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Hepatocytes and IL-15: A Favorable Microenvironment for T Cell Survival and CD8$^+$ T Cell Differentiation[1]

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Human intrahepatic lymphocytes are enriched in CD1d-unrestricted T cells coexpressing NKR. Although the origin of this population remains controversial, it is possible to speculate that the hepatic microenvironment, namely epithelial cells or the cytokine milieu, may play a role in its shaping. IL-15 is constitutively expressed in the liver and has a key role in activation and survival of innate and tissue-associated immune cells. In this in vitro study, we examined whether hepatocyte cell lines and/or IL-15 could play a role in the generation of NK-like T cells. The results show that both HepG2 cells and a human immortalized hepatocyte cell line increase survival and drive basal proliferation of T cells. In addition, IL-15 was capable of inducing Ag-independent up-regulation of NKR, including NKG2A, Ig-like receptors, and de novo expression of CD56 and NKp46 in CD8$^+$CD56$^-$ T cells. In conclusion, our study suggests that hepatocytes and IL-15 create a favorable microenvironment for T cells to growth and survive. It can be proposed that the increased percentage of intrahepatic nonclassical NKT cells could be in part due to a local CD8$^+$ T cell differentiation. *The Journal of Immunology,* **2009, 182: 6149–6159.**

The liver is a particular organ from the immunological point of view, being described as possessing immunostimulatory properties (1–3). Several studies have shown that liver sinusoidal endothelial cells (4), activated human hepatic stellate cells (5), and murine hepatocytes (6) can induce in vitro Ag-specific activation and T cell proliferation. Also, murine hepatocyte cell lines have been shown to promote expansion and differentiation of NK cells (3). In vivo cellular interactions between lymphocytes and epithelial cells may take place due to the fenestrated structure of hepatic sinusoids, combined with the lack of basement membrane and the low blood flow, which is unique between solid organs (7, 8). Intrahepatic lymphocytes have a distinctive phenotypic composition compared with blood and other organs. They are enriched in NK and NKT cells, and the CD4/CD8 ratio shows a skewing toward CD8$^+$ T cells with a memory/activated phenotype (9–11).

The factors responsible for this distinctive intrahepatic population are uncertain, but could include selective recruitment of those lymphocyte subsets from the periphery and/or local generation and differentiation (12, 13). Of particular interest is the fact that NKT cells are far more abundant in the liver than in any other place (10, 14, 15). Unlike in mice, human livers contain few CD1d-restricted NKT cells; instead they are enriched for CD3$^+$CD56$^+$ NKT cells coexpressing an oligoclonal TCR and NKR such as CD94/NKG2 and killer Ig-like receptor (KIR)[4] (16, 17), making even more intriguing the origin of this nonclassical NKT cell population. It has been proposed that the unique hepatic microenvironment, through interactions with either epithelial cells or local cytokines, may shape the development of this distinctive intrahepatic population (11, 13, 18).

IL-15 is a γ-common cytokine that has been reported to be present constitutively in the hepatic microenvironment (13, 19). Up-regulation of IL-15 expression has been reported in pathological conditions, such as hepatitis and hepatocellular carcinoma (13, 20–22). Besides having a key role in NK and NKT cell development and maintenance, IL-15 also promotes activation, survival, and differentiation of both memory and naive CD8$^+$ T cells (21, 23–26). Interestingly, some studies suggest that NKR can be expressed during the differentiation of CD8$^+$TCR$\alpha\beta^+$ cells into effector and memory cells (27, 28). Although the factors necessary for their induction remain uncertain, some studies suggest that IL-15, in the context of Ag stimulation, could up-regulate NKG2A expression in CD8$^+$ T cells (29), which, in parallel to the increasing association of this cytokine to tissue-associated immune responses, raised the possibility that IL-15 could be important in the hepatic context.

In this study, we examined the possibility that hepatocytes and IL-15 could create a favorable microenvironment for T cell survival and differentiation. To that end, we have used two human hepatocyte cell lines (hepatoma and nontumoral) and IL-15 to study parameters of T cell survival, expansion, and differentiation. The data suggest that in vitro, IL-15 and hepatocytes play an important role in the generation of a NK-like phenotype on CD8$^+$ T

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[4] Abbreviations used in this paper: KIR, killer Ig-like receptor; APS, amphotericin B/penicillin/streptomycin; CM, conditioned medium; FBSi, inactivated FBS; FSC, forward light scatter; IHH, immortalized human hepatocyte; PI, propidium iodide; SSC, side light scatter.

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Table I. *List of Abs used*

Ab	Conjugate	Company	Isotype	Clone
CD3	PE	ImmunoTools	IgG2a	MEM-57
CD3	PE-Texas Red	Beckman Coulter	IgG1	UCHT1
CD4	Allophycocyanin	ImmunoTools	IgG1	MEM-241
CD8	Allophycocyanin-Cy7	BD Biosciences	IgG1	SK1
CD56	Allophycocyanin	Beckman Coulter	IgG1	N901
KIR2DL2/S2/L3 (NKAT2)	PE	BD Biosciences	IgG2a	DX27
KIR2DL4 (CD158d)	PE	R&D Systems	IgG2a	181703
NKG2A (CD159a)	PE	Beckman Coulter	IgG2b	Z199
NKp46 (CD335)	PE	Beckman Coulter	IgG1	BAB281
NKG2D (CD314)	PE	Beckman Coulter	IgG1	ON72
CD3			IgG2a	OKT3
CD28		ImmunoTools	IgG1	15E8
HLA-DP, DQ, DR		DakoCytomation	IgG1	CR3/43
Mouse IgG1/IgG2 (isotype control)	FITC/PE	Beckman Coulter	IgG1/IgG2	679.1Mc7/7T4-1F5
Mouse IgG1 (isotype control)		R&D Systems	IgG1	11711
IL-15		R&D Systems	IgG1	34559
Polyclonal rabbit anti-mouse IgG	FITC	DakoCytomation		
Rabbit anti-mouse		DakoCytomation		

cells, both through the induction of cell survival (IL-15 and hepatocytes) and NKR (IL-15). The likelihood that local differentiation of $CD8^+$ T cells takes place within the liver microenvironment deserves further investigations.

Materials and Methods

Reagents and mAbs

RPMI 1640 GlutaMAX, MEM GlutaMAX, DMEM-F12 (with L-glutamine + 15 mM HEPES), bovine insulin, dexamethasone, trypsin/EDTA, FBS, and antibiotic/antimycotic (100×) solution (amphotericin B/penicillin/streptomycin (APS)) were from Life Technologies. CFSE and annexin V-Alexa488 were purchased from Molecular Probes. Human serum was obtained from Cambrex. Human rIL-15 was obtained from R&D Systems. Propidium iodide (PI) was obtained from Sigma-Aldrich. The used mAbs are listed in Table I.

Cell lines

The HepG2 and HeLa cell lines were obtained from European Collection of Cell Cultures and maintained in MEM GlutaMAX supplemented with 10% inactivated FBS (FBSi) and 1% APS solution. The AGS cell line, a human gastric epithelial adenocarcinoma cell line, was obtained from the American Type Culture Collection and maintained in RPMI 1640 GlutaMAX supplemented with 10% FBSi and 1% APS solution. Immortalized human hepatocytes (IHH; clone 10.3) were provided by Dr. T. H. Nguyen (INSERM, University Hospital, Nantes, France) and maintained in DMEM-F12, as described (30). Cells (0.5×10^6) were placed in 75-cm^2 flasks (Nunc) and incubated at 37°C, 5% CO_2, and 95% humidity. When cells reached confluence, 5 ml of a 1% solution of trypsin/EDTA was added for 5–10 min at 37°C. After harvesting, cells were washed with 1× PBS and resuspended in the corresponding medium. For use in cocultures, hepatocyte cell lines were trypsinized as described and placed at 0.5×10^6 cells/well in 24-well plates overnight to recover. Afterward, cells were harvested, irradiated (10,000 rad), and washed before coculture.

Isolation of PBL and T cell subsets

Fresh PBMC or cord blood was obtained from buffy coats after centrifugation over Lymphoprep (Nycomed). Contaminating RBC were lysed in lysis solution (10 mM Tris and 150 mM NH_4Cl (pH 7.4)) for 10 min at 37°C. Partially purified PBL were obtained by routine overnight culture. T cells were enriched by rosetting with sheep RBC (ProBiologica), yielding a population with <1% monocytes or B cells. Pure $CD8^+$ T cells (>95%) were obtained by positive selection using MACS microbeads and columns (Miltenyi Biotec). Pure $CD8^+CD56^-$ T cells were obtained by two-step isolation. First, $CD8^+$ T cells were isolated from PBL using a CD8-negative isolation kit (Miltenyi Biotec). Then, collected cells were subjected to positive isolation with CD8 beads (Miltenyi Biotec), obtaining $CD8^+$ T cells that were >99.5% $CD8^+CD3^+CD56^-$. In cord blood, only CD8-negative isolation was performed, due to the limitation in cell number.

