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Isolation, purification and flow cytometric analysis of human intrahepatic lymphocytes using an improved technique

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Intrahepatic lymphocytes (IHL) with their diverse and distinctive subsets emphasise the importance of the liver as a site of immunological activity, but special care is required for their isolation and characterisation. Protocols for IHL isolation, purification and FACS analysis were devised and compared with published extraction protocols. We have reduced the time that IHL are exposed to potentially damaging enzymes during extraction and purified specific subsets using monoclonal antibody (mAb)-coated magnetic microbeads. This has yielded IHL populations with higher viability than previously described protocols (92–95%, compared with 39–86%). Flow cytometric characterisation of IHL subset immunophenotypes was optimised by combining CD45 staining (fluorescence gating) with traditional light scatter properties. Using a panel of mAb and liver biopsies obtained from 23 cadaveric liver transplant donors, we show that the normal liver contains a heterogeneous IHL population with distinctive phenotypes. CD8$^+$ IHL was the predominant population with a mean CD4/CD8 ratio of 1:1.7. Up to 40% of IHL expressed $\gamma\delta$TCR and a third expressed CD56 NK marker; indicating a site of intense immunological activity. The techniques described will allow these cell types to be isolated, fully characterised and their physiological functions to be determined. The histologically normal liver contains heterogeneous and diverse IHL with large numbers of CD8$^+$, NK, NKT and $\gamma\delta^+$ cells.
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The liver plays a major role in the development of the immune system during embryonic life and remains an immunologically competent organ after birth.[1–6] To avoid pathological consequences, mechanisms that reduce unnecessary activation of the immune system are required in the face of continuous exposure to pathogens, toxins, tumour cells and dietary antigens.[7] The liver has a complex histological structure, which consists primarily of hepatocytes (70% of hepatic cellular component), with intrahepatic lymphocytes (IHL) constituting 16–22% of the remaining nonparenchymal cells (30%).[8–10] IHL have unusual characteristics and so their study in normal and diseased liver will be an important step towards understanding of the patho-

genesis of liver diseases. Local resident IHL may play a crucial role in avoiding immunological over-reactivity, as HLA incompatible allogeneic transplanted liver grafts can survive without the need for immunosuppression.[11]

In human liver, conventional T lymphocytes that express $\alpha\beta$ T-cell receptors (TCR) are accompanied by $\gamma\delta$TCR$^+$ T cells,[5] T cells expressing NK receptors (NKT cells), natural killer (NK) cells[5,12] and B cells.[5]

Immunohistochemical study of liver biopsies has facilitated *in situ* location of IHL to portal tracts and parenchyma and allowed simple phenotyping of lymphoid infiltrates[5,13–16] but does not provide details about lymphocyte subtypes or their functional attributes. For accurate qualitative and quantitative immunophenotyping, cells must be isolated from fresh tissue. However, special care is required to ensure sufficient cell yields accompanied by satisfactory viability for further analysis.

A variety of methods have been attempted for IHL preparation, including mechanical dissociation[17,18]

and/or enzymatic isolation protocols.[5,19–21] Mechanical methods have yielded very low cell numbers, with low viability and contamination with dead cells and cell debris. Enzymatic methods initially failed to improve cell yields due to long periods of incubation with collagenase.[19,22] Moreover, the effect of enzymes on the surface markers was not assessed. Proteolytic enzymes may affect the accuracy of lymphocyte phenotyping, since proteolysis of cell surface molecules might perturb their immunological detection and function.[17,18] Proteolysis may reduce expression of certain lymphocyte surface molecules[23] or inhibit response to stimulation *in vitro*.[24] Furthermore, multiple wash steps and/or overnight incubation may result in further cell loss. Recently, modified procedures for isolation and enrichment of nonparenchymal cells from human livers have been described.[5,21] These approaches improved yield and viability but resulted in time-dependent enzyme-induced alteration of certain markers.[21] Hence, further efforts are needed to improve the purity of single-cell suspensions and to ensure that there are sufficient cell yields with acceptable viability for phenotyping and functional analysis.

Using wedge liver biopsies obtained from adult human donors, we compare three different isolation protocols with respect to their effect on cell viability and degree of IHL purity. We have analysed purified cell subpopulations by flow cytometry to give a detailed account of the various IHL subtypes that reside in normal human livers.

Subjects and methods

All donors included in this study were part of the liver transplantation programme at King's College Hospital. Hospital Ethical Committee approval to collect liver biopsies from cadaveric donors with research consent was granted. Liver retrieval was achieved by a standard operation after *in situ* perfusion with University of Wisconsin (UW) solution.[25] UW has no significant effect on lymphocyte viability and functional characteristics[21,26] and removes the intrasinusoidal lymphocytes that constitute up to 20% of the hepatic lymphocyte pool, while the resident IHL remain.[17] Fresh liver biopsies ($\sim$10 g each) were collected from unused liver segments of 23 donors during liver reduction or splitting at the time of transplantation. Of the donors, 14 (61%) were females and nine (39%) males, with a median donor age of 29 years (range 14–59 years). The liver functions were within normal ranges. All liver biopsies were collected in complete RPMI 1640 medium, which contained 2 mM L-glutamine, 25 mM HEPES, 10% fetal calf serum (FCS), penicillin (50 Us/ml) and streptomycin (50 μg/ml) (Sigma-Aldrich, Dorset, UK).

Isolation Protocols

Each donor biopsy was divided into three equal pieces ($\sim$3 g each) for isolation of IHL using (a) mechanical, (b) enzymatic digestion,[5,21] and (c) a modified enzymatic dispersal protocol.

(a) *Mechanical method*
Hepatic tissue was dissected on ice, washed twice in Hank's balanced salt solution (HBSS) and scraped extensively with a sterile blade into homogenized suspension in a mixture containing 0.5 mg/ml collagenase (type IV, 312 U/mg) (Sigma-Aldrich), 0.02 μg/ml DNase I, 2% FCS, 0.6% bovine serum albumin (BSA) and HBSS at 37°C. The suspension was passed through a 30-μm-nylon mesh filter to remove cell clumps and undissociated tissue. The filtered suspension was centrifuged (500g; 10 min; room temperature) and the cell pellet washed twice in HBSS. The final pellet was resuspended in HBSS and hepatocytes removed by differential grade centrifugation (36g; 1 min; room temperature). The resulting supernatant was then centrifuged once again (500g for 10 min; room temperature) and the pellet was resuspended in complete RPMI 1640 medium. We used 20 μl of the fresh cell suspension to assess IHL numbers and viability using ethidium bromide and acridine orange stains (EB/AO) (see below).

(b) *Enzymatic digestion method*
Hepatic tissue was washed twice in HBSS and gently chopped with a sterile blade into $\sim$1 mm³ pieces in HBSS-containing collagenase IV (500 mg/l (312 U/mg)), DNase I (50 μg/l), FCS (2%) and BSA (0.6%). The tissue suspension was incubated at 37°C for 30 min, and then filtered, washed and centrifuged exactly as above (protocol a.).

