or immobilized anti-CD3 mAb. These cytokines are also produced by similar percentages of peripheral blood T cells. The proportions of peripheral T cells producing cytokines in response to PMA and ionomycin stimulation in our study are similar to those in previous reports (34, 35). In contrast to T cells and NT cells, both hepatic and peripheral NK cells produce TNF-α and IFN-γ only. Pharmacological stimulation of both hepatic and peripheral T cells with PMA and ionomycin was generally more potent than CD3 cross-linking as an activator of cytokine production and resulted in greater numbers of cells expressing all cytokines. This observation has previously been reported for T cell clones (36), and the more complete sustained stimulation by PMA and ionomycin is likely to reflect its independence of the levels of cell surface CD3/TCR expression and/or the involvement of other non-CD3-mediated signaling pathways. The percentages of hepatic NT cells expressing cytokines were similar or slightly higher than the percentages of hepatic NK cell and T cell fractions, and the majority of HL produced IFN-γ and TNF-α, about one-half of the T and NT cells produced IL-2, and up to 15% produced IL-4. These results indicate that the majority, though not all, of the lymphocytes in the normal human liver produce cytokines of the Th1/Tc1 profile, a finding that is consistent with previous reports of proinflammatory cytokine production by hepatocytes and Kupffer cells (32, 37, 38) and our demonstration of large numbers of cytotoxic lymphocytes in the liver. To our knowledge, this is the first report of cytokine production at the single cell level by HL subsets. The predominant inflammatory pattern of cytokine secretion found for human hepatic NT cells contrasts with that of murine peripheral NKT cells expressing NK1.1 receptors, which most notably produce large amounts of IL-4 (5, 7). These findings suggest that human hepatic NT cells are either functionally distinct from murine peripheral $NK1.1^+$ T cells, they include only small numbers of $NK1.1^+$ T cell homologues, or they are polarized by the liver to predominantly produce Th1/Tc1 cytokines only.

Cytokine production by mature T cells is generally polarized to Th1/Tc1 or Th2/Tc2 profiles, which appear to be mutually exclusive and are mutually inhibitory (39). The results of the present study indicate, however, that a significant number of hepatic T cells (mean 6%) can simultaneously produce IFN-γ and IL-4. Dual Th1/Th2 cytokine production has previously been reported for Vα24-JαQ TCR-bearing (15, 18) and γδ (40) T cells. In the present study, up to 11% (mean 5.1%) of hepatic $V\alpha24^+$ T cells and 1–3% hepatic $\gamma\delta^+$ T cells could simultaneously produce IFN-γ and IL-4 upon PMA and ionomycin stimulation. Since 6% of all hepatic $CD3^+$ cells can simultaneously produce these cytokines, but only ~2.7% of hepatic $CD3^+$ cells express the Vα24 TCR chain, it is clear that other, yet undefined, Th0 cells exist.

Although it has not been directly demonstrated at the level of single cells, murine NK1.1$^+$ T cells can also secrete both IFN-γ and IL-4 and are thought to be Th0 cells (5–7). In vitro and in vivo studies have shown that the cytokine profiles of these cells depend both on the nature of activating stimulus and on the nature of the cytokines and other soluble factors in the local microenvironment. Activation of NK1.1$^+$ T cells by CD3 cross-linking or with CD1 results in the production of both IFN-γ and IL-4, whereas stimulation of NK1.1 results in the production of IFN-γ only (6, 7, 10). IL-12 stimulates NK1.1$^+$ T cells to produce IFN-γ and inhibits their production of IL-4 (7, 25, 27), while IL-4 production by these cells requires IL-7 and is promoted by glucocorticoids (24, 26, 28). Because of their similarities to NK1.1$^+$ T cells, it is likely that human hepatic NT cells will be similarly regulated. One would therefore expect the microenvironment of the liver to have an important influence on their functional properties. Our finding that the majority of human HL taken freshly from their hepatic environment mainly produce Th1/Tc1 cytokines, suggests that the liver, a primary location of NT cell activity, provides a predominantly inflammatory microenvironment. Interestingly, the majority (mean 82%) of hepatic T cells that produced IL-4 also produced IFN-γ, indicating that most IL-4-producing T cells in the liver are of the Th0 type, rather than conventional Th2 cells.

