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ON THE ORIGIN OF HUMAN CD8$^+$ NK-LIKE T CELLS: A ROLE FOR IL-15 AND HEPATOCYTES?

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Summary/ Sumário

Summary

The liver is a particular organ from the immunological point of view. It has been suggested that its singular sinusoidal architectural structure provide a high hepatic permeability to both naïve and activated T cells, allowing a direct interaction with hepatocytes, which seems to be unique between solid organs. Besides, it contains a distinctive resident lymphocyte population - the intrahepatic lymphocytes (IHL). The lymphocyte composition of normal adult human liver is dominated by NK cells, NKT cells and activated/memory $CD8^+$ T cells, containing the richest population of NKT cells of the entire body. Interestingly, human hepatic NKT cells are mainly CD1d-unrestricted NK-like $CD8^+$ T cells expressing CD56 and other NK receptors (NKR). The origin of the intrahepatic lymphocyte population is not consensual, although it is conceivable to suggest that the hepatic microenvironment should allow and favor the selective accumulation of the lymphocyte subsets mainly enriched in the liver. Interleukin-15 (IL-15) has been shown to be constitutively present within the hepatic milieu. Interestingly, this cytokine is crucial for NK and NKT cell development, survival and function and also for the proliferation and survival of $CD8^+$ T cells, playing a major role on innate and tissue-associated immune cells – the lymphocyte populations enriched within human liver. Herein, by using *in vitro* co-cultures we have found that both tumoral and non-tumoral human hepatocyte cell lines were capable of increasing survival and driving basal proliferation of *ex vivo* T cells, suggesting hepatocytes as putative players in T cell homeostasis. Also, IL-15 was shown to sustain T cell survival and promote increased levels of $CD8^+$ T cell proliferation. Remarkably, we have found that IL-15 was *per se* capable of driving purified $CD8^+CD56^-$ T cells into a TCR-independent program of differentiation towards the acquisition/upregulation of typical NK receptors, resulting in the generation of $CD8^+CD56^+$ T cells co-expressing other NKR. Although $CD8^+$ T cells expressing NKR have been described in the literature, the signals that drive their origin have remained largely unknown. In this context, our work points to IL-15 as a factor involved in NKR acquisition by $CD8^+$ T cells. The *in vitro* IL-15-genarated $CD8^+NKR^+$ T cells were shown to display mainly an effector memory phenotype, increased apoptosis resistance and cytotoxic potential. Also, we have found that IL-15-cultured $CD8^+$ T cells were capable of exhibiting both TCR-mediated and NK-like non-MHC restricted cytotoxicity and to be endowed with the capacity to secrete an array of cytokines. Remarkably, those phenotypic and functional features are reminiscent of the NKT cell population enriched in human liver. Overall, these findings suggest that hepatocytes and IL-15 could have a role in the homeostasis and shaping of intrahepatic lymphocyte population, creating not only a favorable microenvironment for T cell survival

but also for a local IL-15-driven $CD8^+$ T cell differentiation, contributing for the enrichment of NK-like T cells in human liver. Interestingly, by dissecting the functional outcome of NKR triggering in those cells, we have found that NKG2D mAb crosslinking is able *per se* to induce increased degranulation levels, acting as a functional cytotoxic receptor. Remarkably and intriguingly, engagement of KIR and NKG2A induced the production of Tc1/Tc2 cytokines like IFN-γ, TNF-α, IL-10 and IL-1β. This suggests that, by acting in concert with certain tissue-specific signals, IL-15 might generate NK-like T cells by the acquisition of functional NK receptors capable of modulating both cytotoxicity and cytokine release. Noteworthy, the secretion of a varied pattern of cytokines might suggest that those cells could ultimately display an immunoregulatory role. The unveiling of IL-15 as a putative environmental factor involved in the acquisition of NKR from $CD8^+$ T cells points to IL-15 as a missing link between $CD8^+$ T cells and NK-like T cells, blurring the distinction between cell types and bringing “innate” and “adaptive” close together, highlighting the dynamic concept of the immune system.