(c) *Modified enzymatic dispersal protocol*
Hepatic tissue used for this protocol was washed and perfused twice with HBSS (37°C) to remove remaining intravascular lymphocytes. The liver tissue was injected with warm HBSS (37°C) containing collagenase IV (500 mg/l (312 U/mg)), DNase I (50 μg/l), FCS (2%) and BSA (0.6%) through the blood vessels on the surface whenever possible and directly into the tissue aiming to loosen it. Then, the tissue was gently teased apart with a sterile blade into $\sim$1 mm³ pieces in warm collagenase/HBSS solution (37°C). The resulting tissue suspension was incubated at 37°C for 15 min with frequent shaking. Following enzymatic digestion, ice-cold HBSS was added immediately to stop the action of the enzymes. The tissue suspension was filtered, washed and centrifuged exactly as before, but with all steps and solutions at 4°C.

The final cell pellets from each protocol were resuspended in FCS + 10% dimethyl sulphoxide (DMSO), stored at −20°C, then −80°C for 24 h each,

followed by storage in liquid nitrogen until required.

The cell number and per cent viability assessment was carried out using a 1:20 dilution of IHL suspension in EB/AO.[27] Viability was assessed under UV light using a haemocytometer, as viable cells fluoresce green and nonviable cells orange.

Liver biopsies from additional five cadaveric donors were used for mononuclear cell isolation using protocols a, b and c. The differential counts of the isolated cell populations were performed on counts of at least 200 cells using Papanicolaou-stained cytospin preparations.

Evaluation of the Apoptotic Effect of Collagenase Using Flow Cytometry

To determine if collagenase has any apoptotic effect on lymphocytes, purified spleen lymphocytes were labelled with flow cytometry TACS™ Annexin V-FITC/propidium iodide-PE (PI-PE) apoptosis detection kit (R&D Systems, Europe, Oxon, UK). Spleen lymphocytes were isolated from fresh spleen biopsies obtained from three cadaveric donors during multiorgan retrieval. Briefly, Spleen biopsies were mechanically homogenized in spleen preparation medium (1% disodium EDTA in PBS), filtered (30 μm nylon mesh) and washed in HBSS. Monocytes were positively depleted by incubating the spleen cell suspension with carbonyl iron powder (ISP, Manchester, UK) for 10 min at 37°C to allow iron particles phagocytosis by monocytes and then the monocytes were depleted using Dynal magnetic particle concentrator (MPC, Dynal, Merseyside, UK). The mononuclear cell supernatant was collected in clean Falcon® tube and purified by layering the supernatant cell suspension on lymphoprep™ (Nycomed Amersham, UK) (800g; 20°C; 20 min). The cloudy buffy coat was removed into a clean tube and washed twice in PBS and pelleted. After resuspending the cell pellet in HBSS, a small aliquot was kept as control. To the remaining cell suspension, a mixture of warm HBSS (37°C) containing collagenase IV, (500 mg/l (312 U/mg)), DNase I (50 μg/l), FCS (2%) and BSA (0.6%), as that used for IHL isolation, was added. The cell suspension was divided into three aliquots that were incubated at 37°C for 20, 50 and 90 min. Following enzymatic incubation, ice-cold HBSS was added immediately to the cell suspensions to stop the action of the enzymes and pelleted (500g; 4°C; 10 min). The cell pellets were resuspended, washed and centrifuged twice in ice-cold complete RPMI as mentioned above. The final pellets were resuspended in complete RPMI. Aliquots (100 μl) from each cell suspension were incubated with Annexin-V/PI-PE for 15 min at room temperature according to the manufacturer's instruction. Flow cytometry was performed using a Coulter EPICS-XL I set up for two-colour detection and XL system II software (Beck-man Coulter, High Wycombe, UK). Single Annexin V-FITC$^+$, double Annexin V-FITC$^+$/PI-PE$^+$, single PI-PE$^+$ and double negative cells staining indicated early apoptotic changes, late apoptotic changes, necrotic cells and viable cells, respectively.

Evaluation of the Apoptotic IHL Component in the Final Cell Product of Isolation Protocols b and c

Aliquots (100 μl) from the final IHL cell suspension isolated from the five additional donor liver specimens using protocols b and c were labelled with flow cytometry TACS™ Annexin V-FITC/PI-PE apoptosis detection kit as mentioned above.

Purification Protocols

A total of 10 donor IHL cell suspentions isolated using protocol c were retrieved from liquid nitrogen, left to thaw on ice and washed twice in cold complete RPMI 1640 medium. Cells were pelleted (500g; 4°C; 10 min) and resuspended in FACS buffer (PBS/BSA/sodium azide). Cell count and viability were evaluated before and after purification.

(1) Purification by simple filtration (Group I)
The filtrate was used directly for FACS analysis without any further purification.

(2) Filtration and positive selection of IHL using immunomagnetic microbeads
Positive selection was used to obtain a cell suspension free of parenchymal and other non-IHL, dead cells and cell debris.

(a) Using anti-CD2 beads (Group II)
CD2 costimulatory molecule is expressed on hepatic T and NKT cells.[5,12] Anti-CD2 immuno-magnetic microbeads (Dynal, Merseyside, UK: 6.6×10^8 beads/ml) and releasing buffer were used according to the manufacturer's instructions.

(b) Using anti-CD56 beads (Group III)
CD56 is expressed on all hepatic NK and NKT cells.[5,12] MACS anti-CD56 microbeads and separation column (Miltenyi Biotec, Surrey, UK; mouse IgG2b, Clone: NCAM 16.2) were used according to the manufacturer's instructions. Briefly, the magnetically labelled CD56$^+$ cells were retained in the column while the unlabelled CD56$^-$ cells continued through. After removal of the column from the magnetic field, the magnetically retained CD56$^+$ cells were eluted as a positively selected cell fraction.

Evaluation of Purification Protocols Using Flow Cytometry

Purified IHL populations were stained using CY5-conjugated CD45-specific monoclonal antibody

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(mAb) (CD45-CY5 (j.33 clone)). Cells from Group I and Group II were counterstained with mAb conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) flourochromes that included CD3-PE or CD3-FITC (UCHT1), CD19-PE (HD37), CD14-FITC (TÜK4), CD4-FITC (13B8.2), CD8-PE (B9.11) and CD161-PE (191B8) (Beckman Coulter, Buckinghamshire, UK and BD Biosciences, Oxford, UK). The cells from Group III were counterstained with CD3-PE or CD3-FITC, CD19-PE, CD14-FITC or CD161-PE.

IHL from Groups I–III were gated according to the forward and side light scatter properties of normal lymphocytes and also by CD45-CY5 fluorescence.

