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Interleukin 12 (IL-12) is increased in tumour bearing human liver and expands $CD8^+$ and $CD56^+$ T cells in vitro but not in vivo

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Abstract

Human liver is enriched with $CD8^+$T- and $CD3^+CD56^+$ natural T (NT)-lymphocytes, important anti-tumour effectors, similar to murine NKTs. IL-12 promotes anti-tumour functions of NKTs. We quantified IL-12 and $CD56^+/CD8^+$T lymphocytes in normal and tumour bearing liver. We also examined the effect of IL-12 on the expansion/activation of peripheral blood cells in vitro.

IL-12 was detected in normal ($n = 13$, median 2032 pg/100 mg protein) and increased in tumour bearing liver ($n = 9$, 3678 pg, $p < 0.01$). Infiltrating monocytes appear to be the principal producers. Culture with IL-12 selectively expanded $CD8^+$T and $CD3^+CD56^+$NT cells and polarised their cytokine responses to Th1-type. However, there was no in vivo expansion of these cells in tumour bearing liver. Changes observed in culture required addition of IL-2. We therefore quantified IL-2 in hepatic tissue. IL-2 was detected in normal liver (median 4700 pg/100 mg protein). Surprisingly, there was no increase in tumour-infiltrated liver (4910 pg).

The presence of IL-12 may create an environment in healthy liver that promotes the accumulation of $CD8^+$T and $CD56^+$NT cells. Therefore, the development of metastases in the presence of high levels of IL-12 may be due to an insufficient IL-12 response. Alternatively, lack of IL-2 rather than a defect in IL-12, may be responsible for insufficient expansion/activation of tumour specific cytotoxic T lymphocytes.

Keywords: Cytokine; T lymphocytes; Tumour immunity; NKT

Abbreviations: IFN, Interferon; IL, Interleukin; mAb, Monoclonal antibody; NKT, Natural killer T cells; PBMCs, Peripheral blood mononuclear cells; PMA, Phorbol-12-myristate acetate; TGF, Transforming growth factor; Th1, T-helper type 1

1. Introduction

The adult human liver is constantly exposed to a large antigenic load which includes pathogens, toxins and tumour cells. Because of its location and rich blood supply, it is the primary site of metastases from colorectal carcinoma [1]. It is not surprising therefore that in addition to classical $CD8^+$ cytotoxic T lymphocytes of the adaptive immune system, the liver is a particularly rich source of lymphocytes that have a critical role in the early innate immune response against malignancy. These cells—natural killer (NK) cells ($CD3^-CD56^+$) and natural T (NT) cells ($CD3^+CD56^+$) account for greater than 50% of the hepatic lymphocyte pool, compared to approximately 15% in the peripheral blood [2–4]. NK cells in vivo spontaneously kill MHC class I deficient tumour cells

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and their metastases. They display potent cytotoxicity and produce cytokines upon interaction of their cell surface receptor with ligands [5,6]. Recently, tumour immunology has been revolutionised by the discovery of the role of NKT cells in the innate immune response to malignancy [7–10]. Murine NKT cells, particularly those expressing the invariant TCRα chain Vα14 Jα218, have been shown to have a direct antimetastatic role in certain inbred strains of mice [11]. Human NT cells display several phenotypic and functional similarities to murine NKT cells. They can rapidly lyse a range of tumour cell lines in vitro, rapidly secrete both IFN-γ and IL-4, and they express NK cell stimulatory, co-stimulatory and inhibitory receptors [12,13]. It is therefore likely that they may have similar antimetastatic roles to murine NKT cells. The hepatic microenvironment, specifically the cytokine milieu, has the potential to influence the function of these cells [14].

Interleukin 12 is a heterodimeric cytokine composed of two covalently linked p35 and p40 subunits, both of which are required for biological activity. It is produced primarily by antigen presenting cells such as monocytes, macrophages and dendritic cells and is central to the inflammatory response. It enhances the cytotoxic activity of Th1 cells, NKT and NK cells and induces the production of inflammatory cytokines including interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) [15–21]. Recently, a major antimetastatic role has been ascribed to IL-12. It has been shown to induce anti-tumour activity and tumour rejection in several animal models following systemic treatment with recombinant protein [22–25] or gene therapy for orthotopic carcinoma models [26,27]. There is a large body of evidence to show that the antimetastatic effect of IL-12 in mice is mediated through NK and NKT cells [9,11,28,29]. Mice deficient in the Vα14 T cell receptor α chain, which is expressed by NKT cells can no longer mediate IL-12 induced rejection of tumours [11]. Vα14 cells are an essential target of IL-12 and mediated IL-12 induced cytotoxicity by an NK-like effector mechanism. The anti-tumour effect of IL-12 can be replaced by the adoptive transfer of IL-12 activated Vα14 NKT cells, but not by IL-12 activated NK cells [10]. IL-12 is currently being assessed in clinical trials for the treatment of human malignancy [30]. However, the level of IL-12 in human liver is not known, nor has the implications of this on the accumulation of NT cell populations in malignant liver been studied. The aim of this study was to determine IL-12 protein levels in normal liver and, to compare this to levels in tumour infiltrated human hepatic tissue, analysed ex vivo in the absence of stimulation. We also investigated the effects of IL-12 on human T lymphocyte subpopulation expansion in vitro and quantified these subpopulations in normal and tumour bearing human liver.