Sumário

O fígado é um órgão sólido com propriedades únicas do ponto de vista imunológico. A sua estrutura endotelial particular permite que o fígado possua uma elevada permeabilidade quer a linfócitos activados, quer naive, permitindo a interacção directa com os hepatócitos, o que parece ser único entre os órgãos sólidos. Para além disso, o fígado possui uma população residente de linfócitos – linfócitos intrahepáticos (IHL). A composição linfocitária de um fígado adulto normal é distinta do sangue periférico e outros órgãos, sendo essencialmente dominada por células NK, células NKT e linfócitos T $CD8^+$ com fenótico activado/ de memória, contendo a proporção mais elevada de células NKT de todo o corpo. De realçar, as células NKT hepáticas humanas são caracterizadas por serem maioritariamente linfócitos T $CD8^+$ que expressam CD56 e outros receptores de células NK (NKR), sendo designados de células NKT não restritas à molécula CD1d ou "NK-like T cells". A origem da população intrahepática linfocitária não é consensual, podendo ser resultado de recrutamento específico de determinados subtipos de linfócitos ou de génese local a partir de células hematopoiéticas reminescentes do fígado fetal. Contudo, independentemente da origem, parece concebível que o microambiente hepático favoreça a existência dos subtipos de linfócitos maioritariamente enriquecidos no fígado. A Interleucina-15 (IL-15) é uma citocina crucial para o devenvolvimento, sobrevivência e função das células NK e NKT e para a proliferação e homeostasia dos linfócitos T $CD8^+$, desempenhando assim um papel chave sobre as células imune inatas – as populações linfocitárias mais enriquecidas no fígado humano, local onde é expressa constitutivamente. Neste estudo, pelo estabelecimento de co-culturas *in vitro*, foi possível observar que linhas celulares de hepatócitos humanos possuem capacidade de aumentar a sobrevivência e de suster uma proliferação basal de linfócitos T humanos *ex vivo*, sugerindo que os hepatócitos podem desempenhar um papel na homeostasia de linfócitos T circulantes e residentes. Em paralelo, a citocina IL-15 demonstrou ter uma importante função na promoção da sobrevivência e na indução de proliferação de linfócitos T, particularmente linfócitos T $CD8^+$. Para além disso, e com particular interesse, os dados obtidos indicaram a IL-15 como capaz de induzir linfócitos T $CD8^+CD56^-$ purificados a entrar num programa de diferenciação conducente à acquisição de receptores NK, resultando consequentemente na formação de linfócitos T $CD8^+CD56^+$ com co-expressão de outros receptores NK – "NK-like T cells". Embora a existência de linfócitos T $CD8^+$ que expressam NKR tenha vindo a ser sido descrita na literatura, os sinais fisiológicos que se encontram na base da sua origem permanecem largamente desconhecidos. Deste modo, é de realçar a importância do trabalho presente nesta

dissertação no sentido de apontar a IL-15 como um sinal envolvido na acquisição de linfócitos T $CD8^+$. A análise fenotípica e funcional dos linfócitos $CD8^+$ após cultura com IL-15 revelou que as células $CD8^+NKR^+$ apresentam essencialmente um fenótipo de efectores-memória, elevada resistência à apoptose e elevado potencial citotóxico. Em paralelo, foi igualmente observado que a população de linfócitos cultivados com IL-15 demonstram capacidade citotóxica quer mediada pelo TCR, quer não restrita ao MHC ou citotoxicidade "NK-like", assim como capacidade de secreção de uma larga gama de citocinas. De realçar, as características fenotípicas e funcionais apresentadas por estas células são evocativas das células NKT enriquecidas no fígado humano. De modo geral, os resultados apresentados nesta dissertação sugerem que os hepatócitos e a IL-15 possam integrados desempenhar uma papel na homeostasia e modulação da população linfocitária intrahepática, pelo estabelecimento de um microambiente favorável quer para a sobrevivência dos linfócitos T, quer conducente a uma diferenciação local dos linfócitos T $CD8^+$ conduzida pela IL-15, podendo deste modo contribuir para o enriquecimento das células NKT presentes no fígado. De realçar, a análise funcional dos receptores NK presentes nas células $CD8^+NKR^+$ geradas na presença de IL-15 demonstrou que o *cross*linking do receptor NKG2D foi capaz de desencadear o aumento dos níveis de desgranulação, funcionando *per se* como um receptor citotóxico funcional nessas células. Por seu lado, o *cross*linking dos receptores KIR e NKG2A resultou na indução da produção de citocinas Tc1/Tc2, nomeadamente IFN-γ, TNF-α, IL-10 and IL-1β, constituindo dados novos e interessantes. A integração destes resultados sugere que, a actuação concertada da IL-15 com determinados sinais específicos no contexto de tecidos pode levar à formação de células NK-like através da aquisição de receptores NK capazes de modular funções citotóxicas e de secreção de citocinas. De destacar, a capacidade de produção de uma vasta gama de citocinas pode levar a sugerir que estas células possam desempenhar uma função immunorreguladora. A descoberta da IL-15 como um possível factor ambiental envolvido na aquisição de receptors NK por linfócitos T $CD8^+$ sugere que esta citocina possa ser uma ponte de ligação entre linfócitos T $CD8^+$ e células NKT, tornando menos definida a distinção entre os dois tipos celulares e aproximando o "inato" e "adaptativo" no contexto de um sistema imune dinâmico.