Phenotyping of Intrahepatic Lymphocytes

In all, 100 μl aliquots of IHL suspension that were isolated from 19 donor livers using the optimised protocol were stained with combinations of CD45-CY5, CD3-FITC, CD3-PE, CD19-PE, CD14-FITC, CD4-PE, CD4-FITC (13B8.2), CD8-PE, CD8-FITC, CD16-PE, CD56-PE (N901 (NKH1)), CD161-PE (191B8), $\alpha\beta$TCR-PE (BMA 031), $\gamma\delta$TCR-PE (IMMU510), TCRVα24 (C15) and CD45RO-PE (UCHL1) (Beckman Coulter, Bucks, UK and BD Biosciences, Oxford, UK). The lymphocyte populations were identified using CD45-CY5 fluorescence gating as above.

IHL phenotyping was performed using a Coulter EPICS-XL I set up for three-colour detection and XL system II software (Beckman Coulter, High Wycombe UK).

Flow cytometry data were analysed using WinMDI software and statistical analysis performed using SPSS-version 9.0. Nonparametric test (Mann–Whitney U-test) for independent samples was used to test differences among study groups.

Results

Isolation and Purification Methods

The mechanical method yielded low cell numbers (mean; 0.8×10^6/100 mg) with a mean 39% viability (ie viable: dead cell ratio $\sim$1:3). The original enzymatic digestion protocol improved yield and viability (1.3×10^6/100 mg and 86.4% viability), while the modified enzymatic digestion protocol had a lower yield, but the highest viability (1.16×10^6/100 mg (range $0.6-1.6 \times 10^6$) and 92.6% viability) (Table 1). Papanicolaou-stained cytospin preparations of the cell populations isolated using protocols a, b and c after purification with simple filtration, revealed that the lymphocyte population accounted for 84 ± 2.8% (range 81.5–88.5%) for protocol b compared with 85.5 ± 3.1% (range 81–89%) for protocol c ($P > 0.05$), with 16 ± 2.7% (range 11.5–18.5%) and 14.5 ± 3.5% (range 11–19%) hepatocytes and nonparenchymal (liver sinusoidal endothelial cells, dendritic cells, Kupffer cells and polymorphonuclear cells), respectively. The morphological characteristics of the cell population isolated using the mechanical protocol showed less parenchymal and nonparenchymal cells with high proportion of degraded cells, nuclei and cell debris (Figure 1).

The incubation of spleen lymphocytes with collagenase for different periods of time revealed no significant difference between 20 min exposure and the control with minimal apoptotic changes with 2.4% Annexin-V$^+$ and 2.3% Annexin-V$^+$/PI$^+$ compared to 1.2 and 0.7% for control, respectively. Significant early and late apoptotic changes were observed for 90 min incubation time as 9.5% of the cell population were Annexin-V$^+$ and 9.8% were Annexin-V$^+$PI$^+$ compared to 2.8 and 4.4% for 50 min incubation time and 2.4 and 2.3% for 20 min incubation time (Figure 2).

Table 1 Time of exposure to collagenase, cell yields and viability of IHL with different isolation techniques

	Isolation protocols		
	Mechanical (n = 23)	*Enzymatic dispersal* (n = 23)	*Modified enzymatic dispersal* (n = 23)
Total time of protocol (min)	40–50	60–70	60–70
Dissection in collagenase	7–10	5	5
Incubation with collagenase	0	30	15
Time in collagenase after incubation	15	15	0[a]
Total time of collagenase exposure	20–25	50	20
Cell yield/100 mg[b]	0.8×10^6 ($0.2-1.2 \times 10^6$)	1.3×10^6 ($0.9-3.2 \times 10^6$)	1.16×10^6 ($0.6-1.6 \times 10^6$)
Viability %mean (range)[b]	39 (12–68)	86.4 (68–92)	95.4 (74–98)
MNC purity (%mean $\pm$ (range))	62.6 ± 10.6 (48–72.5)	84 ± 2.8 (81.5–88.5%)	85.5 ± 3.0% (81–89%)
Parenchymal and nonparenchymal cells and cell debris (%mean $\pm$ (range))	37.4 ± 10.5 (27.5–52)[c]	16 ± 2.7 (11.5–18.5%)	14.5 ± 3.5 (11–19%)

[a]The action of collagenase was stopped immediately after incubation (modified protocol) by adding cold buffer (4°C).

[b]Mann–Whitney *U*-test was used to test the difference between different protocols. The difference was statistically significant between the mechanical isolation and both enzymatic dispersal and modified protocols ($P < 0.01$) in cell yield and viability; and there was no significant difference between enzymatic dispersal and modified protocol ($P > 0.05$).

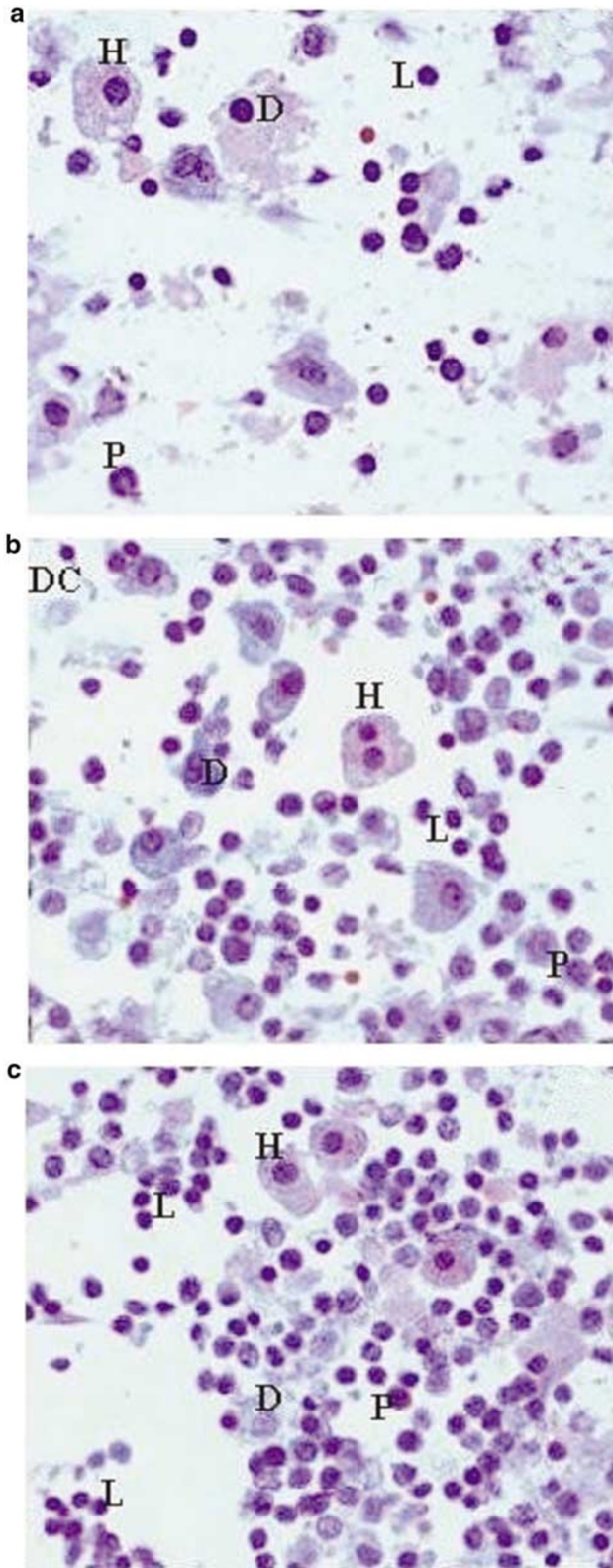[c]The majority were degraded cells and nuclei.