Culture conditions

For coculture studies, PBL, purified T cells, or pure $CD8^+$ T cells were labeled with CFSE, as described below. One million cells were cultured alone, with irradiated cell lines, IL-15, or a combination of both for 7 days. Cocultures with irradiated HepG2, IHH, HeLa, or AGS cell lines were performed at a lymphocyte:cell line ratio of 4:1 in 24-well plates (Nunc) in a final 1 ml of RPMI 1640 supplemented with 3% human serum and 1% APS solution, at 37°C, 5% CO_2, and 95% humidity. IL-15 was added at a final concentration of 10 ng/ml. In some experiments, cocultures were performed in the absence of cell contact using either cell culture inserts (0.2 μm of pore diameter; Nunc) or conditioned medium (CM) from overnight cultures of HepG2 or IHH. Supernatants were centrifuged twice before addition to PBL cultures. For blocking experiments, HepG2 cells were first preincubated for 1 h at 4°C with saturating amounts of anti-IL-15 (clone MAB2471) or the respective mouse IgG1 isotype control, both from R&D Systems. For NKR expression studies, 10^6 $CD8^+CD56^-$ T cells/ml were labeled with CFSE, as described below, and cultured in RPMI 1640 supplemented with 10% human serum and 1% APS solution, at 37°C, 5% CO_2, 95% humidity, during 6 and 12 days in the absence or presence of

Table II. *Sets of primers used for NKR mRNA amplification by RT-PCR*

	Sense Primer	Antisense Primer	Product (bp)
KIR2DL2	CCA CTG CTT GTT TCT GTC AT	CAG CAT TTG GAA GTT CCG C	370
KIR2DL3	CCT TCA TCG CTG GTG CTG	CAG GAG ACA ACT TTG GAT CA	252
KIR2DL4 v1	CGG GCC CCA CGG TTC GCA	AGG CAG TGG GTC ACT CGC	249
KIR2DL4 v2	GGG CCC CAC GGT TCG CG	AGG CAG TGG GTC ACT CGG	249
KIR3DL2	CGG TCC CTT GAT GCC TGT	GAC CAC ACG CAG GGC AG	368
NKG2A	CCA GAG AAG CTC ATT GTT GG	CAC CAT CCT CAT GGA TTG G	325
NKG2D	GAA GAC TTT AGA TTC CTC TCT GCG G	GAC TAC TGG ACA TCT TTG CTT TTG C	175
β-actin	GAA GAT CCT CAC CGA GCG C	AGG GTA CAT GGT GGT GCC G	352
CD56	TTC TTC GCT GCT GAT GTT CC	TTG TGA ATG TGC CAC CTA CC	437

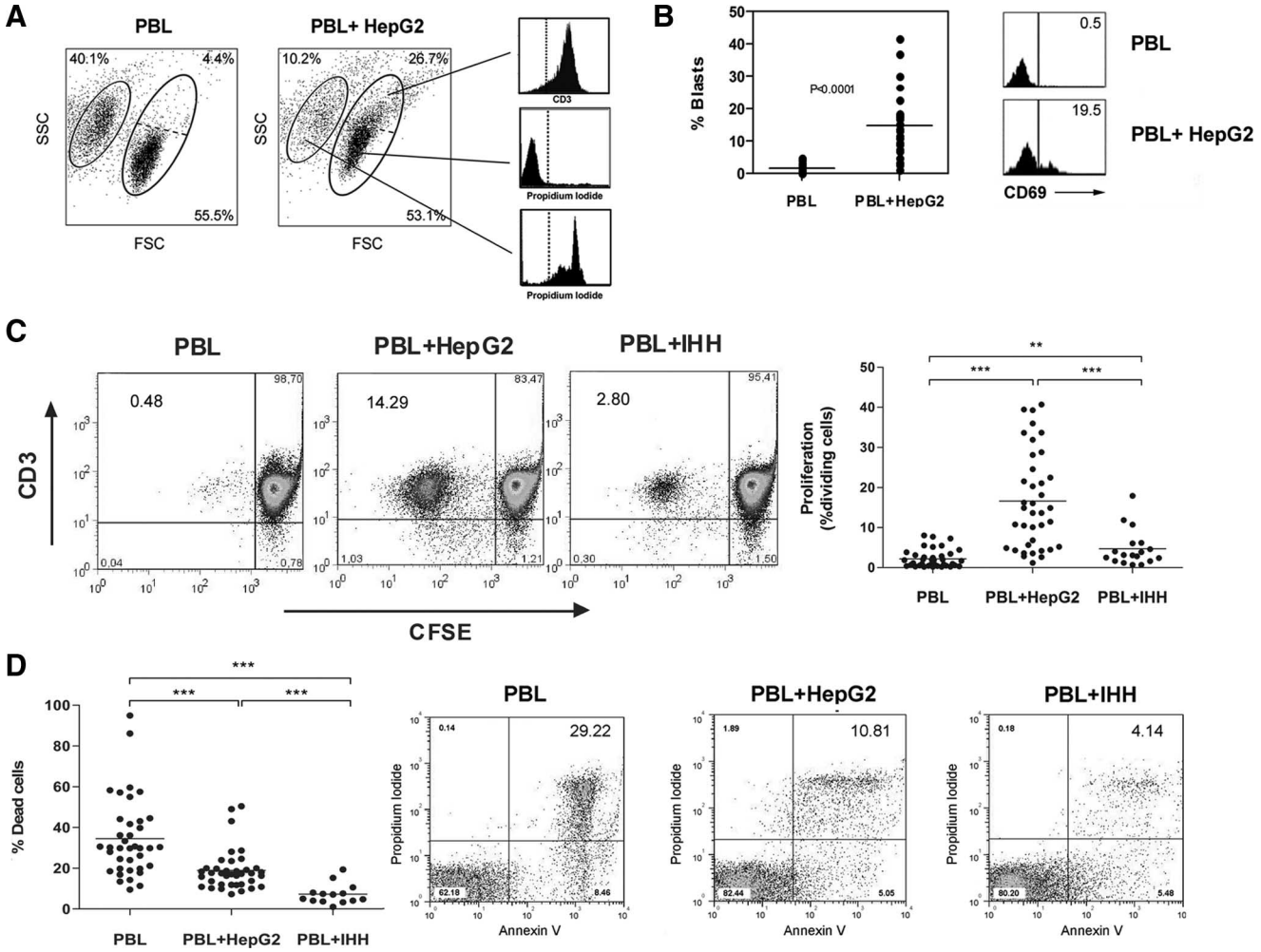

FIGURE 1. Hepatocytes promote T cell growth and survival. PBL (1×10^6) labeled with CFSE were cocultured either alone or with hepatocyte cell lines (HepG2 or IHH) (0.25×10^6) for 7 days, and then harvested, stained, and acquired in a FACSCalibur. *A*, Dot plots (FSC vs SSC) of PBL culture alone (*left*) or in the presence of HepG2 cells (*right*) show that the hepatocyte cell line promotes T cell survival (40.1 vs 10.2% dead cells) and blast formation (4.4 vs 26.7%). *Insets*: *Upper histogram*, Illustrates that the selected blast cells correspond mainly to $CD3^+$ T cells; *middle histogram*, illustrates that the gated population of lymphocytes is viable as determined by negativity to PI labeling; *lower histogram*, illustrates that the gated population of lymphocytes is dying as determined by PI labeling. *B*, *Left graph*, Shows the percentage of blast cells in each experiment performed (1.49 ± 0.24 vs 14.82 ± 1.76). *Right histograms*, Illustrate the up-regulation in PBL CD69 expression after coculture with HepG2 cells. *C*, *Left dot plots*, Illustrate the proliferation of $CD3^+$ T cells after PBL coculture with HepG2 or IHH cells, by CFSE quenching. *Right*, Graph showing the overall percentage of dividing PBL for the different conditions (PBL, 2.18 ± 0.36; PBL + HepG2, 16.61 ± 1.95; PBL + IHH, 4.70 ± 1.07). *D*, *Left*, The overall percentage of gated dead cells (PBL, 34.56 ± 3.10; PBL + HepG2, 18.93 ± 1.61; PBL + IHH, 7.26 ± 1.33) for the different culture conditions. *Right*, A representative double labeling of annexin V and PI dot plots for the different culture conditions. Results are presented as mean ± SEM and represent at least 18 different experiments. Values of p are shown (***, $p < 0.0001$; **, $p < 0.01$).