Abbreviations

Ab	Antibody
Ag	Antigen
AICD	Activation induced cell death
APC	Antigen presenting cell
APS	Amphotericin B/Penicillin/Streptomycin
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	5-(and-6)-Carboxyfluorescin diacetate succinimidyl ester
CM	Conditioned medium
CTL	Cytotoxic T lymphocytes
FACS	Fluorescence activated cell sorter
FBSi	Inactivated fetal bovine serum
IFN	Interferon
Ig	Immunoglobulin
IHH	Immortalized human hepatocytes
IHL	Intrahepatic lymphocytes
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
JNK	Jun N-terminal kinase
KIR	Killer Immunoglobulin-like receptor
mAb	Monoclonal antibody
MIP	Macrophage inflammatory protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NF-kB	Nuclear factor-kappa B
NK cell	Natural Killer cell
NKG2	Natural Killer cell lectin-like receptor gene-2
NKR	Natural Killer receptor
NKT	Natural Killer T
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin

PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-kinase
RBC	Red blood cells
STAT	Signal Transducers and Activators of Transcription
TCR	T cell receptor
TNF	Tumor necrosis factor

I – GENERAL INTRODUCTION

1. Liver and immune system

The liver has been for a long time considered an inert organ from the immunological point of view in normal conditions. However, this view has been changed since immunologists begin to recognize that the liver possesses distinct properties compared with the other solid organs (1-3). Indeed, due to its unique location and anatomy and the existence of a distinctive intrahepatic lymphocyte population the concept of the liver as a lymphoid organ with unique immunological properties has emerged (2, 4-5).

From the anatomical point of view, the liver is strategically located implying an important immunological function: it stands at a hemodynamic confluence where systemic blood mixes with portal venous blood from the gastrointestinal tract. The portal venous blood, returning from the intestine, corresponds to 75%-80% of its blood supply and is rich in microbial products, environmental toxins and food antigens. Besides, the metabolic function of the liver implies that it is continuously exposed to metabolites that could then represent neo-antigens (1, 6). Accordingly, the liver must be selective about the antigens to which it responds to prevent inappropriate immune activation and yet be capable of dealing effectively with pathogens. This requires a delicate balance between immunity and tolerance in the liver. Indeed, tolerance is a key concept of liver immunity.

Given its strategic location and blood supply, the liver is ideally placed to interact with most lymphocytes circulating in the blood, and it is tempting to speculate that this organ has evolved to filter and tolerate circulating T cells by presenting blood-borne and food-derived antigens absorbed by the intestine and carried by the portal vein (10).

1.1. Liver microanatomy and cell composition

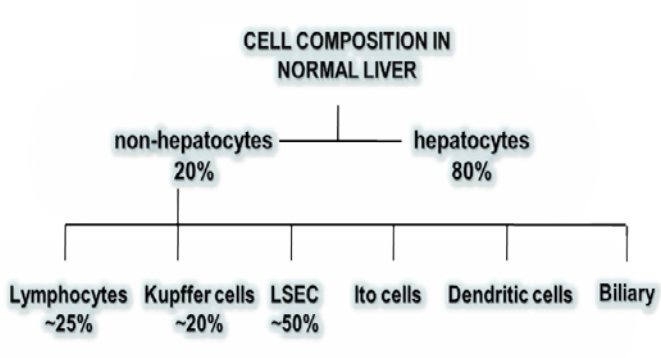


Figure 1. Liver cell composition.

Percentage proportions of different cell types in the normal liver. Adapted from (2)

The liver is composed of different cell populations with distinct functions. **Hepatocytes** constitute the major cell type in the parenchyma (about 70-80% of cells in the liver) (Figure 1), and are arranged into cell plates separated by narrow sinusoids (1-2, 11).