Figure 1 The morphology of liver cell products isolated using protocols a, b and c. Papanicolaou-stained cytospin preparations comparing the morphology of intrahepatic mononuclear cells isolated using mechanical (**a**), enzymatic digestion (**b**) and modified enzymatic dispersal (**c**) protocols (original magnification ×400). H; hepatocytes, L; lymphocytes, DC; dendritic cells, P; polymorphonuclear cells, D; degraded cells.

Small difference was found in apoptotic IHL population isolated from UW-perfused donor liver specimens using the enzymatic dispersal and the modified enzymatic dispersal protocol. Early (Annexin-V$^+$) and late apoptotic (Annexin-V$^+$/PI$^+$) IHL were 2.6% (range 0.5–4.1%) and 3.8% (range 0.7–5.8%) for the enzymatic dispersal protocol compared to 2.3% (range 0.5–3.3%) and 3.6% (range 0.5–4.7%) for the modified protocol, respectively. While dead or necrotic cells (PI$^+$) accounted for 7.7% (range 2.6–9.1%) for the enzymatic dispersal protocol compared to 2.3% (range 0.3–3.1%) for the modified protocol (Figure 3).

The use of anti-CD2 and anti-CD56 immunomagnetic microbeads enabled further purification of the IHL population (Table 2). There were less than 5% dead cells compared with 12% without beads, and less contamination with CD14$^+$ cells; 3.9, and 0.5%, respectively, compared to 15.2% without beads. Using CD45-CY5 to gate IHL in addition to microbeads isolation resulted in higher estimates of CD3, CD4, CD8 and CD161 positive cells and less contamination with other non-IHL cells (Figure 4). Indicating that these populations had previously been under estimated.

Phenotyping of Intrahepatic Lymphocytes in Donor Livers

Intrahepatic T lymphocytes (CD3$^+$)
Phenotype characterisation of IHL was performed using three-colour FACS analysis on freshly isolated IHL from 19 adult donor livers. The majority of the IHL population expressed CD3, with a mean value of 78.2% (Table 3). CD4$^+$ accounted for an average of 29.6% of the CD3$^+$ IHL and 43.9% expressed CD8 coreceptor, with a mean CD4:CD8 ratio of 0.7 (Table 3). A double-positive (CD8$^+$CD4$^+$) subset accounted for 2.7% of CD3$^+$, 10.2% of CD4$^+$ and 6.7% of the total CD8$^+$ IHL. A double-negative (CD8$^-$CD4$^-$) subset accounted for an average 22.7% of CD3$^+$ IHL (Table 4, Figure 5).

The majority of CD3$^+$ IHL expressed $\alpha\beta$TCR (mean 80.8%) and $\gamma\delta$TCR was expressed on 18.8% (Table 4). Most CD4$^+$ IHL expressed $\alpha\beta$TCR (mean 81.7%) while 15.9% expressed $\gamma\delta$. The majority of CD8$^+$ IHL expressed $\alpha\beta$TCR (mean 83.7%) while 18.4% expressed $\gamma\delta$TCR. CD45RO was expressed on an average 66% of CD4$^+$ and 35.2% of CD8$^+$ IHL (Table 4).

NKT cells (CD3$^+$CD56$^+$, CD3$^+$CD161$^+$CD56$^+$ and CD3$^+$Vα24$^+$)
Three-colour FACS analysis using CD45CY5 fluorescence gating of freshly isolated and filtration purified IHL revealed that approximately one-third of IHL lymphocytes expressed the CD56 NK marker (range 14.6–43.8%; Table 3). T cells that express CD56 (CD3$^+$CD56$^+$) accounted for 23.1% of the CD3$^+$ population and 15.9% of the total IHL (Table 4).
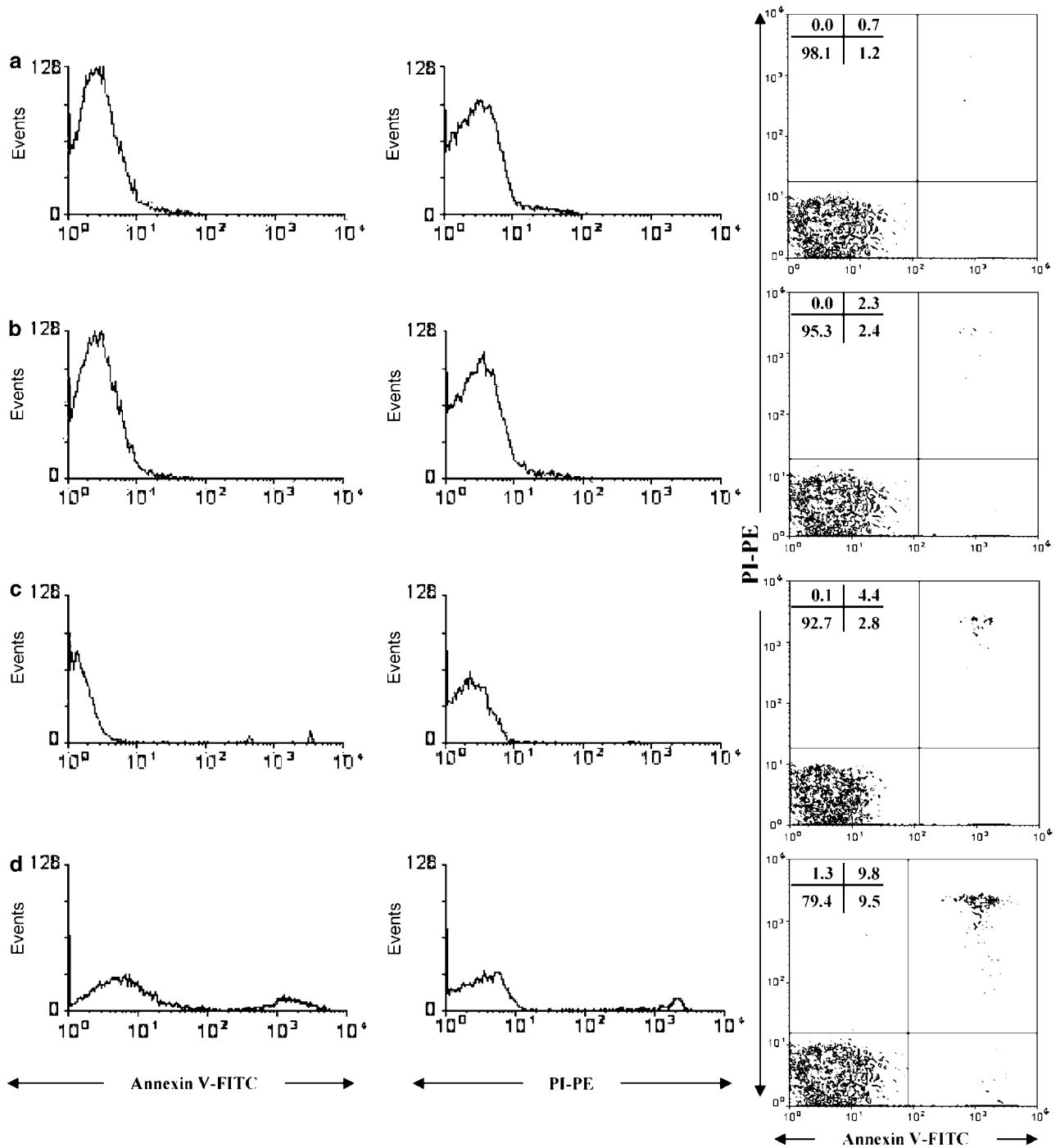
Figure 2 The apoptotic effect of collagenase on spleen lymphocytes. Flow cytometry histogram and contour plots demonstrating the apoptotic effect of collagenase on spleen lymphocytes comparing the incubation of collagenase with spleen lymphocytes for different periods of time. In comparison to control (**a**) there is minimal early (Annexin-V$^+$) and late apoptotic changes (Annexin-V$^+$PI$^+$) after 20 min (**b**) and 50 min (**c**) incubation. There is increasing apoptotic changes with increased incubation time to 90 min (**d**). The values inside represent the mean proportions of sorted cells.