2. Results

2.1. IL-12 protein levels in normal and tumour liver

Soluble protein was extracted from 13 normal and 9 tumour samples and yield was estimated using the BCA protein assay. Mean protein yield per 100 mg liver tissue powder was 8.1 mg (range 2.6–17.5). All samples tested positive for IL-12 protein. IL-12 was detected in normal liver, in the absence of in vitro stimulation at a median concentration of 2032 pg/100 mg protein. Similar levels were present in stimulated PBMCs (median 1981 pg/100 mg protein). Significantly lower levels were detected in resting PBMCs (1012 pg/100 mg protein, $p < 0.05$). IL-12 in tumour-infiltrated liver was significantly raised compared to normal liver (median 3678 pg/100 mg protein, $p < 0.01$) (Fig. 1).

2.2. Localisation of IL-12 producing cells in hepatic tissue

Immunohistochemical staining of hepatic tissue showed that IL-12 protein was mainly distributed within portal tracts. The cells staining positively for IL-12 have a mononuclear morphology and are therefore likely to be infiltrating monocytes (Fig. 2).

2.3. Effect of IL-2 and IL-12 on expansion of PBMCs

Following stimulation of PBMCs with anti-CD3 and 21 day culture with medium alone, IL-2 or a combination of IL-2 and IL-12, the cells were phenotypically characterised by surface mAb staining and three colour flow cytometry. The combination of IL-2 and IL-12 resulted in the selective expansion of $CD8^+$T cells.

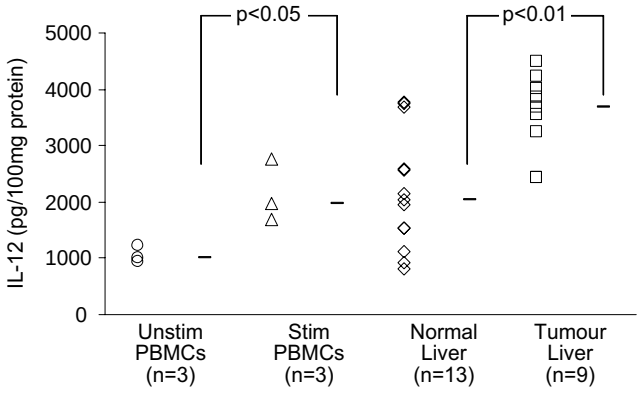

Fig. 1. Levels of IL-12 in normal and tumour bearing liver. IL-12 is significantly increased in tumour bearing liver. Levels in normal liver are similar to those of stimulated PBMCs, which are significantly greater than resting PBMCs. Each data point represents an individual sample. Horizontal bars represent the median.