The blood percolates through sinusoidal spaces in the liver rather than through capillaries, and the endothelial barrier consists of the **sinusoidal endothelial cells (LSEC)** that are morphologically unique: (i) they do not form junctions with adjacent endothelial cells, but rather form an incomplete barrier between the sinusoidal lumen and the perivascular parenchymal tissue; (ii) are perforated by several holes or fenestrations; and (iii) lack a basal membrane, thus defining a perisinusoidal space between LSEC and hepatocytes, the space of Disse (1, 11-12) (Figure 2).

Ito cells, also described as *hepatic stellate cells* (HSC), store 80% of total body retinol (vitamin A) as cytoplasmic lipid droplets (13-14) and are found in the space of Disse (Figure 2). Interestingly, the liver contains a local population of macrophages, the **Kupffer cells (KC),** that constitute about 20% of liver cells, **dendritic cells (DC)** and a resident population of lymphocytes: **intrahepatic lymphocytes (IHL)** (Figure 1). IHL and DC are described to mainly line the lumen of the hepatic sinusoids (Figure 2) (1, 10, 12). However, those cells have also been described throughout the parenchyma (IHL), as well as in the portal tracts (DC, IHL) (4, 10). The mean yield of intrahepatic lymphocytes is about 10^6 IHL/mg of tissue, representing a lymphocyte population of between 10^9−10^{10} cells and constituting about 25% of the non-parenchymal cell population in the liver (Figure 1) (2). The IHL population is generally constituted by natural killer (NK) cells, natural killer T (NKT) cells, T cells and B cells, differing widely in number and composition from the lymphocyte population present in blood and lymph nodes, being thus unique *(as will be below discussed)*.

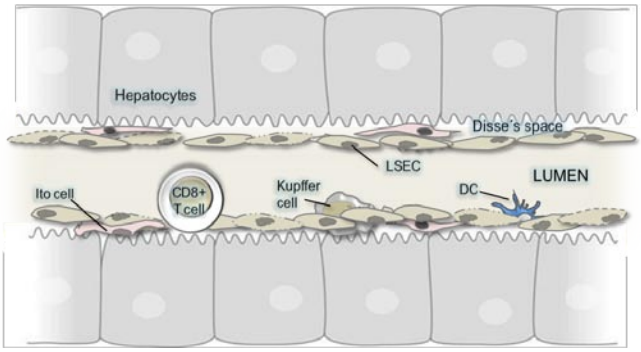


Figure 2. Liver microanatomy

Schematic representation of hepatic sinusoids showing a lymphocyte interacting with hepatocytes through fenestrated LSEC. *Stellate* cells existent in Disse´s space and Kupffer and dendritic cells are also represented. Adapted from (1).

The liver is, this way, distinct from other lymphoid and non-lymphoid organs in terms of its structure and vascular architecture and the combination of the particular architectural characteristics provide this organ an important feature: a high permeability to lymphocytes. Indeed, the unique structure of hepatic sinusoids and conditions of low velocity blood flow, created by the large *cross*-sectional area of the sinusoids and by the presence of Kupffer cells patrolling in the lumen, may be the principal properties responsible for the singular ability of the liver to be highly permeable, which favors an increased possibility of interaction with hepatocytes (12, 15) Besides, it has been proposed that leukocyte rolling, a prerequisite for their binding to other endothelia, is dispensable in the liver in the view of the previously described distinctive architectural characteristics (12, 16-17), and can be substituted by other widely expressed adhesion molecules such as ICAM-1 (10, 18). This allows that, contrarily to other solid organs, not only activated but also naïve T cells could interact directly interact with hepatocytes.

Remarkably, a study of Warren and colleagues has demonstrated that **both resident lymphocytes (IHL) as well as circulating naive $CD8^+$ T cells make direct contact with hepatocytes** through extensions penetrating the endothelial fenestrations that perforate the LSEC (19) (Figure 2).

1.2. Liver as an immunological active place: Intrahepatic primary T cell activation, apoptosis and liver tolerance

1.2.1. Intrahepatic primary T cell activation and tolerance

The immunological importance of liver is reinforced by evidence suggesting another striking feature: it has been pointed as a place where primary activation can occur. The paradigm of primary T cell activation implies that it is mediated in lymphoid tissues by