Anti-CD56 immunomagnetic beads followed by CD45-CY5 fluorescence gating showed that CD3$^+$CD56$^+$ accounted for an average 44.7% of the CD56$^+$ IHL population. CD3$^+$CD161$^+$CD56$^+$ accounted for 10.7% of the total IHL, 69.8% of CD3$^+$CD56$^+$ IHL and 15.9% of the CD3$^+$ IHL population (Table 3). CD3$^+\alpha$24$^+$ accounted for 1.3% of the CD3$^+$ IHL population.

NK Cells (CD3$^-$CD56$^+$)
This analysis revealed that the human liver contains a proportion of IHL bearing the NK phenotype
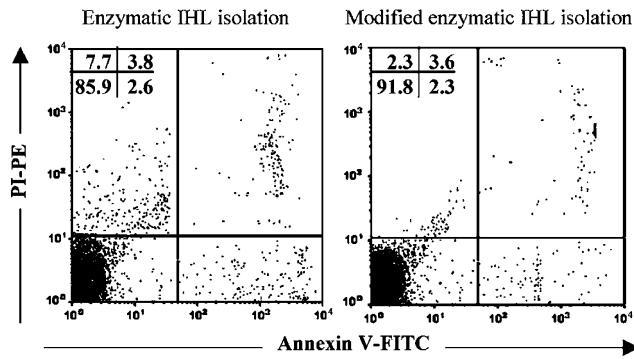
Figure 3 Isolation of the apoptotic IHL component. Flow cytometry contour plots demonstrating the apoptotic IHL component in the cell population isolated using the enzymatic digestion (left) and the enzymatic dispersal (right) protocols. Both protocols isolated approximately similar small proportions of apoptotic cells represented by Annexin-V$^+$ and Annexin-V$^+$PI$^+$ cells with slightly higher proportion for the enzymatic digestion protocol with more dead or necrotic cells (PI$^+$) than the modified protocol. The values inside represent the mean proportion of sorted cells.

necrotic and dead cell proportions compared with the modified protocol. The low proportion of apoptotic cells isolated using both protocols might be ascribed to the wash out of intrasinusoidal recruited apoptotic lymphocytes during liver retrieval and back table UW solution perfusion. The incubation of splenic lymphocytes with collagenase revealed increasing apoptotic changes with more than 1-h incubation, while small difference was found between 20 and 50 min incubation. However, several reports showed that long exposure to collagenase might also affect the accuracy of subsequent phenotyping.[17,18,23,21]

The optimised isolation protocol yielded sufficient pure and viable single cells for flow cytometry. However, the isolated cell population using different protocols including the modified dispersal protocol contained high numbers of parenchymal and nonparenchymal cells. Additionally, cryopreservation, thawing and frequent washing steps may lead to more cell death and hence cell debris. Therefore, we attempted further purification protocols that included simple filtration and positive selection for lymphocyte subsets using magnetic microbeads. The combined purification protocols resulted in highly viable single-cell suspensions. The optimised isolation protocol has been applied successfully for immune phenotyping in small size as well as percutaneous liver biopsies from patients with hepatitis C and alcohol-related chronic liver diseases and extrahepatic biliary atresia that has been and being carried out in our laboratory.

To improve the accuracy in estimating IHL subsets during flow cytometry, we have evaluated fluorescence gating by labelling the cells with a mAb specific for CD45 (the common leukocyte antigen). Using cells isolated with the optimised protocol, CD45-CY5 gating resulted higher estimates of IHL and less contamination with other nonparenchymal cells than simple light scatter parameters. The use of fluorescence gating could eliminate the need for purification protocols that may lead to selective subpopulation loss. Additionally, fluorescence gat-

(mean 19.7% of the total IHL; Table 3). NK (CD3$^-$CD56$^+$) accounted an average 55.3% of the total CD56$^+$ IHL population. CD3$^-$CD161$^+$CD56$^+$ accounted for 58.8% of NK IHL.