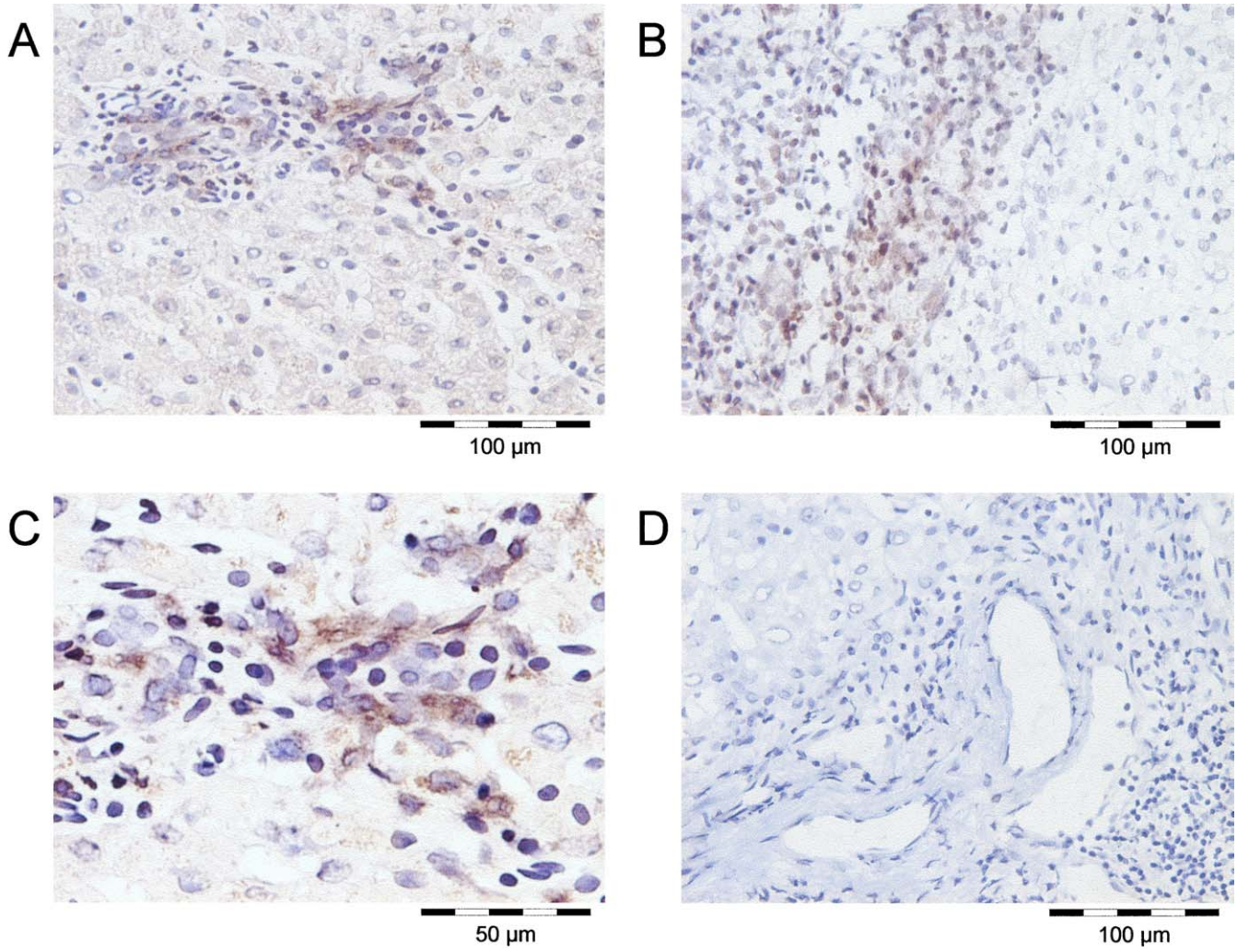

Fig. 2. Localisation of IL-12 expressing cells in hepatic tissue. (A) Staining pattern in normal tissue. IL-12 was mainly distributed within portal tracts. (B) Increased levels of IL-12 in tumour bearing liver may be a reflection of an increased inflammatory infiltrate within the portal tract. (C) Normal liver at a higher magnification shows the cells staining positively for IL-12 have a mononuclear morphology and are therefore likely to be infiltrating monocytes. (D) IgG negative control.

These cells were expanded from a median value of 26.9% of total lymphocytes to 56.6% following culture with IL-2 and IL-12. A greater than twofold expansion of NT cells ($CD3^+CD56^+$) was also observed following culture with IL-2 and IL-12 in combination (4.18%–11.06%). There were no expansions of either of these cell types following culture with IL-2 alone. $CD4^+$T cells expanded from a median value of 47.8% on day 0 to 51.7% following culture with IL-2 in combination with IL-12. This contrasts with an expansion to 75.3% following culture with IL-2 alone. $CD4^+CD8^+$ cells expanded from a median value of 0.87% of total lymphocytes prior to culture to a median of 1.89% following culture with IL-2 and to 6.2% following culture with IL-2 and IL-12 in combination (Figs. 3 and 4). Stimulated cells cultured in IL-12 alone were not viable following 21 day culture.

2.4. Effect of IL-2 and IL-12 on expansion of NT cell subsets

The $CD3^+CD56^+$NT cells that expanded following culture with IL-2 and IL-12 in combination were further characterised. $CD4^+$NT cells expanded from a mean of 1.3% of all NT cells prior to culture to a mean of 75% following culture with IL-2 and IL-12. Prior to culture 54.6% of all NT cells were $CD8^+$, however following culture they represented only 28% of all NT cells. Almost 60% of all NTs were $\alpha\beta TCR^+$ and 36% were $\gamma\delta TCR^+$ prior to culture but following culture this had changed to 94% and 3.5%, respectively. $CD57^+$ NT cells were also depleted on culture, with 38% of NT cells expressing this marker prior to culture but only 6.4% following culture. Similarly the expression of CD161 was reduced from 26% prior to culture to 3% following