IL-15 or a combination of CD3/CD28 cross-linking Abs. For CD3/CD28 stimulation, pure $CD8^+CD56^-$ T cells were preincubated with 10 μg of anti-CD3 (clone OKT3) and 4 μg of anti-CD28 (ImmunoTools) for 30 min at 4°C and washed twice. Then, labeled $CD8^+CD56^-$ T cells were added to 24-well culture plates that were previously coated with rabbit anti-mouse (RAM) Abs (DakoCytomation) overnight at 4°C in 1× PBS.

Flow cytometry determinations

Cell stainings were normally performed at 4°C for 30 min in 1× PBS or staining buffer (PBS, 0.2% BSA, and 0.1% NaN_3) in 96-well round-bottom plates (Greiner Bioscience). Irrelevant mouse mAb were used as negative controls to define background staining. In second-step stainings, rabbit anti-mouse FITC-conjugated Abs were used. T cell death was determined by two methods, as follows: 1) a decrease in cell size according to forward light scatter (FSC)/side light scatter (SSC) parameters; 2) double annexin V and PI staining by using Ca^{2+}-based staining buffer (10 mM HEPES/140 mM NaCl/2.5 mM $CaCl_2$). T cell activation and division were studied by three methods, as follows: 1) determination of cell size and complexity according to FSC/SSC parameters (blasts); 2) CD69 expression; and 3) CFSE fluorescence loss. For proliferation studies, 10^7 cells/ml cells were labeled with CFSE at a final concentration of 10 μM for 10 min, with occasional mixing, at 37°C. Then, cells were washed twice with PBS/20% FBSi and resuspended in RPMI 1640. Analysis of cells immediately following CFSE labeling indicated a labeling efficiency higher than 99%. Rounds of cell division were determined by sequential halving of CFSE-fluorescence intensity. After staining, cells were washed and acquired in a FACSCalibur or a FACSCanto (both from BD Biosciences). For each sample, 50,000 events were acquired using FSC/SSC characteristics and analyzed using CellQuest or FlowJo softwares.

RNA isolation and RT-PCR amplification

For IL-15 and IL-15Rα mRNA expression, total RNA was isolated from HepG2 cells and from PBMC using the total RNA purification kit, Versagene RNA purification system (Gentra), according to manufacturer's instructions. Specific primers for IL-15 (sense, 5′-CCGTGGCTTTGAGT AATGAG-3′ and antisense, 5′-CAGATTCTGTTACATTCCC-3′) and IL-15Rα (sense, 5′-GGCGACGCGGGGCATCAC-3′ and antisense, 5′-TCG CTGTGGCCCTGTGGATA-3′) were used and RNA amplified, as previously described (19, 31). For NKR mRNA expression, total RNA was isolated from $CD8^+CD56^-$ T cells prior to and after culture with IL-15 using a RNeasy mini kit from Qiagen, according to manufacturer's

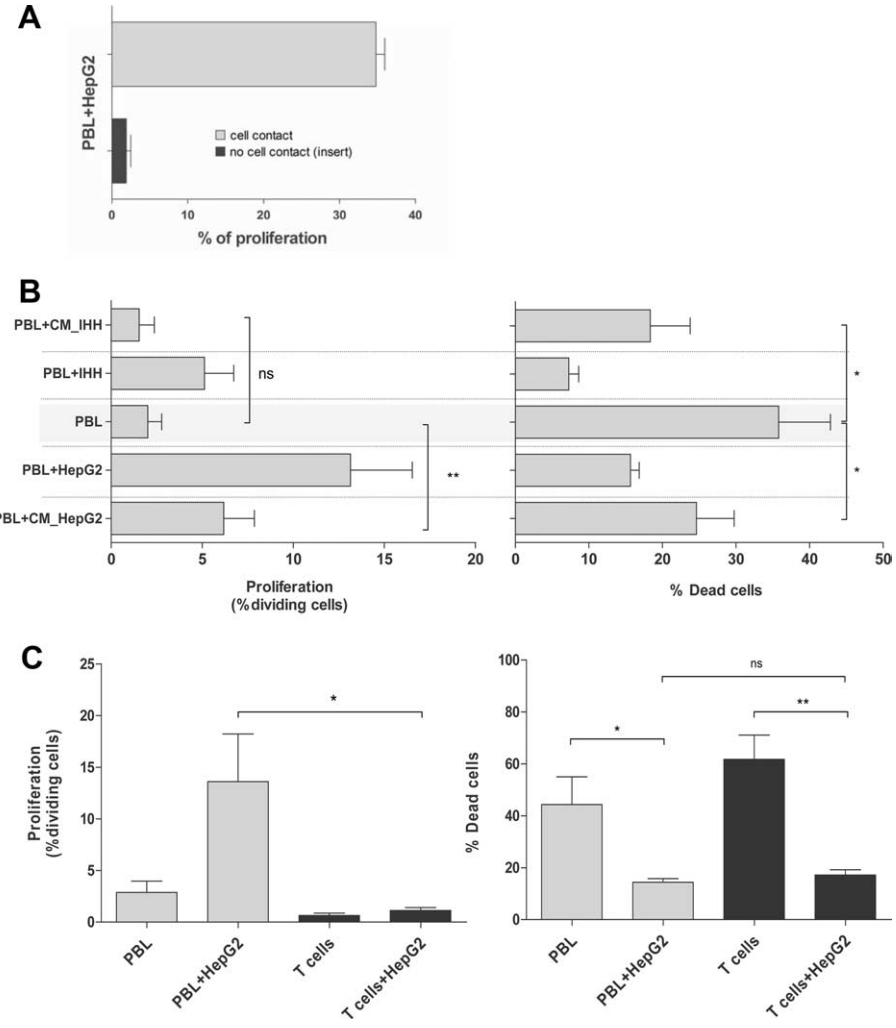

FIGURE 2. Hepatocyte-driven T cell proliferation is mainly cell contact dependent and, contrarily to cell survival, requires accessory cells. PBL were labeled with CFSE and cultured for 7 days in the following conditions: alone, with HepG2 (or IHH) cells, with HepG2 cells separated by a cell culture insert, and with CM from cultures of HepG2 (or IHH). Afterward, cells were harvested and acquired in a FACSCalibur. *A*, The graph shows the percentage of proliferating PBL (CFSE) in cocultures with HepG2 cells without (▨, 34.80 ± 1.16) or with (■, 4.43 ± 1.04) cell inserts. *B*, *Left graph*, Percentage of proliferating PBL in the presence of hepatocyte cell lines (HepG2 or IHH) or the corresponding CM (PBL, 1.79 ± 0.60; PBL + CM_HepG2, 6.18 ± 1.69; PBL + CM_IHH, 1.54 ± 0.82); *right graph*, percentage of dead cells in the presence of hepatocyte cell lines or with the corresponding CM (PBL, 33.49 ± 5.27; PBL + CM_HepG2, 24.63 ± 5.12; PBL + CM_IHH, 18.36 ± 5.42). In both graphs, only paired experiments were used, and Student's *t* tests were performed accordingly. Results are presented as mean ± SEM and represent from 2 to 18 different experiments. *C*, PBL and purified T cells were labeled with CFSE, cultured either alone or with HepG2 cells for 7 days, and analyzed. *Left graph*, Percentage of proliferating PBL vs T cells after culture with HepG2 cells (13.60 ± 4.61 vs 1.12 ± 0.30); *right graph*, percentage of dead PBL vs T cells after culture with HepG2 cells (14.43 ± 1.30 vs 17.70 ± 2.02). Results show the mean ± SEM of at least 11 different paired experiments. Values of p are shown (**, $p < 0.01$; *, $p < 0.05$; ns = nonsignificant).

instructions. Primers used for NKR and amplification conditions have been previously described (32, 33) (Table II).

Statistical analysis

Statistical analyses were performed using Excel or GraphPad Prism 5 software. Student's *t* test was used to test the significance of the differences between group means. Statistical significance was defined as $p < 0.05$.