professional antigen presenting cells (APC) that (i) efficiently process antigens, (ii) possess a specialized endocytic compartment in which MHC class II molecules bind antigen peptides, (iii) express high levels of MHC class I and class II molecules, invariant chain, CD80 and CD86 co-stimulatory molecules, as well as several other adhesion molecules involved in T cell activation as CD54 (ICAM-1) (20). Within the resident liver cells there are professional APC as dendritic cells and Kupffer cells (resident macrophages). Besides, and interestingly, in the last years, LSEC (1, 21-22), Ito (stellate) cells (1, 14) and also **hepatocytes** (1, 23) have been pointed as cells with potential APC ability. Indeed, resting hepatocytes express MHC class I molecules (24) and even though MHC class II molecules, CD40L and co-stimulatory molecules such as CD80 are not constitutively expressed, they were shown to be upregulated following inflammation (1). In this line, Bertolino and colleagues have found that hepatocytes can actually act as very efficient APC for high avidity naïve $CD8^+$ T cells *in vitro*, without T cell priming in lymph nodes (1, 23). Interestingly, the results suggest that T cells activated by hepatocytes died by neglect due to an inefficient expression of IL-2 and survival genes, resulting from inefficient co-stimulation during activation (23). Indeed, although several cell types can present antigens to naïve and activated T cells within the liver, this process has been mainly associated with hepatic tolerance. It has been suggested that regardless of the liver APC involved in presentation, there is a competition with DC in lymphoid tissues for activation of naïve T cells but that these two activation pathways lead to two different outcomes: while T cells activated in the **lymph nodes** acquire full effector function and participate in immunity, T cells activated in the **liver** are thought to become unresponsive and/or are deleted, a process resulting in antigen-specific tolerance (25). This model could thus help to explain why effective immune responses to some pathogens can occur within the liver while this organ still maintains an intrinsic ability to induce tolerance (10).

Therefore, the liver is an exception of the paradigm of T cell recirculation that predicts that naïve T cells can only be activated in the lymph nodes. Besides, it has been shown that naïve T cells recognizing their cognate antigen in the liver behave differently than in other solid organs, as they can be retained and activated *in situ* (26).The ability of the liver to support primary activation of T cells is increasingly perceived as an important mechanism inducing tolerance within the naïve T cell population. Supporting this view, several studies suggest that intrahepatic antigen presentation to naïve T cells in the absence of inflammation leads to tolerance rather than immunity (1, 10).

1.2.2. T cell apoptosis in the liver: for the sake of tolerance?

The liver is considered by some authors as a preferential place of $CD8^+$ T cell apoptosis after systemic activation (12, 27-28). Crispe and colleagues have suggested that $CD8^+$ T cells activated in the periphery travel preferentially to the liver to be eliminated by apoptosis in order to maintain the peripheral homeostasis of those cells (27); which seems to be related with the increased capacity of this organ to selectively retain those cells (12). According to this hypothesis, some authors envisioned the liver as a "graveyard" for activated $CD8^+$ T cells (27). However, even though it has been shown that that the liver seems to selectively sequester activated circulating $CD8^+$ T cells (29-30), it is now mainly believed that the major source of apoptotic cells in the liver are probably cells that were defectively activated by non-professional APC in the liver, namely hepatocytes, in order to maintain tolerance, and Crispe *et al* (2006) now agree that the model of "liver as a graveyard" should be revised (30).

1.3. Intrahepatic lymphocyte (IHL) population

The population of intrahepatic lymphocytes (IHL) is characterized by a unique repertoire of lymphocytes, differing phenotypically and in number from the peripheral blood and lymph nodes. The liver is mainly enriched in natural killer (NK) cells, γδT cells and NKT cells (2, 31-34) and memory/activated CD8+ T cells in comparison to peripheral blood and lymphoid tissues. The frequency of NK cells, NKT cells and γδ T is variable representing about **65%** of all IHL, while only about **17%** in the peripheral blood (3, 8) (Figure 3). Consequently, the liver is considered one of the richest sources for innate immune cells (34). Indeed, the presence of various intrahepatic subpopulations with multiple effector functions suggests that those cells may have important specialized functions in local immune responses (15).