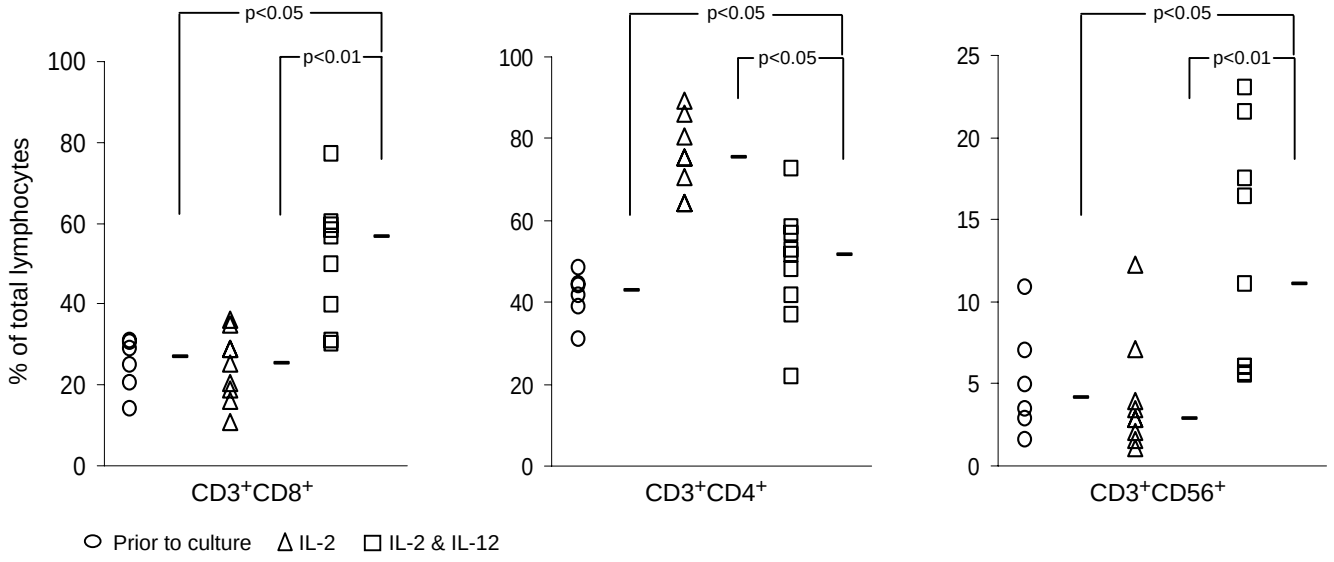

Fig. 3. Cell surface phenotype was examined by flow cytometry, prior to (○) and following 21 day culture with IL-2 (△) or IL-2 in combination with IL-12 (□). $CD3^+CD8^+$ and $CD3^+CD56^+$ cells were significantly expanded by addition of IL-12. Conversely, addition of IL-12 inhibits IL-2 mediated expansion of $CD3^+CD4^+$ cells.

culture. This depletion of CD161 and CD57 is likely a reflection of the loss of activated cells pre-programmed for apoptosis. Forty-five percent of all NT cells expressed CD94 prior to culture and this remains largely unchanged at 42% following culture. In order to determine if these changes were attributable to IL-12, we also examined the effect of culture with IL-2 in the absence of IL-12. Culture with IL-2 alone resulted in similar changes as those described for IL-2 in combination with IL-12 (Table 1).

2.5. Effect of IL-12 on cytokine secretion by NT and T cells

The effect of IL-2 and IL-12 on cytokine production by NT cells and conventional $CD56^-$T cells after stimulation with plate-bound anti-CD3 mAb was tested by flow cytometry. Fig. 5 shows that the Th1 cytokine, IFN-γ was produced by a third of NT cells (median 32.98%) following culture with IL-12 alone. This is in contrast to IFN-γ production by 14.8% of NT cells cultured in medium alone. Culturing in a combination of IL-2 and IL-12 resulted in IFN-γ production by the majority of NT cells (median 57.5%). However, a similar effect was seen following culture with IL-2 alone (median 62.6%). In contrast, IL-12 did not promote IFN-γ production by conventional T cells. Secretion of the Th2 cytokine IL-4 was not enhanced by culture with IL-12.

2.6. Levels of $CD8^+$ and $CD56^+$ T cells in normal and tumour liver

Using flow cytometry, we estimated the percentages of hepatic lymphocytes expressing CD8 and CD56 in normal and tumour bearing liver. Two thirds of hepatic T cells from normal donor liver were $CD8^+$ (median 66.7%, range 59.5–75.6%). Surprisingly, these levels were not increased in tumour bearing liver (median 67.5%, range 46.1–90.2%). Similarly, there was no significant difference in the level of hepatic T cells co-expressing CD56 in tumour bearing compared to normal liver (median 19.7% in tumour, 21.4% in normal) (Fig. 6).

2.7. IL-2 protein levels in normal and tumour liver

As the changes observed in cell culture appear to be related to contact with IL-2, we determined the ex vivo level of IL-2 in liver tissue. Soluble protein was extracted from five normal and five tumour samples and yield was estimated using the BCA protein assay. All samples tested positive for IL-2 protein. IL-2 was detected in normal liver, in the absence of in vitro stimulation at a median concentration of 4700 pg/100 mg protein (range 4490–12160). Surprisingly, there was no significant increase in levels of IL-2 in tumour-infiltrated liver (4910 pg/100 mg protein, range 2240–8390).

3. Discussion

The human liver is emerging as an important site of anti-tumour immunity and its lymphocyte repertoire represents a significant part of this defence mechanism. The hepatic lymphocyte environment is a particularly rich source of NK and NT cells, key mediators of anti-tumour immunity [3,4,31–34]. The factors that influence the accumulation of NK and NT cells are likely to