Results

HepG2 cells and IHH induce T cell activation and proliferation and increase survival

Previous studies have suggested that primary mouse hepatocytes could deliver Ag-dependent activation signals to T cells (1–3). However, studies involving an Ag-independent influence of human hepatocytes on T cells are scarce. Accordingly, we went to evaluate the influence of the hepatoma cell line HepG2 on parameters of T cell activation, proliferation, and survival. Analysis of FSC/SSC properties, after 7 days of coculture, showed an increase in the percentage of blast cells when compared with PBL alone. Analysis of the phenotype of the blast cells showed that they were mainly $CD3^+$ T cells (Fig. 1*A*, *upper histogram inset*). All blast cells analyzed were viable based on lack of PI labeling (Fig. 1*A*, *middle histogram inset*). The increase in the percentage of T cell blasts was observed for each single experiment performed and was statistically significant (Fig. 1*B*, graph). The activated phenotype was also confirmed by an increase in CD69 expression in PBL after coculture with HepG2 cells (Fig. 1*B*, histograms). Although an increase in cell size as a result of T cell activation usually precedes mitosis, delivery of activation signals is not always accompanied by cell division and proliferation, resulting in most cases in apoptosis. To assess whether HepG2 cells could also support cell division, we measured CFSE halving. As shown in Fig. 1*C*, ~15% of $CD3^+$ T cells present in the PBL preparations proliferated after the coculture. Noteworthily, nontumoral human hepatocytes (IHH) were also able to drive T cells into proliferation, although to a lesser extent, ruling out a possible unspecific effect resulting from the use of a tumoral cell line (Fig. 1*C*, dot-plots). Accordingly, both HepG2 and IHH were capable of inducing T cell proliferation in a statistical significant way when compared with PBL alone (Fig. 1*C*, graph). In addition, both hepatocyte cell lines were able to reduce markedly the percentage of dead PBL in culture as determined by PI labeling (see Fig. 1*A*, *lower histogram inset*). As summarized in the graph of Fig. 1*D*, the percentage of dead PBL was significantly reduced by interaction with HepG2 cells (~2-fold) and with nontumoral IHH cells (~4-fold). Further analysis by double labeling with annexin V and PI clearly showed the capacity of HepG2 and IHH cells to inhibit apoptosis (Fig. 1*D*, dot-plots).

HepG2-induced T cell proliferation is cell-contact dependent and, contrarily to cell survival, requires the presence of accessory cells

To ascertain whether the effect observed on T cell proliferation involved or not direct cell contact with HepG2 cells, cultures using

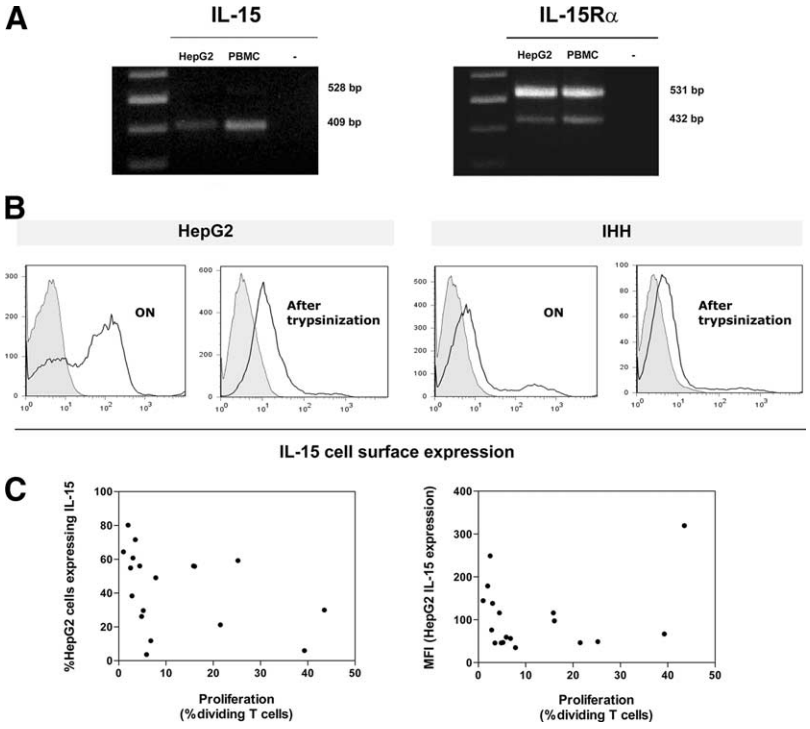

FIGURE 3. Expression of IL-15 and IL-15Rα by hepatocyte cells and lack of correlation with T cell proliferation (*A*). Total RNA was isolated from HepG2 cells, and expression of IL-15 and IL-15Rα was analyzed by RT-PCR, as described in *Materials and Methods*. Graph shows mRNA expression for IL-15 (band of 409 bp, and a faint band of 528 bp) and IL-15Rα (bands of 531 and 432 bp), both in HepG2 cells and PBMC. *B*, HepG2 or IHH cells were cell surface stained with mouse anti-human IL-15 mAbs (clone MAB2471), or with the corresponding isotype control, followed by a rabbit anti-mouse FITC at 4°C, washed, and acquired in a FACSCalibur. Histograms show the levels of cell surface IL-15 expression in HepG2 (*left*) and IHH (*right*) cells immediately after trypsin treatment or following overnight (ON) incubation, as indicated in the figure. Mouse IgG1 Abs were used as control (solid gray). *C*, Cocultures with CFSE-labeled PBL and HepG2 cells were performed, as described above. The graphs show the lack of correlation between the percentages of IL-15-expressing HepG2 cells (*left*) or the mean fluorescence intensity (MFI) of IL-15 expression by HepG2 cells (*right*) and PBL proliferation. Before the coculture, IL-15 cell surface expression levels by HepG2 cells were measured by flow cytometry. Each dot corresponds to a single experiment.

cell culture inserts were performed. As shown in Fig. 2*A*, T cell proliferation in cultures with inserts was markedly reduced when compared with cultures with cellular contact. To exclude the possibility that the porous membranes used in these experiments could be obstructed, blocking the diffusion of released soluble factors, experiments using HepG2 and IHH CM were performed. Interestingly, HepG2 CM was able to induce T cell proliferation and survival above background levels (Fig. 2*B*). In contrast, CM from IHH cells showed no effect in driving T proliferation, but still was capable of decreasing T cell death (Fig. 2*B*). Finally, to find out whether the HepG2-induced T cell proliferation and survival were a direct effect on T cells, PBL preparations were depleted of accessory cells (monocytes and B cells) and pure $CD3^+$ T cells cultured with HepG2 cells. Under these conditions, HepG2 cells were no longer capable of activating T cells (Fig. 2*C*, *left graph*). Addition of the removed accessory cells to the cocultures of HepG2 cells, namely monocytes, partially restored cell proliferation, but never to the levels seen in cocultures of HepG2 cells and PBL (data not shown). Although accessory cells appear to be required for the observed HepG2-induced T cell proliferation, preincubation of PBL preparations with Abs against MHC-class II molecules, in order to block any possible presentation of hepatocyte-derived Ags, did not abolish proliferation (data not shown). Importantly, even though HepG2 cells were incapable of driving pure T cells into proliferation, they still retained their capacity to induce survival (Fig. 2*C*, *right graph*). Unlike HepG2 cells, two other epithelial-like cell lines (HeLa and AGS) did not exert either proliferation or survival activities toward $CD3^+$ T cells present in PBL preparations (see below).

HepG2 cells and IHH express IL-15 at the cell surface

A number of reports indicate that IL-15 is expressed in liver tissue (13, 19, 22). Therefore, we went to examine IL-15, and its private receptor unit, IL-15Rα, expression in HepG2 cells at the mRNA level. As shown in Fig. 3*A*, RT-PCR of IL-15 yielded mainly a product of 409 bp, corresponding to the secretory isoform that can be expressed at cell surface. A faint band at 528 bp corresponding to the IL-15 cytoplasmic isoform was also observed (Fig. 3*A*). RT-PCR of IL-15Rα originated two products of 531 and 432 bp (Fig. 3*A*), corresponding to two mRNA isoforms (including or lacking exon 3, respectively). These results were observed both in HepG2 cells and PBMC (used as control), revealing that HepG2 cells express both IL-15 and IL-15Rα at the mRNA level. Because expression of IL-15 has been shown to be highly controlled both at transcriptional and posttranscriptional levels, we investigated whether HepG2 cells could express IL-15 at the cell surface. Flow cytometry analysis revealed the existence of IL-15 at the plasma membrane in a significant fraction of HepG2 cells (Fig. 3*B*). Analysis of IL-15Rα expression also revealed the presence of this receptor at the plasma membrane (data not shown). Of note, IL-15 cell surface expression was also observed in IHH cells (Fig. 3*B*). IL-15 cell surface expression was markedly reduced after treatment with trypsin, being again re-expressed after overnight culture, suggesting that a large fraction is most likely bound to IL-15 Rα. Considering previous studies showing that IL-15 can be *trans*-presented by monocytes and fibroblasts to T cells, leading to cell activation and proliferation (34–37), we examined whether membrane-bound IL-15 could play a role in the HepG2-induced T cell proliferation and/or survival observed in the coculture experiments. Despite cell surface expression of IL-15, preincubation of HepG2 cells with anti-IL-15 Abs before the coculture with PBL did not abrogate the HepG2-induced T cell proliferation or T cell

Table III. *Anti-IL-15 Abs do not abrogate HepG2-mediated T cell proliferation and survival*