Discussion

In this study, a multistep protocol for IHL isolation, purification and FACS analysis has been devised and compared with published protocols for mechanical dissociation[5,17,18] and enzymatic dispersal.[5,19–21] The mechanical protocol resulted in poor viability, which was improved by enzymatic dispersal. However, the modified protocol yielded the highest percentage of viable cells. This may be ascribed to less mechanical disruption facilitated by injecting warm HBSS (37°C) containing collagenase, short exposure to collagenase and cold centrifugation compared to the original enzymatic dispersal method. While the original dispersal protocol isolated slightly higher proportions of apoptotic IHL, higher

Table 2 Implications of different purification/selection techniques

Protocol	Viability (% mean)	% Mean $\pm$ s.d.[a]					
		CD19$^+$	CD14$^+$	CD3$^+$	CD4$^+$	CD8$^+$	CD161$^+$
(1) Filtration	88.1	7.3$\pm$0.3	15.2$\pm$0.5	36.8$\pm$1.8	7.2$\pm$4.7	19.2$\pm$4.6	18.7$\pm$5.3
(2) Filtration and fluorescence gating[b]	88.1	6.6$\pm$0.2	5.8$\pm$0.6	50.0$\pm$2.5	13.4$\pm$7.3	25.1$\pm$3.8	25.2$\pm$2.7
(3) CD2 beads and fluorescence gating	95.4	0.8$\pm$0.4	4.2$\pm$0.5	77.5$\pm$4.8	18.8$\pm$5.6	36.4$\pm$8.7	18$\pm$3.3
(4) CD56 beads and fluorescence gating	97.3	0.3$\pm$0.081	0.5$\pm$0.1	—	—	—	52.6$\pm$2.8

[a]Values represent the percentage of each subset out of the gated population.

[b]Light scatter gating was used to sort cells in protocol 1 and fluorescence gating (CD45-CY5) to sort cells in the other three protocols.
Mann–Whitney U-test was used to test the difference between different protocols. The difference was statistically significant between prtocol 1 and 2 in all subsets; and significant between protocol 1 and 2 vs 3 in CD19$^+$, CD14$^+$, CD3$^+$, CD8$^+$ cells ($P < 0.01$).
The difference was significant between protocol 1 and 4 in tested subsets; and between 2 and 4 protocols CD19$^+$ and CD14$^+$ cell contamination. CD161$^+$ subsets are not compared in protocol 1 and 2 vs 3 or 4 due to the selective nature of the magnetic beads in protocols 3 and 4.
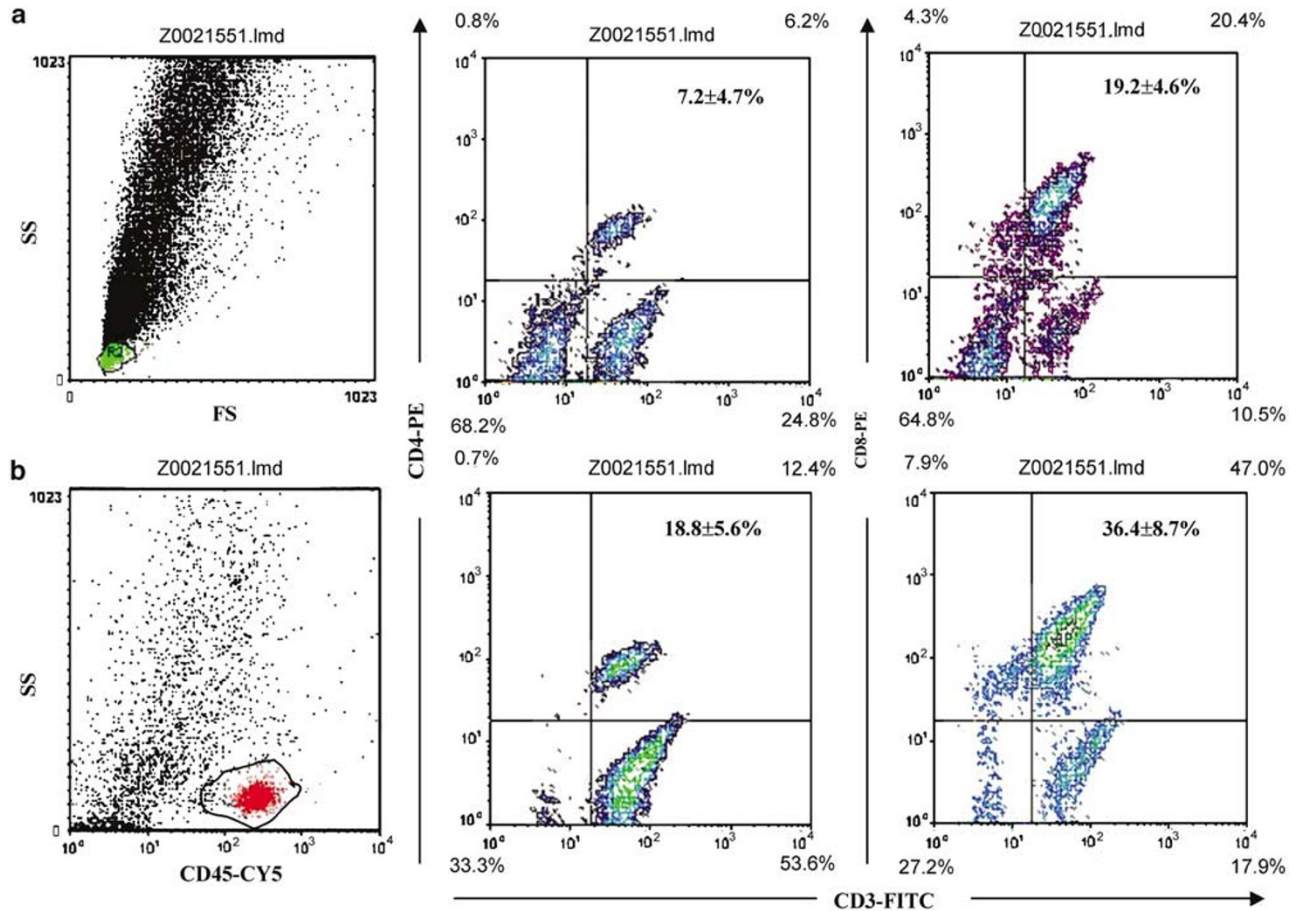— Not tested.

Figure 4 Flow cytometry plots demonstrating the differences in CD4$^+$ IHL and CD8$^+$ IHL proportions in the same cell suspension using different purification and cell sorting protocols. (**a**) Simple filtration protocol and cell sorting (gate) using the traditional light scatter (forward- and side-scatter) parameters (lymphogate-green), and two-colour FACS analysis. (**b**) Purification protocol using anti-CD2 immunomagnetic beads and cell sorting (gate) using fluorescence gating (anti-CD45-CY5-red) and three-colour FACS analysis. The values outside and inside represent the FACS reading of cell subsets in the studied sample and the mean values of CD4$^+$ and CD8$^+$ cells in all samples, respectively. These plots show that CD4$^+$ and CD8$^+$ IHL populations are underestimated in protocol (**a**) compared to (**b**) ($P < 0.01$).