The results of the present study indicate that the normal adult human liver is a site of potent immunological activity with multiple cytotoxic functions and patterns of cytokine secretion. Crucial to this activity is the presence of uniquely high numbers of NT cells in the liver. A role for NT cells as effectors in innate immune responses is suggested by their ability to respond rapidly to various Ag-nonspecific stimuli and to spontaneously lyse target cells. A second innate immune activity may lie in the Ag specificities of their TCRs. Hepatic NT cells include Vα24-JαQ TCR-bearing cells that recognize glycosylceramide Ags presented by CD1d (10, 16, 17). Hepatic NT cells are also particularly rich in $\gamma\delta$TCR$^+$ cells, a subset of which have been implicated in CD1c-restricted responses (41, 42). Furthermore, other human CD1-restricted T cells, which are known to express the CD8$^+$ or CD4$^-$CD8$^-$ phenotypes that are predominant among hepatic NT cells, and to recognize glycolipid components of bacterial cell walls (43, 44), may be also present in the NT cell fraction. Thus, the liver may have a role in immune responses to lipid Ags that are relatively fixed in structure and would constitute targets of innate recognition. Hepatic NT cells are also likely to function at the interface between the innate and acquired immune systems. Their ability to rapidly produce large amounts of both Th1 and Th2 cytokines upon primary stimulation suggests that they may regulate secondary adaptive immune responses. Defective regulation of the Th1/Th2 balance by these NT cells is thought to have a role in immune-mediated diseases (45–47).

Hepatic NT cells may also have a role in the destruction of autologous cells. The liver has been postulated to be a site for the elimination of activated T cells by apoptosis. Huang et al. (48, 49) reported that, following activation of peripheral CD8$^+$ T cells with Ag, a down-regulation of TCR expression is associated with their disappearance from the lymph nodes and spleen to the liver, where they undergo apoptosis. The mechanism of apoptotic death could be through the interaction of Fas (CD95, APO-1) with its ligand (FasL), since *lpr* and *gld* mice (which are deficient in Fas and FasL, respectively) accumulate large numbers of CD4$^-$CD8$^-$ $\alpha\beta$TCR$^+$ T cells expressing low TCR levels in the liver (50). Our findings of the liver being a site of potent cytotoxicity are consistent with this hypothesis and suggest that the liver might provide an "inflammatory" environment, as observed in the present study, that induces the Ag-nonspecific cytotoxic functions (LAK activity) of NT cells. Hepatic NT cells might also lyse autologous cells via TCR ligation. CD1-restricted T cells (15, 41) and $\gamma\delta$TCR$^+$ T cells (51) can recognize and respond to target molecules in the absence of any identifiable cognate Ag. Thus, the up-regulation of CD1 or heat shock protein expression by moribund target cells under certain conditions, such as the microenvironment of the liver, might render them susceptible to lysis by Vα24JαQ, $\gamma\delta$, or other NT cells. An important function of such autoreactivity is the cytolytic destruction of tumors, and several studies have implicated murine NKT cells expressing the V14Jα281 TCR (21, 22) and human hepatic CD3$^+$CD56$^+$ NT cells (52) as important antitumor effector cells. Hepatic NT cells are therefore likely to be future targets for manipulation in the treatment of malignancies and immune-mediated diseases.

Acknowledgments

We thank Michael Curry, Lucy Golden-Mason, Tina Deignan, Sheila O'Toole, and Aoife Coffey for assistance in obtaining normal liver specimens.