	Percentage of Dividing Cells		Percentage of Dead Cells	
Exp #	+IgG1	+anti-IL-15	+IgG1	+anti-IL-15
1	25.8	25.7	29.8	35.9
2	10.0	8.8	10.1	9.14
3	8.5	7.7	19.8	21.9
4	4.6	3.9	18.5	18.0
5	4.7	6.7	14.7	16.2

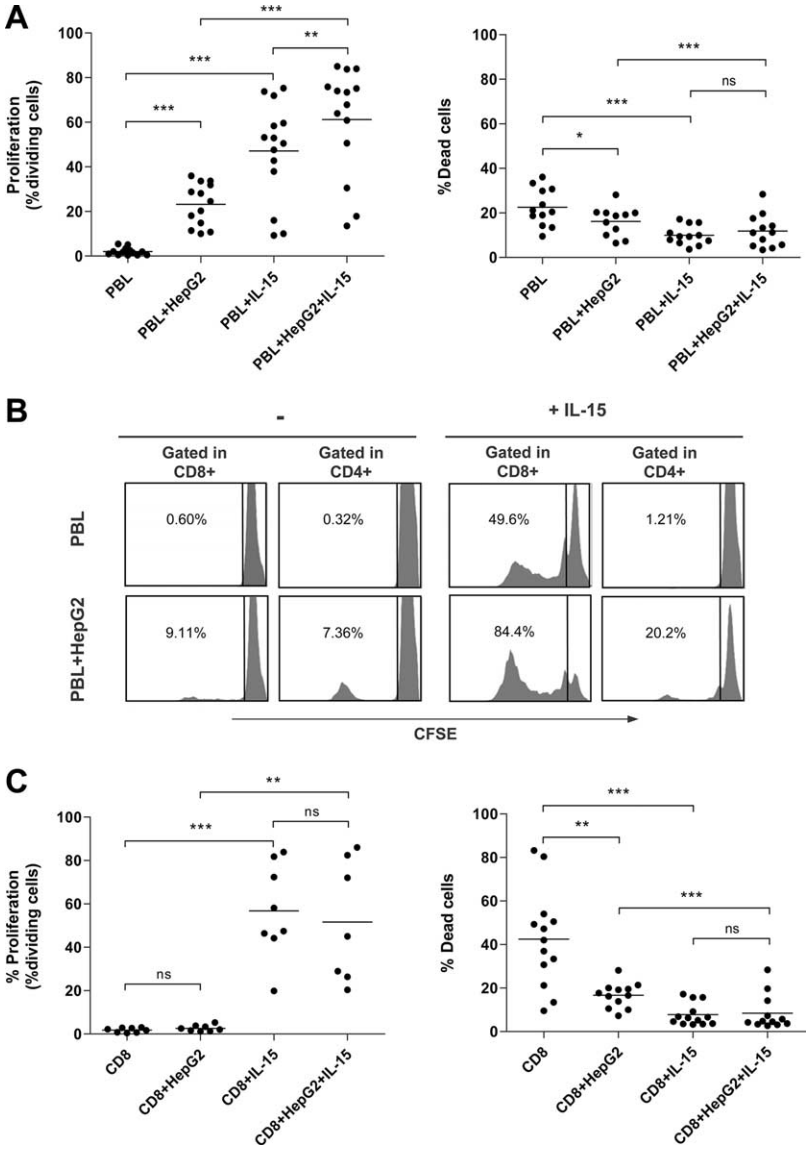

FIGURE 4. IL-15 and HepG2 effect on proliferation and survival of PBL and $CD8^+$ T cells. PBL and pure $CD8^+$ T cells were labeled with CFSE and cultured either alone, with HepG2 cells, with 10 ng of IL-15, or with both HepG2 and IL-15 for 7 days. Then, cells were harvested, acquired in a FACSCalibur, and analyzed. *A*, *Left graph*, Shows the percentage of PBL proliferation for each condition (PBL, 2.06 ± 0.47; PBL + HepG2, 23.24 ± 2.64; PBL + IL-15, 47.10 ± 5.91; PBL + HepG2 + IL-15, 61.18 ± 6.45). *Right graph*, Shows the percentage of dead PBL for each condition (PBL, 22.53 ± 2.42; PBL + HepG2, 16.24 ± 1.97; PBL + IL-15, 9.95 ± 1.24; PBL + HepG2 + IL-15, 11.85 ± 2.13). *B*, Histograms illustrating the percentage of proliferating $CD4^+$ and $CD8^+$ T cells gated in PBL, for each condition. *C*, *Left graph*, Shows the percentage of $CD8^+$ T cells that proliferated, as determined by CFSE loss, for each condition ($CD8^+$ alone, 1.82 ± 0.37; CD8 + HepG2, 2.64 ± 0.51; CD8 + IL-15, 57.75 ± 7.70; CD8 + HepG2 + IL-15, 51.60 ± 10.61). *Right graph*, Shows the percentage of dead $CD8^+$ T cells for each condition (CD8, 42.49 ± 6.20; CD8 + HepG2, 16.70 ± 1.65; CD8 + IL-15, 7.80 ± 1.41; CD8 + HepG2 + IL-15, 8.48 ± 2.34). Results are presented as mean ± SEM and represent at least eight different paired experiments. Values of p are shown (***, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$; ns = nonsignificant).

survival (Table III). These results are in agreement with the lack of correlation between either the percentage of IL-15-positive HepG2 cells or the mean fluorescence intensity of IL-15 and the level of T cell proliferation (Fig. 3*C*, *left* and *right graphs*, respectively).

Soluble IL-15-mediated T cell proliferation and survival: effect of HepG2 cells

Even though membrane-bound IL-15 present in HepG2 cells apparently does not play a role in T cell proliferation, some authors have reported the existence of soluble IL-15 in the liver microenvironment (13, 19). Thus, we went to see the effect of this cytokine in T cell proliferation and survival, in the presence or absence of HepG2 cells. Exogenously added IL-15 induced a fraction of T cells to proliferate, driving on average 50% of the cells into cell division (Fig. 4*A*, *left graph*). As illustrated in Fig. 4*B*, the IL-15 effect was observed prominently on $CD8^+$ T cells. In this particular experiment, the presence of HepG2 cells together with IL-15 led to a synergistic $CD8^+$ T cell proliferation, indicating that HepG2 and IL-15 could act together in inducing mainly $CD8^+$ T cell proliferation in PBL preparations. On average, the combined use of HepG2 cells and IL-15 resulted in an increase in proliferation when compared with IL-15 alone (Fig. 4*A*, *left graph*). IL-15 also induced a marked decrease in cell death, which was not influenced by the presence of HepG2 (Fig. 4*A*, *right graph*). In marked contrast, the use of two other epithelial-like cell lines, HeLa and AGS, showed that both cell types had a negative impact on IL-15-induced T cell proliferation and survival (Fig. 5). Also, as referred above, and unlike HepG2 cells, these cell lines per se did not induce T cell proliferation or survival (Fig. 5). Finally, we examined the effect of IL-15 and HepG2 cells on $CD8^+$ T cells. As previously shown with pure $CD3^+$ T cells (Fig. 2*C*), HepG2 cells were incapable of driving pure $CD8^+$ T cells into proliferation, yet they were capable of increasing survival. In contrast, IL-15 induced a large fraction of pure $CD8^+$ T cells to enter cell division, as expected (Fig. 4*C*). When the combined effect of IL-15 and HepG2 cells was examined, no significant differences were observed comparing to IL-15 alone (Fig. 4*C*), suggesting that the effect of IL-15 on proliferation and survival is not significantly altered in the presence of HepG2 cells when pure $CD8^+$ T cells are used.