Table 3 IHL subsets in donors

IHL subsets	%Mean ± s.d. (range) (n = 19)
Total T CD3$^+$	78.2 ± 11 (46.8–86.1)
Total NK CD3$^-$CD56$^+$	19.7 ± 6.4 (10.7–30.3)
Total CD3$^+$CD56$^+$	15.9 ± 7 (7.5–28.2)
Total CD56$^+$	35.6 ± 11.7 (18.2–51.6)
CD3$^-$CD56$^+$/CD56	55.3 ± 15.5 (21.3–66.2)
CD3$^+$CD56$^+$/CD56	44.7 ± 15.5 (33.8–78.7)
Total CD3$^-$CD161$^+$CD56$^+$	11.6 ± 3.7 (6.2–17.6)
Total CD3$^+$CD161$^+$CD56$^+$	10.7 ± 4.8 (5.2–19.4)
Total CD3$^+$Vα24$^+$	1.0 ± 0.6 (0.9–2.0)

CD3$^+$CD56$^+$, CD3$^+$CD161$^+$CD56$^+$ and CD3$^+$Vα24$^+$ are considered NKT cells.[35]

ing reduces the technical challenge, which in turn reduces time and cost.[28]

We demonstrated that histologically normal adult human liver contains high numbers of IHL that are charaterised by their heterogeneity and distinctive phenotypes. CD8$^+$ IHL were the predominant population with CD4$^+$/CD8$^+$ ratio 1:1.7, which is a reversal of peripheral blood lymphocytes, where CD4$^+$ T cells are the predominant subpopulation.[5,29] Double-positive (CD8$^+$CD4$^+$) and double-negative (CD8$^-$CD4$^-$) subsets constituted proportions of CD3$^+$ IHL population. CD45RO (effector/memory phenotype) was expressed on the majority of CD4$^+$ IHL. While these results are generally consistent with previous reports,[5,19] the detailed proportions of these subpopulations are different. CD4$^+$ and CD8$^-$CD4$^-$ T cells were higher and CD8$^+$ T cells were lower in this study compared with one study,[5] while CD3$^+$ cells were higher compared to another study.[19] Such cells, represent a constitutive population of the liver and not specific recruitment into the livers of brain-stem dead donors. Thus, the differences could be ascribed to the quality of donor liver, variations in IHL isolation protocols and the fluorescent gating used during flow cytometry.

While the vast majority of T cells present in the liver express αβTCR, we have shown that the histologically normal human liver contains high proportions of γδ$^+$ T cells (18.8%). The peripheral
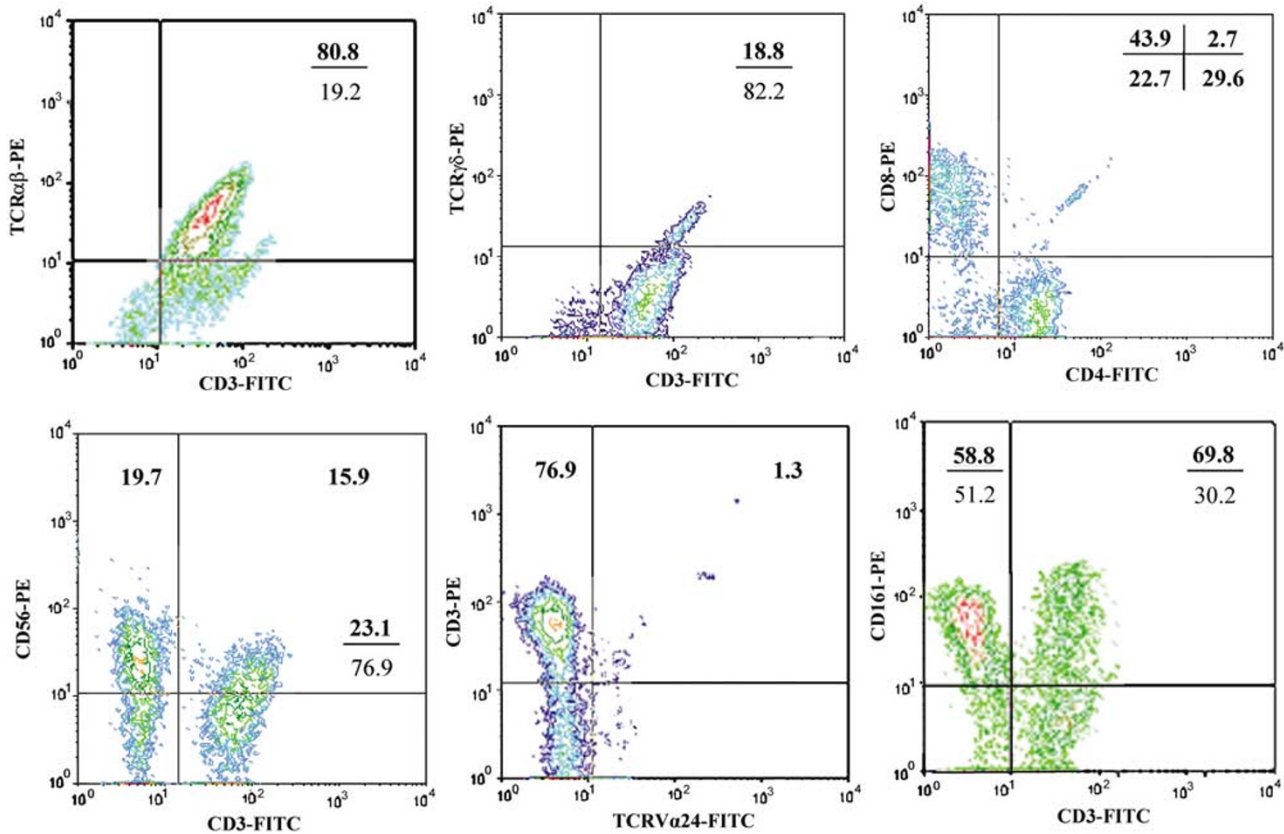
Figure 5 Intrahepatic Lymphocyte subsets. Flow cytometry contour plots show the diversity of IHL isolated from histologically normal donor liver specimens. The values inside represent the mean values of sorted subsets. The majority of CD3$^+$ IHL express $\alpha\beta$TCR (ab) with about one-fifth express $\gamma\delta$TCR (gd). CD8$^+$ IHL is the predominant population. The donor liver contains IHL populations that express CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ phenotype. Approximately, one-third of IHL express CD56 marker. The donor liver contains large populations of NK cells CD3$^-$CD56$^+$ IHL and NKT cells (CD3$^+$CD56$^+$, CD3$^+$Vα24$^+$ and CD3$^+$CD161$^+$). The last FACS plot shows the expression of CD161 by CD56$^+$ IHL selected using anti-CD56 immunomagnetic beads. The majority of NK and CD3$^+$CD56$^+$ IHL express CD161 marker.