References

1. Hata, K., D. H. Van Thiel, R. B. Herberman, and T. L. Whiteside. 1992. Phenotypic and functional characteristics of lymphocytes isolated from liver biopsy specimens from patients with active liver disease. *Hepatology 15:816.*
2. Norris, S., C. Collins, D. G. Doherty, F. Smith, G. McEntee, O. Traynor, N. Nolan, J. E. Hegarty, and C. O'Farrelly. 1998. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J. Hepatol. 28:84.*
3. Ohteki, T., and H. R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of CD4$^+$8$^-$ and CD4$^-$8$^-$ subsets of natural killer 1.1$^+$ T cell receptor-$\alpha\beta^+$ cells in the liver of mice. *J. Exp. Med. 180:699.*
4. Tsukahara, A., S. Seki, T. Iiai, T. Moroda, H. Watanabe, S. Suzuki, T. Tada, H. Hiraide, K. Hatakeyama, and T. Abo. 1997. Mouse liver T cells: their change with aging and in comparison with peripheral T cells. *Hepatology 26:301.*
5. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W. E. Paul. 1995. Role of NK1.1$^+$ T cells in a Th2 response and in immunoglobulin E production. *Science 270:1845.*
6. Arase, H., N. Arase, and T. Saito. 1996. Interferon γ production by natural killer (NK) cells and NK1.1$^+$ T cells upon NKR-P1 cross-linking. *J. Exp. Med. 183: 2391.*
7. Chen, H., and W. E. Paul. 1997. Cultured NK1.1$^+$CD4$^+$ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. *J. Immunol. 159:2240.*
8. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4$^+$ and CD4$^-$8$^-$ T cells in mice and humans. *J. Exp. Med. 180:1097.*
9. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1$^+$ T lymphocytes. *Science 268:863.*
10. Kawano, T., J. Cui, Y. Koezuka, I. Taora, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of V_α14 NKT cells by glycosylceramides. *Science 278:1626.*
11. Ortaldo, J. R., R. T. Winkler-Pickett, H. Yagita, and H. A. Young. 1991. Comparative studies of CD3$^-$ and CD3$^+$CD56$^+$ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. *Cell Immunol. 136:486.*
12. Ferrini, S., A. Cambiaggi, R. Meazza, S. Sforzini, S. Marciano, M. C. Mingari, and L. Moretta. 1994. T cell clones expressing the natural killer cell-related p58 receptor molecule display heterogeneity in phenotypic properties and p58 function. *Eur. J. Immunol. 24:2294.*
13. Phillips, J. H., J. E. Gumperz, P. Parham, and L. L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science 268:403.*
14. Mingari, M. C., C. Vitale, A. Cambiaggi, F. Schiavetti, G. Melioli, S. Ferrini, and A. Poggi. 1995. Cytolytic T lymphocytes displaying natural killer (NK)-like activity: expression of NK-related functional receptors for HLA class I molecules (p58 and CD94) and inhibitory effect on the TCR-mediated target cell lysis or lymphokine production. *Int. Immunol. 7:697.*
15. Exley, M., J. Garcia, S. P. Balk, and S. Porcelli. 1997. Requirements for CD1d recognition by human invariant Vα24$^+$ CD4$^-$CD8$^-$ T cells. *J. Exp. Med. 186: 109.*
16. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med. 188:1521.*