Soluble IL-15 induces NKR expression in $CD8^+$ T cells

Even though $CD8^+$ T cells present in the human liver are characterized by the presence of a number of NKR, little is known about the factors that originate or regulate their expression. Thus, we

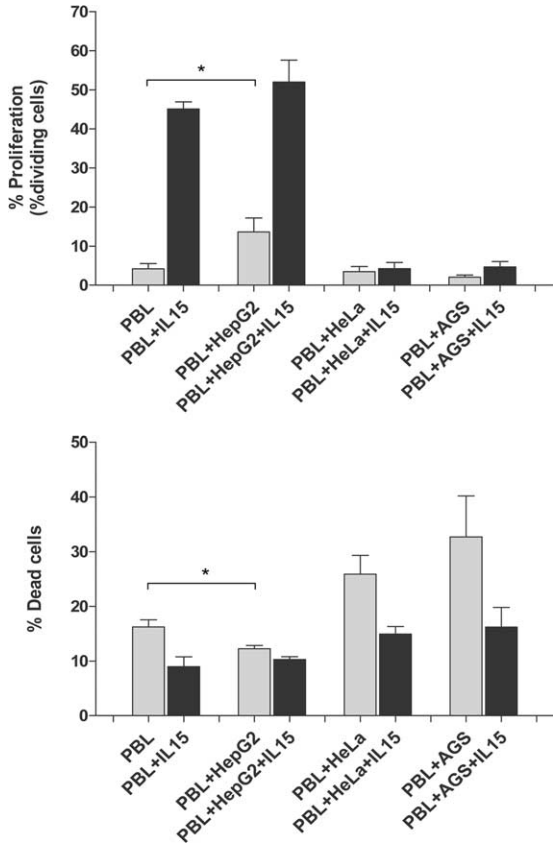

FIGURE 5. HeLa cells and AGS cells do not induce T cell proliferation, and survival PBL (1×10^6) labeled with CFSE were cocultured either alone or with HepG2, HeLa, or AGS cell lines (0.25×10^6) for 7 days in the presence or absence of IL-15 (10 ng), and then harvested, stained, and acquired in a FACSCalibur. *Upper graph*, Percentage of PBL proliferation in the presence of each cell line with or without IL-15 (PBL, 4.19 ± 1.37; PBL + IL-15, 45.12 ± 1.81; PBL + HepG2, 13.6 ± 3.57; PBL + HepG2 + IL-15, 51.97 ± 5.66; PBL + HeLa, 3.45 ± 1.31; PBL + HeLa + IL-15, 4.25 ± 1.55; PBL + AGS, 2.04 ± 0.56; PBL + AGS + IL-15, 4.68 ± 1.37); *lower graph*, percentage of dead PBL after culture with each cell line in the presence or absence of IL-15 (PBL, 16.22 ± 1.29; PBL + IL-15, 8.97 ± 1.77; PBL + HepG2, 12.26 ± 0.58; PBL + HepG2 + IL-15, 10.28 ± 0.52; PBL + HeLa, 25.87 ± 3.43; PBL + HeLa + IL-15, 14.94 ± 1.34; PBL + AGS, 32.66 ± 7.51; PBL + AGS + IL-15, 16.19 ± 3.61). Results are presented as mean ± SEM and represent at least four different experiments. Values of p are shown (*, $p < 0.05$).

wanted to ascertain whether IL-15 could play a role in the expression of NKR by $CD8^+$ T cells in vitro. To that purpose, pure $CD8^+CD3^+CD56^-$ T cells were obtained and cultured in the presence and absence of IL-15, and NKR (KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL2, NKG2A, NKG2D, and CD56) expression was analyzed by RT-PCR. As shown in Fig. 6*A*, slight mRNA expression for some NKR, like KIR3DL2 and NKG2A, but not CD56, was observed in the isolated $CD8^+CD56^-$ T cells at day 0. Expression of KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL2, and NKG2A was markedly up-regulated in $CD8^+$ T cells after 6 days of culture with IL-15, being more evident after 12 days (Fig. 6*A*). Noteworthily, IL-15 induced $CD8^+CD56^-$ T cells to express CD56 mRNA, suggesting a de novo expression of this typical NKR. To ascertain whether NKR up-regulation at the mRNA level was paralleled by changes in expression at the plasma membrane, flow cytometry analysis was performed for KIR2DL2/3/S2, KIR2DL4, NKG2A, and CD56. In these sets of experiments, NKp46 expression was also studied because it is considered to be a bona fide marker for NK cells. As summarized in Fig. 6*B*, $CD8^+CD56^-$ T cells expressed barely detectable levels of some NKR at the cell surface, but culture in the presence of IL-15 induced up-regulation of all NKR screened. Noteworthily, IL-15 induced de novo cell surface expression of CD56, which is also suggested for NKp46 (Fig. 6). Similarly to the mRNA expression, the increase in NKR expression was more evident from day 6 to day 12. Simultaneous determination of NKR expression and CFSE halving by flow cytometry suggested that up-regulation of some NKR (e.g., KIR2DL2/3 and NKG2A) could be due to expansion of pre-existing NKR^+ $CD8^+$ T cells at the start of the culture, while confirming de novo expression of CD56 and NKp46 by the dividing $CD8^+$ T cells (Fig. 6*C*). However, if we consider that NKR-expressing cells did not show a proliferative advantage over non-NKR-expressing cells (Fig. 6*C*) and that an increase in mRNA expression for those NKR was observed (Fig. 6*A*), it is likely that the up-regulation in NKR expression was not only the result of the proliferation of NKR-expressing cells. In fact, further experiments using CD3/CD28 cross-linking as the activation stimulus for pure $CD8^+$ T cells revealed that even though $CD8^+$ T cells proliferated to levels comparable, or even higher, to those seen with IL-15, there was not induction of NKR expression on proliferating T cells (Fig. 6*D*). Additional experiments with pure $CD8^+$ T cells from cord blood samples, known to have very low numbers of NKR-expressing $CD8^+$ T cells (38), were performed. Double-labeling (CFSE vs NKR) flow cytometry results shown in Fig. 6*E* strongly suggest that IL-15 induces de novo expression of CD56, KIR2DL4, and NKp46 in cord blood $CD8^+$ T cells, reinforcing the results obtained with peripheral blood $CD8^+$ T cells.

NKG2D expression in pure $CD8^+$ T cells after culture with IL-15

Contrasting with the other NKR analyzed, high levels of mRNA for NKG2D were found at day 0 in all $CD8^+$ T cell samples studied (Fig. 6*A*). Flow cytometry analysis of cell surface expression of NKG2D in $CD8^+CD56^-$ T cells cultured for 6 days showed that there was not an increase in the percentage of NKG2D-expressing $CD8^+$ T cells after IL-15, or CD3/CD28, stimulation when compared with $CD8^+$ T cells at day 0. However, an increase was observed when compared with $CD8^+$ T cells cultured for the same period (Fig. 7*A*). Simultaneous determination of NKG2D expression and CFSE halving by flow cytometry 6 days after stimulation with IL-15, but not with CD3/CD28 cross-linking, showed that there was up-regulation of NKG2D mean fluorescence intensity on a fraction of the $CD8^+$ T cells (Fig. 7*B*).

Discussion

In this study, we have found that the hepatoma cell line HepG2 is able to deliver Ag-independent activation signals to a fraction of peripheral blood T cells, driving them into proliferation. This effect is mainly cell contact dependent and requires the presence of accessory cells, namely monocytes. However, blocking experiments showed that Ag presentation via cell surface MHC class II is not involved. The fact that two other epithelial-like cell lines (HeLa and AGS) were incapable of inducing T cell proliferation rules out allorecognition of hepatocyte-derived Ags as the cause of T cell proliferation. Also, the lack of expression of MHC class II molecules by HepG2 cells (39) excludes a possible direct allorecognition event. Importantly, HepG2 cells are also able to support survival, an effect that was more noticeable and consistently observed than proliferation itself. The fact that the HepG2-induced survival was directly exerted on T cells, without the need of accessory cells, suggests differences between proliferation and survival stimuli delivered by HepG2 cells.

Experiments with nontumoral IHH cells closely reproduced the effects of HepG2 cells, predominantly at the level of survival, suggesting that the tumoral nature of HepG2 cells is not responsible for the observed effect. In physiological terms, the increase in survival is perhaps more important and may resemble the in vivo situation in healthy livers. Of note, some of the effects on proliferation and survival were partially reproduced when HepG2 and IHH CM were used, suggesting that factors secreted by hepatocytes are endowed with cell proliferation and survival activities. In general, these results suggest that hepatocytes may play an important role in local T cell homeostasis by contributing to cell survival and inducing basal levels of proliferation. This positive effect gives a novel view on the relationship between hepatocytes and T cells that contrasts, or complements, the view of the liver as a "graveyard" for Ag-activated lymphocytes (40). Nevertheless, we do not exclude the possibility that other nonhematopoietic cells, such as enterocytes and fibroblasts, could have the ability to induce survival and proliferation of T cells (18, 41).

IL-15 is thought to play a wide role in the immune system, particularly in the activation and survival of innate and tissue-associated immune cells (42, 43). Also, a number of reports suggested that IL-15 is produced in the liver (13, 19, 22). In this work, we have clearly demonstrated that tumoral and nontumoral hepatocytes constitutively express IL-15. Trypsin treatment drastically diminished the expression of cell surface IL-15, which was subsequently recovered after overnight culture. In the context of previous studies showing that trypsin treatment causes the IL-15R complex to lose its ability to bind IL-15-IgG2b fusion protein (34), it may be suggested that IL-15 detected at the cell surface of the hepatocyte cell lines is possibly bound to the IL-15Rα. Even though HepG2 cells constitutively express IL-15 at the cell surface, our results indicate that it was not involved in the HepG2-mediated T cell proliferation and survival. The possibility that IL-15 present at the cell surface of hepatocytes may play a more evident role in CD8$^+$ T cell survival and proliferation under pathological conditions remains to be elucidated. Alternatively, it is possible that membrane-bound IL-15 present at the cell surface of hepatocytes, rather than being involved in regulation of T cell survival and proliferation, could play a role in hepatocyte physiology through reverse signaling (44). Indeed, recent reports indicate that IL-15 predominantly promotes a wound healing-type response in the liver by increasing hepatic regenerative activity (22).