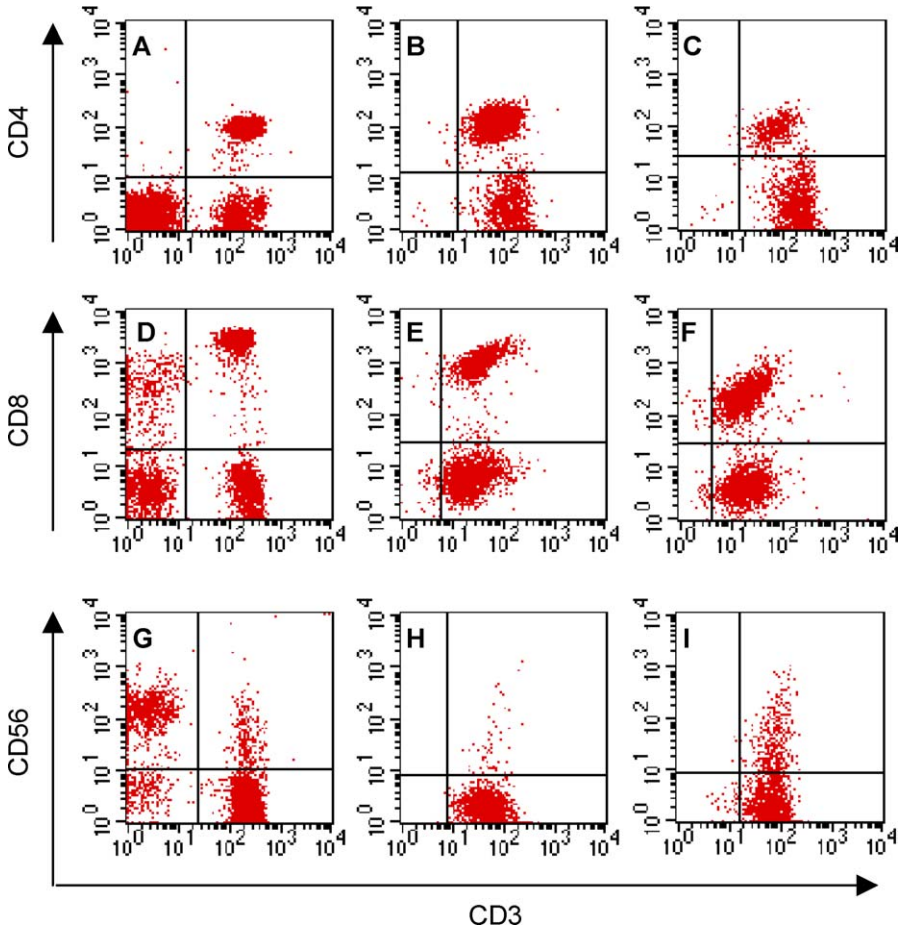

Fig. 4. Representative flow cytometric profiles. The top panel shows $CD3^+CD4^+$ cells prior to culture (A), following culture with IL-2 (B) and with IL-2 and IL-12 (C). The middle and lower panels show the same plots for $CD3^+CD8^+$ (D–F) and $CD3^+CD56^+$ (G–I), respectively.

include adhesion molecules, chemokines and cytokines [35]. IL-12 is a critical cytokine in anti-tumour immunity [17,22–29], but a quantitative difference in levels in normal and tumour bearing liver has not previously been demonstrated. In the present study, we quantified IL-12 levels and localised its expression in normal and tumour bearing liver samples and investigated the effects of this cytokine on proliferation and cytokine production by NT cells. Using ELISA, we found significant IL-12 levels in homogenised liver tissue from organ donors. These levels were comparable to those produced by in vitro stimulated PBMCs, suggesting the presence of an inflammatory microenvironment in the healthy liver. IL-12 levels were significantly higher in liver samples from patients with hepatic malignancy, suggesting activation of expression of this cytokine and its possible role in the regulation of the anti-tumour response. Using immunohistochemistry, we have demonstrated that IL-12 producing cells were confined to portal tracts and thus are likely to be infiltrating monocytes. Surprisingly, there was no evidence of a sinusoidal distribution suggesting that hepatic kupffer cells are not the major producers of hepatic IL-12.

Added in vitro to PBMCs, stimulated by antibody crosslinking of CD3, IL-12 alone did not induce expansions of any lymphocyte subsets. In the presence of IL-2, however, IL-12 induced the selective expansion of $CD8^+$T cells, $CD3^+CD56^+$NT cells and double positive $CD4^+CD8^+$T cells. These T cell populations were not expanded by IL-2 alone. Thus, IL-12 in the presence of IL-2 appears to support expansions of inflammatory lymphocytes, known to be capable of cytotoxic function.

Table 1
Characterisation of NT ($CD3^+CD56^+$) sub-populations following culture with IL-2 compared with IL-2 in combination with IL-12

	Pre-culture	Post-culture	
	Medium	IL-2	IL-2 and IL-12
$CD4^+$	3.5 (0.13–24)	67.0 (2.5–85)	65.5 (36–85.7)
$CD8^+$	56.5 (37–70)	44.0 (14.8–67)	29.5 (19.5–69)
αβTCR	67.5 (36.4–99)	90.5 (93–100)	98.0 (97.5–100)
γδTCR	35.0 (9.6–63.6)	5.2 (0–11.6)	3.4 (0–7.2)
$CD94^+$	41.9 (23–50)	39.0 (14.3–60.9)	44.2 (30–49.3)
$CD57^+$	27.0 (18.2–57)	0 (0–20.4)	0.75 (0–3.7)
$CD161^+$	47.1 (18.5–70)	0 (0–3.5)	0.40 (0–5.5)

All experiments were performed on six separate samples and results are presented as median (range) of values.

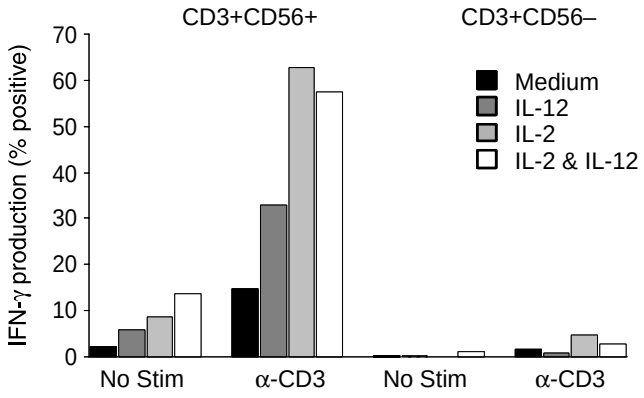

Fig. 5. IFN-γ secretion by NT cells and T cells cultured in medium alone, IL-2, IL-12 and IL-2 in combination with IL-12 for 72 h and subsequently stimulated with anti-CD3 in the presence of Brefeldin A for 4 h. Results are expressed as the median percentage of each cell type showing positive intracytoplasmic staining for IFN-γ and represent five experiments.