17. Spada, F. M., Y. Koezuka, and S. A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med. 188:1529.*
18. Prussin, C., and B. Foster. 1997. TCR Vα24 and Vβ11 coexpression defines a unique NK1 T cell analog containing a unique Th0 population. *J. Immunol. 159:5862.*
19. Norris, S., D. G. Doherty, C. Collins, G. McEntee, O. Traynor, J. E. Hegarty, and C. O'Farrelly. 1999. Natural T cells in the human liver: cytotoxic lymphocytes with dual T cell and natural killer cell phenotype and function are phenotypically heterogeneous and include Vα24-JαQ and $\gamma\delta$ T cell receptor bearing cells. *Hum. Immunol. 60:20.*
20. Ballas, Z. K., and W. Rasmussen. 1990. NK1.1$^+$ thymocytes: adult murine CD4$^-$, CD8$^-$ thymocytes contain an NK1.1$^+$, CD3$^+$, CD5^{high}, CD44^{high}, TCR-Vβ8$^+$ subset. *J. Immunol. 145:1039.*
21. Takeda, K., S. Seki, K. Ogasawara, R. Anzai, W. Hashimoto, K. Sugiura, M. Takahashi, M. Satoh, and K. Kumagai. 1996. Liver NK1.1$^+$ CD4$^+$ $\alpha\beta$ T cells activated by IL-12 as a major effector in inhibition of experimental tumor metastasis. *J. Immunol. 156:3366.*
22. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science 278:1623.*
23. Hashimoto, W., K. Takeda, R. Anzai, K. Ogasawara, H. Sakihara, K. Sugiura, S. Seki, and K. Kumagai. 1995. Cytotoxic NK1.1 Ag$^+$ $\alpha\beta$ T cells with intermediate TCR induced in the liver of mice by IL-12. *J. Immunol. 154:4333.*
24. Vicari, A. P., A. Herbelin, M. C. Leite-De-Moraes, U. V. Freeden-Jeffry, R. Murray, and A. Zlotnik. 1996. NK1.1$^+$ T cells from interleukin-7 deficient mice have a normal distribution but exhibit impaired cytokine production. *Int. Immunol. 8:1759.*
25. Emoto, M., Y. Emoto, and S. H. E. Kaufmann. 1997. Bacille Calmette Guérin and interleukin-12 down-modulate interleukin-4 producing CD4$^+$ NK1$^+$ T lymphocytes. *Eur. J. Immunol. 27:183.*
26. Leite-De-Moraes, M. C., A. Herbelin, J. M. Gombert, A. Vicari, M. Papiernik, and M. Dy. 1997. Requirement of IL-7 for IL-4-producing potential of MHC class I-selected CD4$^-$CD8$^-$TCR$\alpha\beta^+$ thymocytes. *Int. Immunol. 9:73.*
27. Leite-De-Moraes, M. C., G. Moreau, A. Arnould, F. Machavoine, C. Garcia, M. Papiernik, and M. Dy. 1998. IL-4-producing NK T cells are biased towards IFN-γ production by IL-12. Influence of the microenvironment on the functional capacities of NK T cells. *Eur. J. Immunol. 28:1507.*
28. Tamada K., M. Harada, K. Abe, T. Li, and K. Nomoto. 1998. IL-4-producing NK1.1$^+$ T cells are resistant to glucocorticoid-induced apoptosis: implications for the Th1/Th2 balance. *J. Immunol. 161:1239.*
29. Doherty, D. G., J. E. Penzotti, D. M. Koelle, W. W. Kwok, T. P. Lybrand, S. Masewicz, and G. T. Nepom. 1998. Structural basis of specificity and degeneracy of T cell recognition: pluriallelic restriction of T cell responses to a peptide antigen involves both specific and promiscuous interactions between the T cell receptor, peptide, and HLA-DR. *J. Immunol. 161:3527.*
30. Hata, K., D. H. Van Thiel, R. B. Herberman, and T. L. Whiteside. 1991. Natural killer activity of human liver-derived lymphocytes in various liver diseases. *Hepatology 14:495.*
31. Takeda, K., and G. Dennert. 1994. Demonstration of MHC class I-specific cytolytic activity in IL-2-activated NK1$^+$CD3$^+$ cells and evidence of usage of T and NK cell receptors. *Transplantation 58:496.*
32. Takahashi, M., K. Ogasawara, K. Takeda, W. Hashimoto, H. Sakihara, K. Kumagai, R. Anzai, M. Satoh, and S. Seki. 1996. LPS induces NK1. 1$^+$ $\alpha\beta$ T cells with potent cytotoxicity in the liver of mice via production of IL-12 from Kupffer cells. *J. Immunol. 156:2436.*