circulation, spleen and lymph nodes of healthy adults contain 1–10% of T cells expressing $\gamma\delta$ TCR.[30] Thus, in agreement with previous findings the human liver is an important location for $\gamma\delta$ cells, with a higher concentration only found in the intestine.[5] In contrast to $\alpha\beta$TCR$^+$ T cells, which are selected in the thymus, the $\gamma\delta$ T cell repertoire may be generated and selected extrathymically.[31] $\gamma\delta^+$ T cells have an immunoregulatory role through release of IFNγ, TNFα and growth factors,[30,32] can mediate non-MHC-restricted cytolytic activity in a similar manner to NK,[33] and play an important role in eliminating damaged or infected epithelial cells.[30] The presence of large numbers of $\gamma\delta$ T cells in the liver may suggest that, like epithelial $\gamma\delta^+$ T cells,[34] they are involved in the elimination of parasites and microbes or virally infected and tumour cells. Intrahepatic $\gamma\delta$ T cells may also have a role in repair and regeneration in the liver through secretion of growth factors.[6]

In addition to conventional lymphocytes, normal human livers were populated with T cells expressing the NK receptor CD56 (CD3$^+$CD56$^+$) with a proportion of them also expressing CD161

(CD3$^+$CD161$^+$CD56$^+$). There was also a small population (1.2–2.5%) of T cells with CD3$^+$ CD56$^+$Vα24$^+$ phenotype, consistent with a previous report by Norris et al.[12] These CD3$^+$CD56$^+$ cells may also express KIRs and either $\alpha\beta$ or $\gamma\delta$ TCR[12] and have been classified as either NT[35] or NKT cells.[36] Although their precise phenotypic composition and identity compared to their murine counterparts remain controversial, it is clear that these cells have an important physiological role as they are able to kill or induce killing of human tumour cell lines and control hepatitis B and C virus and malaria infection.[36,37] Furthermore, NKT cells may be activated by microbial antigens and may have a role in autoimmune liver diseases pathogenesis.[37]

We showed that the human liver contains a proportion of NK cells (CD3$^-$CD56$^+$; 20%), the majority of them also expressing CD161, which is the human equivalent of mouse NK1.1. Estimates of the NK proportion in normal human liver have varied,[12,38,39] which is most likely due to the differences in isolation techniques, enzyme exposure times or even the quality of the original livers

Table 4 IHL subsets in donors

IHL subsets	%Mean ± s.d. (range) (n = 19)
CD3⁺ population	
CD4⁺CD3⁺	29.6 + 7.6 (23–47.4)
CD8⁺CD3⁺	43.9 + 8.5 (37.9–61.8)
CD4⁺CD8⁺	2.7 + 3.4 (0.3–10.5)
CD4⁻CD8⁻	22.7 + (9.8–38.6)
CD4⁺: CD8⁺	0.7 + 0.2 (0.5–1.3)
$\alpha\beta$TCR	80.8 + 10.3 (58.4–92.9)
$\gamma\delta$TCR	18.8 + 12.9 (3.1–42.8)
CD3⁺CD56⁻	76.9 + 8.6 (62.4–87.8)
CD3⁺CD56⁺	23.1 + 8.6 (12.2–37.7)
CD3⁺CD161⁺CD56⁺	15.9 + 5.9 (8.4–26)
CD3⁺Vα24⁺	1.3 + 0.8 (1.2–2.5)
CD4⁺ population	
CD4⁺CD8⁺	10.2 + 9.7 (2.7–30.5)
$\alpha\beta$TCR	81.7 + 7.1 (70.4–93.8)
$\gamma\delta$TCR	15.9 + 11 (1.01–30.1)
CD45RO⁺	66 + 11 (38.4–73)
CD8⁺ population	
CD8⁺CD4⁺	6.7 + 5.9 (1.4–18.8)
$\alpha\beta$TCR	83.7 + 6.6 (69.1–91.1)
$\gamma\delta$TCR	18.4 + 14.1 (2.1–39.4)
CD45RO⁺	35.2 + 20.9 (9.6–60)

CD3⁺CD56⁺, CD3⁺CD161⁺CD56⁺ and CD3⁺Vα24⁺ are considered NKT cells.

(the current study was performed on high-quality segment IV biopsies during split liver transplantation). Despite these differences, it is obvious that the liver contains one of the human body's highest concentrations of NK cells.[12,35] Hepatic NK cells are important for adaptive and innate immune responses in the liver, are involved in viral hepatitis,[36,40] alcohol-related liver disease,[40] autoimmune liver disease and tumour control.[37] They may be activated by NKT cells and are responsible for the liver injury associated with several liver diseases.[37] By secreting cytokines, hepatic NK can also activate and recruit cytotoxic CD8⁺ T cells and eosinophils and enhance naïve CD4⁺ T cell differentiation into Th1 cells.[35]

The results of this study indicate that the normal human liver contains high numbers of lymphocytes that, due to their diverse phenotypic characteristics, may render the liver an important site for extrathymic lymphocyte development and maturation. Extrathymic lymphocyte development has also been proposed, in both mice and humans, to occur in the small intestine, omentum and uterus.[41,42] IHL express genes that are necessary for T-cell differentiation, such as the recombination activation genes 1 and 2 (RAG1 and RAG2) and the pre-TCR α chain,[4] which suggests that the liver is capable of sustaining development and proliferation of these cells. Hepatic lymphocyte levels are likely to be further boosted or maintained by cytokine-enhanced lymphocyte recruitment from the peripheral circulation[43,44] Such intrahepatic T-cell accumulation may also be a major disposal pathway for apoptosing

CD8⁺ T-cells,[45] and proliferation and apoptosis of murine B220⁺CD4⁻CD8⁻ T cells.[46] In this study, the apoptotic IHL component accounted for approximately 6% of IHL population in human liver. However, *in situ* perfusion with UW solution[25] during the donor liver retrieval might lead to wash out of intrasinusoidal recruited apoptotic lymphocytes.

In summary, we have devised a multi-step modified protocol for IHL isolation, purification and phenotyping that resulted in increased cell viability and accuracy of IHL assessment. The modified protocol isolated slightly less proportions of the apoptotic IHL with higher viable and less dead cell proportions compared with the enzymatic dispersal protocol. Hence, the modified protocol may be the preferred technique for isolation of viable IHL suitable for culture and phenotyping. While the enzymatic dispersal protocol may be the preferred technique for isolation of representative population of liver lymphocytes including the apoptotic component of IHL. It is recommended to use positive or negative cell selection and fluorescence gating during FACS immunophenotyping of lymphocytes isolated from multicellular organs like liver. The data presented in this study also indicate that the human liver contains high numbers of IHL that were characterised by their heterogeneity and diversity. IHL are constituted by large numbers of NK, NKT and CD8⁺ cells, high proportions of CD4⁻CD8⁻, CD4⁺CD8⁺ and TCR$\gamma\delta$⁺ cells. These findings stress the immunological importance of the liver and the techniques described will enable the functional characteristics of each particular IHL subset to be elucidated *ex vivo*.

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