The elevated basal levels of IL-12 in the liver compared with blood suggest a role for this cytokine in the accumulation of $CD8^+$T cells and NT cells in the liver.

The majority of human hepatic and peripheral NT cells express CD8. In order to investigate whether the observed expansion of $CD8^+$T cells was due to the expansion of NT cells, we further characterised the phenotypes of the expanded NT cells. In contrast to the overall expansion of $CD8^+$T lymphocytes, within the NT cell subpopulation $CD4^+$ cells were selectively expanded. Culture with IL-12 also resulted in the selective expansion of αβNT cells with a significant reduction in γδNT cells. This contrasts to a previous study in which the majority of NT cells were $CD8^+\gamma\delta^+$ following culture [14]. Takeda et al. [28] have previously demonstrated that IL-12 activates liver NK1.1 $CD4^+\alpha\beta$ T cells as a major effector in the inhibition of experimental tumour metastases. It is interesting to note that although culture with IL-2 and IL-12 significantly expands $CD3^+CD56^+$ cells when compared to IL-2 alone, the changes in the subpopulations of these NT cells are similar following culture with IL-2 alone compared with IL-2 and IL-12. This implies that although IL-12 is responsible for the expansion of NT cells, it does not contribute to the changes in phenotype. Recently, Dunne et al. [35] reported that in the absence of stimulation, culture of PBMCs with IL-2 resulted in the selective expansion of NT cells, although CD4, CD8 αβ and γδ NT cells were similarly expanded. Our results indicate that upon stimulation, NT cells do not selectively expand among PBMCs in response to IL-2 alone, but CD4 T cells do. Therefore, this would suggest that the combined effects of TCR stimulation and cytokine induction are different than the effects of either treatment alone.

IL-12 released by macrophages and dendritic cells polarises $CD4^+$T cell differentiation to Th1 cells, producing IFN-γ but not IL-4 [15]. In contrast to conventional T cells, NT cells appear to be terminally differentiated, exhibiting effector functions within hours of activation. NT cells are uniquely capable of producing both IFN-γ and IL-4, therefore we investigated the effects of IL-2 and IL-12 on the production of these cytokines by NT cells after stimulation for 4 h by CD3 crosslinking. Our results confirm that NT cells, but not $CD56^-$T cells, can be activated to produce cytokines within 4 h. Preincubation with either IL-2, IL-12, or a combination of IL-2 and IL-12 led to an enhancement of rapid IFN-γ production by NT cells but not of IL-4 production. It is likely that culture with IL-2 alone results in maximal stimulation of these cells, therefore, no additional effect is observed with the addition of IL-12 to the IL-2 culture. Thus, while these cytokines promote Th1 cell differentiation of naive $CD4^+$T cells, they augment the Th1-like functions of NT cells.

Changes in cell phenotype following incubation with IL-12 demonstrate the potential of the cytokine microenvironment to influence the phenotypic profiles of lymphocyte subpopulations in liver. The presence of IL-12 may create an environment in the healthy liver that influences the accumulation of $CD8^+$T cells, double positive $CD4^+CD8^+$T cells and NT cells, all of which are known to have potent cytotoxic activities. This inflammatory environment suggests a role for the liver in immunity against tumours and the elevated levels of IL-12 in tumour bearing compared to histologically normal liver support this theory. However, neither NT nor $CD8^+$T cells are expanded in tumour bearing compared to normal liver. Norris et al. [36] showed no difference in the cytolytic function of these populations in tumour bearing compared to normal liver tissue, suggesting that natural T cells have not acquired increased anti-tumour activity as a result of IL-12 in vivo. Therefore, the presence of metastases in those livers containing high levels of IL-12 may be due to an insufficient IL-12 response, or to a breakdown in the IL-12/NT/CD8/IFN-γ pathway. Surprisingly, IL-2 was not elevated in tumour bearing liver tissue. This lack of IL-2, rather than a defect in IL-12, may be responsible for insufficient expansion/activation of tumour specific cytotoxic T lymphocytes. Undoubtedly, it is the balance and interplay between all cytokines and lymphocytes in the liver that will ultimately determine the outcome of the anti-tumour response.

4. Materials and methods

4.1. Tissue specimens

Normal liver tissue (100–200 mg) was obtained from donor organs at the time of transplantation ($n = 13$). Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy.