33. Satoh, M., S. Seki, W. Hashimoto, K. Ogasawara, T. Kobayashi, K. Kumagai, S. Matsuno, and K. Takeda. 1996. Cytotoxic $\gamma\delta$ or $\alpha\beta$ T cells with a natural killer cell marker, CD56, induced from human peripheral lymphocytes by a combination of IL-2 and IL-12. *J. Immunol. 157:3886.*
34. Prussin, C., and D. D. Metcalfe. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Methods 188:117.*
35. Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood 86:1408.*
36. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med. 182: 1357.*
37. Thornton, A. J., J. Ham, and S. L. Kunkel. 1992. Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures. *Hepatology 15:1112.*
38. Rowell, D. L., L. Eckmann, M. B. Dwindell, S. P. Carpenter, J. L. Raucey, S.-K. Yang, and M. K. Kagnoff. 1997. Human hepatocytes express an array of proinflammatory cytokines after agonist stimulation or bacterial invasion. *Am. J. Physiol. 273:G322.*
39. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
40. Battistini, L., G. Borsellino, G. Sawicki, F. Poccia, M. Salvetti, G. Ristori, and C. F. Brosnan. 1997. Phenotypic and cytokine analysis of human peripheral blood $\gamma\delta$ T cells expressing NK cell receptors. *J. Immunol. 159:3723.*
41. Porcelli, S., M. B. Brenner, J. L. Greenstein, S. P. Balk, C. Terhorst, and P. A. Bleicher. 1989. Recognition of cluster of differentiation 1 antigens by human CD4$^-$CD8$^-$ cytolytic T lymphocytes. *Nature 341:447.*
42. Faure, F., S. Jitsukawa, C. Miossec, and T. Hercend. 1990. CD1c as a target recognition structure for human T lymphocytes: analysis with peripheral blood γ/δ cells. *Eur. J. Immunol. 20:703.*
43. Beckman, E. M., S. A. Porcelli, C. T. Morita, S. M. Behar, S. T. Furlong, and M. B. Brenner. 1994. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature 372:691.*
44. Sieling, P. A., D. Chatterjee, S. A. Porcelli, T. I. Prigozy, R. J. Mazzaccaro, T. Soriano, B. R. Bloom, M. B. Brenner, M. Kronenberg, P. J. Brennan, and R. L. Modlin. 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science 269:227.*
45. Barnaba, V., A. Franco, M. Paroli, R. Benvenuto, G. D. Petrillo, V. L. Burgio, I. Santilio, C. Balsano, M. S. Bonavita, G. Cappelli, et al. 1994. Selective expansion of cytotoxic T lymphocytes with a CD4$^+$CD56$^+$ surface phenotype and a T helper type 1 profile of cytokine secretion in the liver of patients chronically infected with hepatitis B virus. *J. Immunol. 152:3074.*
46. Sumida, T., A. Sakamoto, H. Murata, Y. Makino, H. Takahashi, S. Yoshida, K. Nishioka, I. Iwamoto, and M. Taniguchi. 1995. Selective reduction of T cells bearing invariant Vα24JαQ antigen receptor in patients with systemic sclerosis. *J. Exp. Med. 182:1163.*
47. Wilson, S. B., S. C. Kent, K. T. Patton, T. Orban, R. A. Jackson, M. Exley, S. Porcelli, D. A. Schatz, M. A. Atkinson, S. P. Balk, J. L. Strominger, and D. A. Hafler. 1998. Extreme Th1 bias of invariant Vα24JαQ T cells in type 1 diabetes. *Nature 391:177.*
48. Huang, L., G. Soldevila, M. Leeker, R. Flavell, and I. N. Crispe. 1994a. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunity 1:741.*
49. Huang, L., K. Sye, and I. N. Crispe. 1994b. Proliferation and apoptosis of B220$^+$CD4$^-$CD8$^-$TCRα/β^{int} T cells in the liver of normal mice: implications for *lpr* pathogenesis. *Int. Immunol.* 6:533.
50. Masuda, T., T. Ohteki, T. Abo, S. Seki, M. Nose, H. Nagura, and K. Kumagai. 1991. Expansion of the population of double negative CD4$^-$8$^-$ T$\alpha\beta$-cells in the liver is a common feature of autoimmune mice. *J. Immunol. 147:2907.*
51. Groh, V., A. Steinle, S. Bauer, and T. Spies. 1998. Recognition of stress-induced MHC molecules by intestinal epithelial $\gamma\delta$ T cells. *Science 279:1737.*
52. Shimizu, Y., S. Iwatsuki, R. B. Herberman, and T. L. Whiteside. 1990. Clonal analysis of tumor-infiltrating lymphocytes from human primary and metastatic liver tumors. *Int. J. Cancer 46:373.*