IL-15 has also been shown to be constitutively expressed in the liver in soluble form, and a role in the generation and maintenance of the distinct intrahepatic lymphocyte subsets has been suggested (13). Our results have shown that soluble IL-15 is able to induce T cell survival and proliferation regardless of using PBL preparations or pure CD8$^+$ T cells, and the presence of HepG2 cells appears not to modify significantly the IL-15 proliferation effect, at least in CD8$^+$ T cells. In contrast, the presence of two other cell lines (HeLa and AGS) had a negative impact on the IL-15-induced proliferation. Even though HepG2 cells were ineffective in activating pure CD8$^+$ T cells, they were as efficient as IL-15 in inducing survival. The possibility that both factors could contribute to the increased number of T cells with an activated phenotype observed in the liver deserves further investigations. Although hepatocytes might play an important role in delivering survival signals to CD8$^+$ T cells, IL-15 may be involved in delivering activation and survival signals, which could have implications for T cell differentiation.

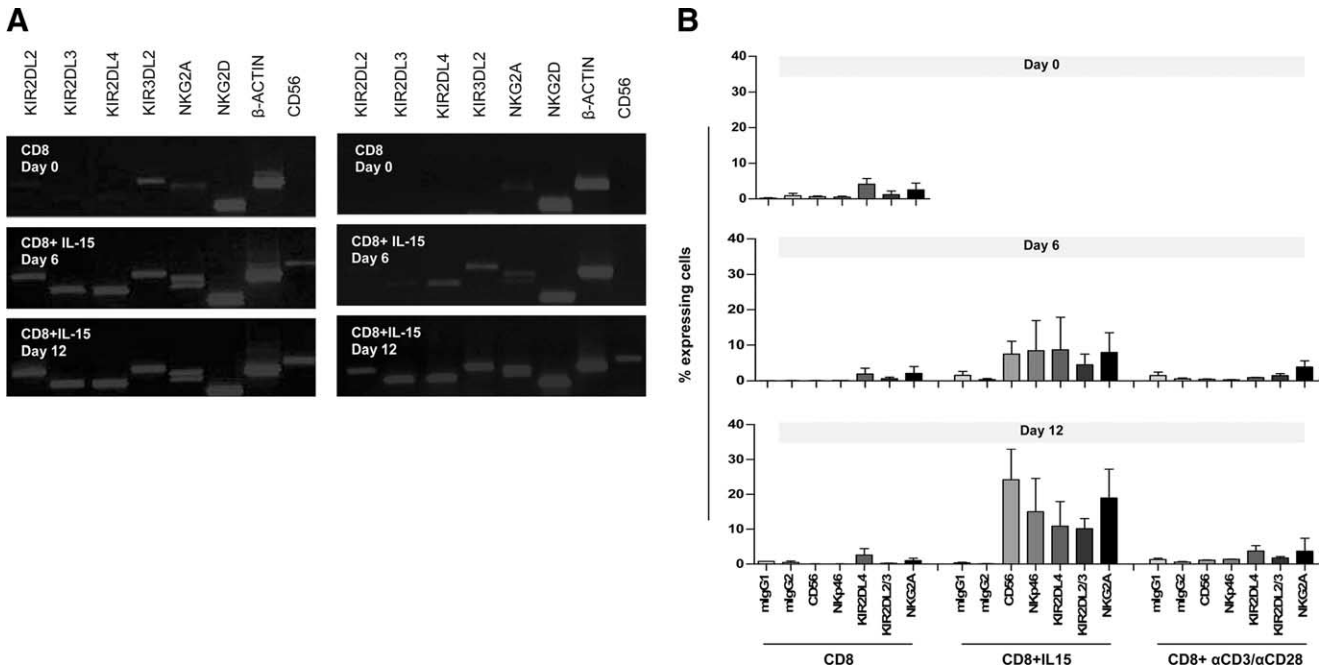

FIGURE 6. IL-15 induces NKR expression in pure CD8$^+$CD56$^-$ T cells. Pure CD8$^+$CD56$^-$ T cells were obtained, as indicated in *Materials and Methods*, and cultured with 10 ng of IL-15. Analysis of NKR expression by RT-PCR and flow cytometry was performed, as indicated. *A*, RT-PCR gels from two different healthy donors showing mRNA expression of different NKR in resting CD8$^+$ T cells (day 0) and after 6 and 12 days of culture with IL-15, as indicated in the figure. β-actin was used as internal control. *B*, Graph showing the percentage (mean $\pm$ SEM) of cells expressing NKR at the cell surface of CD8$^+$ T cells cultured for 6 and 12 days either without stimulus (CD8), with IL-15 (CD8 + IL-15), or with CD3/CD28 cross-linking (CD8$^+$$\alpha$CD3/$\alpha$CD28). NKR expression by CD8$^+$CD56$^-$ T cells at day 0 is included. *C*, Flow cytometry data showing CD8 vs NKR expression in CD8$^+$CD56$^-$ T cells at day 0 (*upper dot plot row*), and CFSE vs NKR expression in CD8$^+$CD56$^-$ T cells at days 6 and 12 after culture with IL-15 (*lower dot plot rows*). *D*, Dot plots showing CFSE vs NKR expression at day 6 in CD8$^+$CD56$^-$ T cells either unstimulated (CD8) or stimulated with CD3/CD28 cross-linking (CD8$^+$$\alpha$CD3/$\alpha$CD28). *E*, *Upper dot plots*, Expression of NKR by cord blood CD8$^+$ T cells at day 0; *lower dot plots*, CFSE vs NKR expression in cord blood CD8$^+$ T cells at day 6 after culture with IL-15.