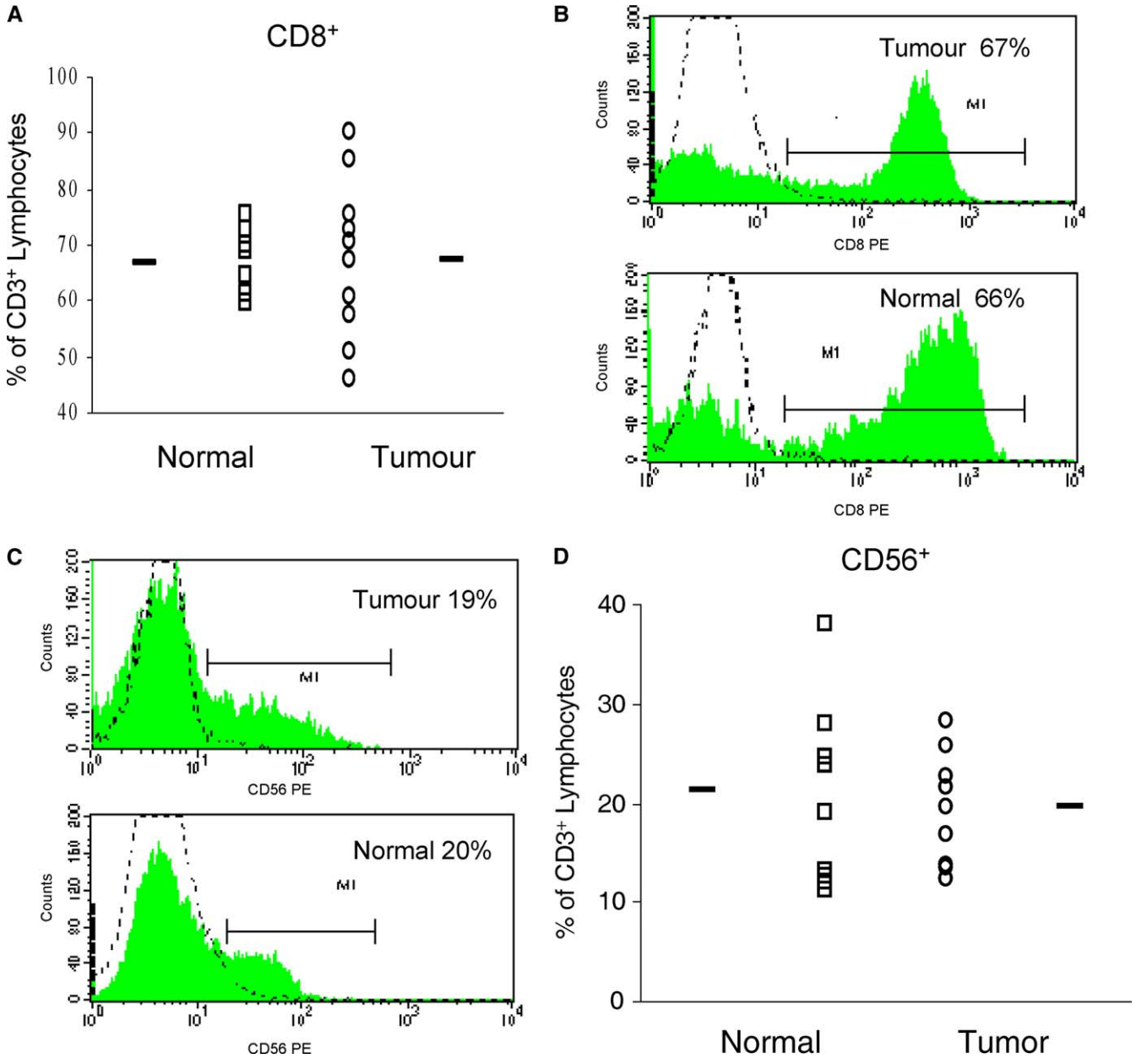

Fig. 6. Expressions of CD8 and CD56 on hepatic T cells ($CD3^+$) from normal and tumour bearing liver. Two thirds of $CD3^+$ cells from normal liver co-express CD8. While the level in tumour bearing liver is more variable, there is no significant difference demonstrated (A). Flow cytometric histogram analysis showing CD8 expression on $CD3^+$ hepatic cells from normal and tumour bearing liver. The dotted line represents the isotyped matched control antibody (B). CD56 is co-expressed on hepatic $CD3^+$ cells at a median value of 20% in both normal and tumour bearing liver (C and D).

Wedge biopsies were obtained from tumour bearing liver at the time of resection for colorectal metastases ($n = 9$). Resected tissue was washed three times in Hanks Balanced Salt Solution (HBSS) to remove residual blood. Hepatic lymphocytes were extracted for flow cytometry by a combination of mechanical and enzymatic disruption as described previously [37]. Whole liver tissue was immediately snap frozen in liquid nitrogen for estimation of cytokine protein.

Peripheral blood was obtained from volunteer controls. Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation (Lymphoprep™, Nycomed, Oslo, Norway). These cells were then used for culture or for the preparation of protein extracts.

The Ethics and Medical Research Committee at St. Vincent's University Hospital, Dublin, granted approval for the study.

4.2. Extraction of protein from liver tissue

Frozen liver samples were powdered using the Braun Mikrodismembrator (Braun Apparate, Melsungen, Germany). Protein was extracted from powdered tissue using 300 μl of lysis buffer (1% Igepal, 0.5% deoxycholic acid, 0.1% SDS in PBS) to which protease inhibitors (10 μl/ml PMSF [phenylmethylsulphonylflouride, dissolved in isopropanol], 3 μl/ml Aprotinin) had been added. All reagents for the extraction of protein were supplied by Sigma Aldrich, Ireland. The tissue powder was homogenised in the lysis buffer by passing several times through a 200 μl pipette tip. The homogenate was then incubated on ice for 30 min, followed by centrifugation at 10,000*g* for 10 min at 4 °C. The supernatant was harvested and total protein was quantified using a bincinconic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The lysates were stored at −80 °C. PBMC preparations ($n = 3$) at a concentration of 2×10^6/ml were stimulated for 6 h using 10 ng/ml phorbol-12-myristate acetate (PMA) and 1 μg/ml ionomycin, in the presence of Brefeldin A (10 μg/ml). Unstimulated cells were used as controls. Following stimulation, the cells were pelleted and the supernatant discarded. Protein was subsequently extracted from the cell pellets in the same manner as described above for hepatic tissue.

4.3. Quantification of IL-12/IL-2 protein

A sandwich enzyme linked immunosorbent assay (ELISA) suitable for use with hepatic tissue was developed to measure cytokine protein levels in liver and blood protein extracts. ELISA antibody pairs for the detection of cytokine protein were obtained from R&D Systems (Oxon, UK). The IL-12 capture and detection antibodies were used at concentrations of 4 μg/ml and 250 ng/ml, respectively, and the IL-2 capture and detection antibodies were used at concentrations of 4 μg/ml and 100 ng/ml, respectively. Recombinant human IL-12 and IL-2 (R&D Systems) were used to create a standard curve (range 0–1000 pg/ml). The assay was performed as per the manufacturer's instructions. Samples and standards were assayed in triplicate. A significant problem encountered in the use of hepatic tissue is the non-specific binding of sample to the plates and the contribution of endogenous alkaline phosphatase and biotin to the signal generated. In order to control for this, isotype matched IgG control antibody (Dako, Cambridge, UK) was used instead of capture antibody at the same concentration (4 μg/ml) for each of the samples examined, to control for the background signal generated by the complex mix of hepatic tissue proteins [38]. The optical density was determined at 410 nm using a spectrophotometer (Dynatech, Guernsey, Channel Islands). The readings obtained from the control wells were subtracted from the readings obtained for the corresponding samples. The concentration of cytokine protein in the unknown samples was calculated against the standard curve and expressed as pg/100 mg protein. The detection limit of the assays under these conditions was determined as 15.6 pg/ml.

4.4. Localisation of IL-12 protein expression in hepatic tissue

Sections (7 μm) were cut from frozen blocks of hepatic tissue embedded in OCT, placed on glass slides coated with 2% 3-amino-propyl-triethoxy-saline in acetone (APES, Sigma Aldrich) and dried overnight at room temperature. Immunoperoxidase staining of sections was carried out using the Vectastain® Elite ABC Kit (Vector Laboratories) according to the manufacturer's guidelines. The primary antibody used was a monoclonal anti-IL-12 (R&D systems, MAB 219, 20 μg/ml). IgG control antibody (Dako, Cambridge, UK, 20 μg/ml) was used in place of the primary antibody as a negative control. The HRP–substrate (diaminobenzidine tetrahydrochloride, DAB, Sigma Aldrich) reaction was allowed to proceed for 7 min and sections were counterstained for 30 s in Mayer's haemotoxylin (BDH).

4.5. In vitro cell culture

PBMCs from nine healthy volunteers were cultured at a concentration of 0.5×10^6/ml in 12 well plates (Becton-Dickinson, Oxford, UK) coated with anti-human CD3 antibody (5 μg/ml in 0.1 M Na_2HPO_4, pH 9 [Pharmingen, San Diego, CA, USA]). Human recombinant IL-2 alone (50 units/ml [Sigma Aldrich]) or IL-2 in combination with human recombinant IL-12 (0.5 ng/ml [R&D Systems]) was added to the wells every three days. Cells were restimulated with anti-CD3 every seven days. Cells were harvested after 21 days culture. Cells were counted by staining with ethidium bromide and acridine orange.

4.6. Phenotypic analysis of cells

Monoclonal antibodies (mAb) specific for human CD3, CD4, CD8, CD56, CD161, CD57, CD94, γδTCR, αβTCR, were obtained from Becton-Dickinson (Oxford, UK). Cells surface expression of lymphocyte antigens on peripheral blood cells and hepatic lymphocytes was examined by mAb staining and analysis by three colour flow cytometry (FACScan, Becton-Dickinson), before and after culture.

4.7. Cytokine Production by NT and T cells following culture

PBMCs from five healthy volunteers were cultured at a concentration of 0.5×10^6/ml in 25 cm^2 flasks (Corning, Cambridge, MA, USA) with IL-12, IL-2 and a combination of IL-2 and IL-12 at the same concentrations as used above for 72 h. Cells cultured in medium alone served as controls. Following culture, PBMCs were stimulated for 4 h by CD3 crosslinking using anti-human CD3 antibody (Pharmingen, San Diego, CA, USA) in the presence of Brefeldin A (10 μg/ml; Sigma, Poole, UK) for 4 h at 37 °C in 5% CO_2. This results in the accumulation of cytokine proteins in the rough endoplasmic reticulum. To determine levels of spontaneous cytokine production, unstimulated cells (i.e. no anti-CD3) were simultaneously incubated under the same conditions in the presence of Brefeldin A. Cells surface expression of lymphocyte antigens CD3, CD56 and intracellular cytokine expression of IFN-γ and IL-4 was examined by mAb staining and analysis by three colour flow cytometry (FACScan, Becton-Dickinson).

4.8. Statistics

Differences between groups were assessed using Mann–Whitney *U*. A p value of <0.05 was taken as significant.

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