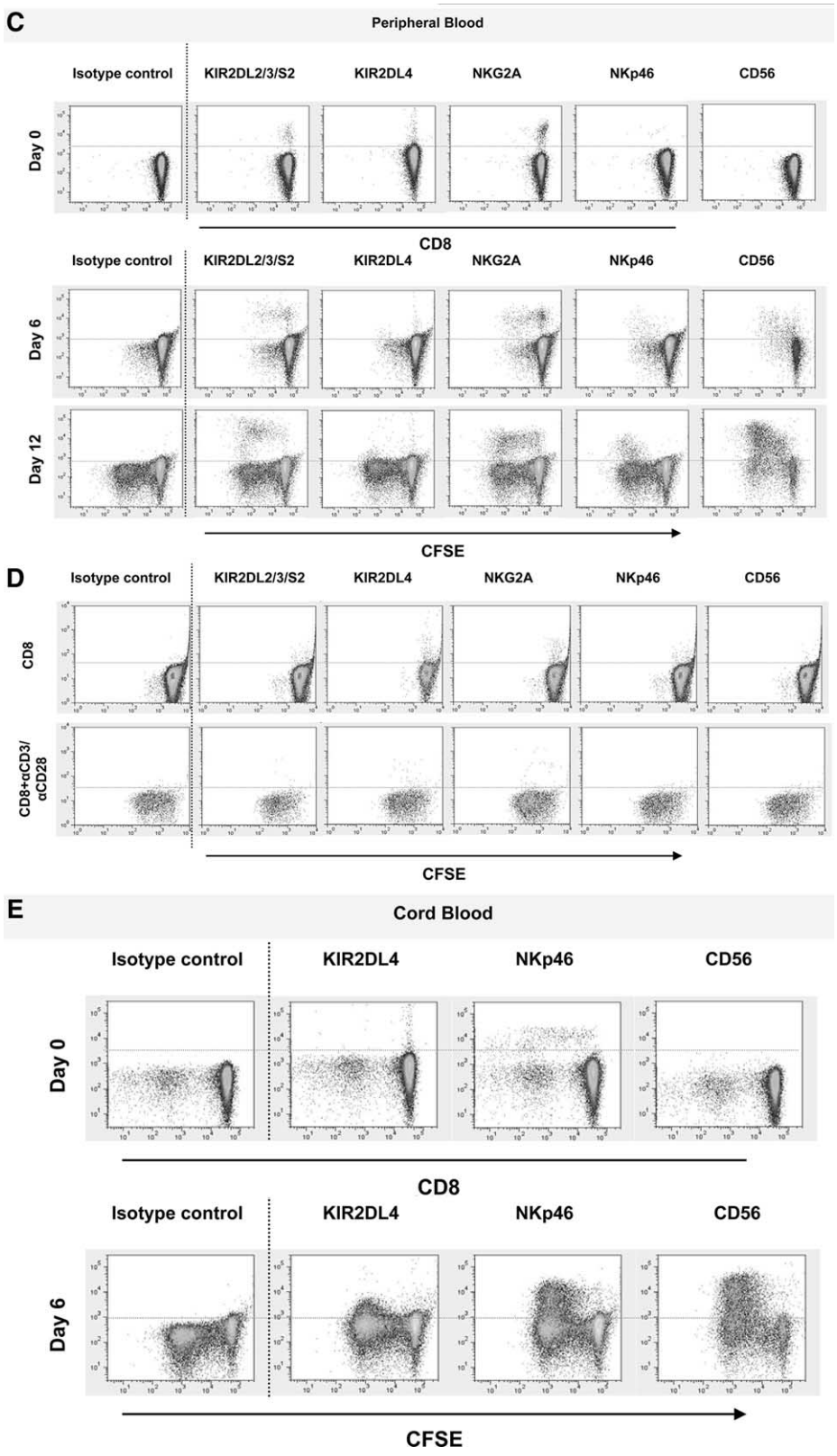

FIGURE 6. (*continued*)

To investigate further the possible role of IL-15 in $CD8^+$ T cell differentiation, we studied the effect of this cytokine on NKR expression in long-term cultures of pure $CD8^+CD56^-$ T cells. NKR expression by T cells has been previously associated with Ag-driven expansion. Indeed, it has been reported that IL-15 could induce CD94/NKG2A expression, but not of NKR belonging to the Ig-like family, in T cells activated by superantigens or allogenic cells (45). Thus, whereas CD94/NKG2A cell surface expression on T cells is modulated by exposure to Ag, the external signals controlling expression of inhibitory KIR have been to date unknown (46). In the present study, we have shown that IL-15 is capable of inducing Ag-independent up-regulation of NKR in $CD8^+$ T cells, including Ig-like receptors

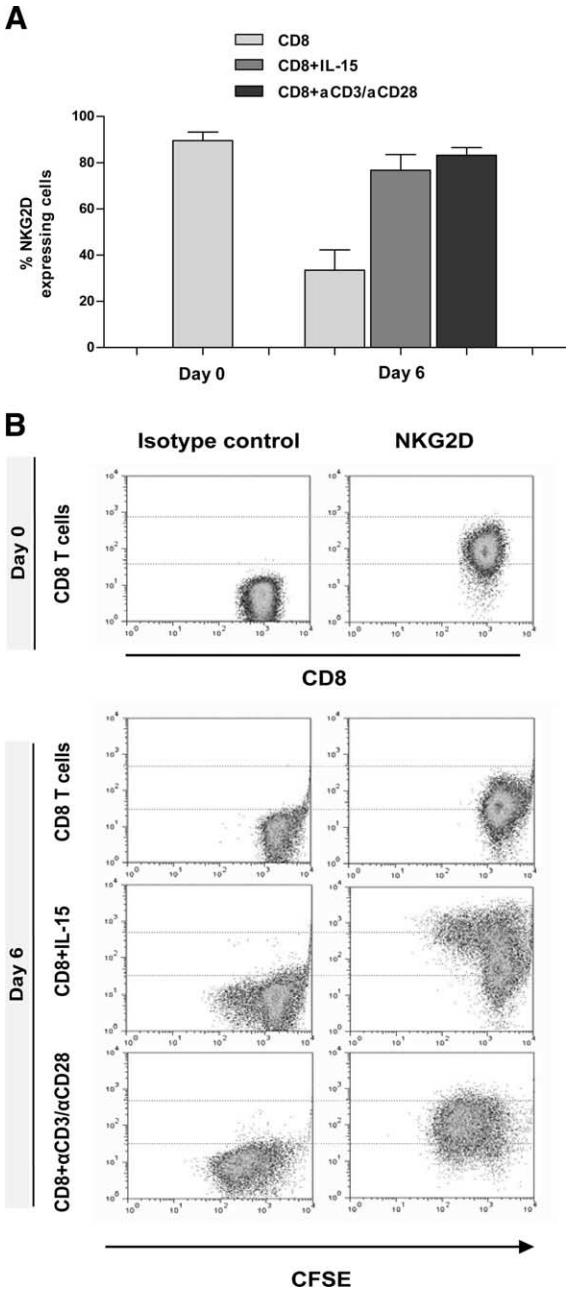

FIGURE 7. NKG2D receptor expression in pure $CD8^+$ T cells induced by IL-15. Pure $CD8^+CD56^-$ T cells were obtained, as indicated in *Materials and Methods*, and stimulated with IL-15 or CD3/CD28 cross-linking. *A*, Graph shows the percentage (mean ± SEM, $n = 3$) of NKG2D-positive $CD8^+$ T cells at days 0 and 6 in the indicated culture conditions. *B*, Dot plots show CFSE vs NKG2D expression in $CD8^+CD56^-$ T cells cultured for 6 days either without stimulus (CD8), with IL-15 (CD8 + IL-15), or with CD3/CD28 cross-linking ($CD8^+$αCD3/αCD28). NKG2D expression by $CD8^+CD56^-$ T cells at day 0 is included.

(KIR2DL2/3, KIR2DL4, and KIR3DL2), lectin-like receptors (NKG2A), and de novo expression of CD56 and NKp46. To our knowledge, this is the first evidence of an Ag-independent NKR up-regulation by IL-15 in resting human peripheral blood $CD8^+CD56^-$ T cells. Regarding NKG2D, high expression levels were already detected in the isolated $CD8^+$ T cells, which is in accordance with previous reports indicating that NKG2D is constitutively expressed by all human $CD8^+$ T cells (47). We have shown that IL-15 induces up-regulation of the level of NKG2D in a fraction of $CD8^+$ T cells without increasing the percentage of NKG2D-positive cells. Up-regulation of NKG2D has previously been shown in effector intestinal CTLs by IL-15 (48).

One striking observation arising from this work was the de novo expression of CD56 and NKp46 on $CD8^+CD56^-$ T cells cultured with IL-15. To our knowledge, only a previous report has shown the ability of IL-15 to drive neonatal T cells to acquire CD56 (49). This result is of particular importance if we consider that NKp46 is considered a truly bona fide NK cell marker. In this context, it is worth mentioning that the large majority of NKT cells present in the human liver are nonclassical $CD3^+CD56^+$ NKT cells with an oligoclonal TCR, not restricted by CD1d, that express other NKR (16, 17, 43). The likelihood that a fraction of NKT cells present in the human liver could originate locally from circulating T cells in the context of hepatic IL-15 is an interesting possibility that deserves to be investigated. In this context, it is worth mentioning reports showing that some of NKR analyzed in this study are expressed by intrahepatic T lymphocytes (50, 51). This does not rule out the possibility that liver IL-15 could also mediate development of NKT cells from hepatic hematopoietic stem cells, as suggested by others (13). Because IL-15 is expressed in other organs, our findings raise the question of whether IL-15-induced NKR expression could be a widespread effect. In our view, the capacity of IL-15 to induce NKR expression by $CD8^+$ T cells could depend on the following: 1) the presence of physiological levels of bioactive IL-15; 2) the presence of resident T cells in continuous contact with IL-15; and 3) the presence of additional survival signals provided by epithelial cells; requirements that seem to be met by the liver and perhaps by other tissues. Indeed, earlier studies established a close relationship between intestinal epithelial cells and human $CD8^+$ T cell proliferation/differentiation (18, 41), and a recent study has shown a link between the presence of IL-15 in the small intestine and the expression of NKR by intraepithelial human cytotoxic lymphocytes (43).

Collectively, our results have shown that hepatocytes contribute in a decisive manner to maintain T cell survival, whereas IL-15 is involved in proliferation, survival, and differentiation, namely at the level of expression of bona fide NKR by $CD8^+$ T cells. These results draw attention to the importance that IL-15 may have when acting in the context of tissue-specific signals through the initiation of a differentiation program that will result in the generation of NKR diversity on selected subsets of T cells, and may contribute to the high levels of nonclassical NKT cells in the human liver.

Note added in proof. During the revision of this manuscript, induction of natural cytotoxic receptors on umbilical cord blood T cells by IL-15 has been reported (Tang, Q., B. Grzywacz, H. Wang, N. Kataria, Q. Cao, J. E. Wagner, B. R. Blazar, J. S. Miller, and M. R. Verneris. 2008. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only NKp30 is functional. *J. Immunol.* 181: 4507–4515), reinforcing the present results.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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