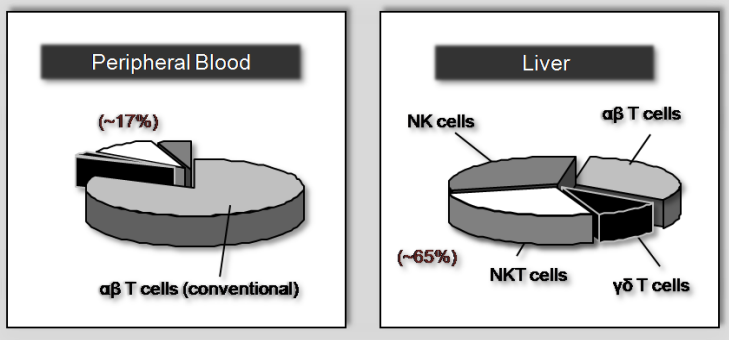


Figure 3. Intrahepatic lymphocyte composition

Percentage proportions of different intrahepatic lymphocytes (IHL) compared with percentages present in peripheral blood. The liver is enriched in NK, NKT and γδ T cells (about 65%) compared with peripheral blood (about 17%). Adapted from (8).

NK cells comprise a large percentage of IHL, accounting for **up to 50%** of the total lymphocyte pool in the non-diseased liver compared to less than 20% of peripheral blood lymphocytes (2, 8, 34-36) (Figure3). Hepatic NK cells are not only enriched in the liver but also naturally activated as they show higher cytotoxicity against tumor cells than splenic or peripheral blood NK cells in rodents (37) and in humans (38). Over the past several years, many studies have shown that hepatic NK cells play an important role in innate immune responses against tumors, viruses, intracellular bacteria, and parasites (34).

NKT cells are lymphocytes that express both TCR and NK receptors (NKR), including different cell types (see Box 1). In humans, this subset can account for up to 55% (mean: **~30%**) of all hepatic $CD3^+$ cells, while only about 2% of peripheral T cells (8, 35-36). This makes the liver a predominant location of NKT cells which are far more abundant in this organ than in any other place, and accumulating evidence is pointing to NKT cells as having a particular role in liver immunity (4, 39). In mice, the majority of NKT cells express a TCR consisting of an invariant α-chain, Vα14, and one of a limited number of β-chains, that recognizes glycolipid antigens presented by the MHC class I-like protein CD1d (8, 40-42), known as CD1d-restricted or invariant NKT cells (see Box 1), constituting about 30–50% of murine intrahepatic lymphocytes (40, 43-44). In marked contrast, the **human liver** only contains a small number of those invariant ($V\alpha24^+$, in humans) NKT cells (about 4%) (35-36, 45), being rather substantially enriched in T cells with a variant TCR expressing NK receptors as CD56, CD161, CD94, and killer Ig-like receptors (KIR) (8, 31, 36). This CD1d-unrestricted NKT cell population enriched in the human liver is normally characterized as **$CD3^+CD56^+$** cells and can be called **NK-like T cells** (see Box 1). Interestingly, the majority of hepatic $CD3^+CD56^+$ NKT cells express the CD8 co-receptor and an αβ TCR, while up to 35% of those were found to express γδ TCR (35-36, 39).

γδ T cells carry an oligoclonal or invariant TCR, but recognize a diverse array of ligands, including soluble non-peptide antigens and stress-inducible proteins without need of MHC restriction. They are likely to serve as a first-line defence against noxious antigens from the gut, and immune regulatory functions are attributed to this subset as well (2, 34, 46). γδ T cells represent a minority of T cells in lymphoid organs and peripheral blood, but a high percentage of γδ T cells is found in intraepithelial lymphoid compartments of skin, intestine, and genitourinary tract. Interestingly, liver lymphocytes are also enriched in γδ T cells (8). In normal livers, γδ T cells account for 3% to 5% of total liver lymphocytes but up to 35% (15%-25% in average) (8) of total liver T cells, contrarily to less than 5% of peripheral blood T cells, making the liver one of the richest sources of γδ T cells in the

body (8, 34). The largest percentage of hepatic γδ T cells express CD8 or are double negative ($CD8^-CD4^-$) and could also express several NK receptors (8, 36). However, despite their unusual large percentage in normal livers, the role of hepatic γδ T cells has not yet been completely elucidated. It has been described that the percentage of γδ T cells is significantly increased in the liver of tumor-bearing mice and elevation of γδ T cells was also found in the livers of patients with viral hepatitis infection, but not in patients with nonviral hepatitis. Thus, emerging evidence suggests that γδ T cells may play a prominent role in innate defenses against viral and bacterial infection and against tumor formation (34, 46), supporting the view of liver as a specialized site of innate immune responses.

Besides being present in the liver in a much smaller percentage, the **conventional αβ T cells** show a skewing of the $CD4^+/CD8^+$ ratio towards $CD8^+$ lymphocytes (about 60-90% of T cells in the liver express CD8), contrarily to peripheral blood and spleen (8, 35). Also, they are predominantly activated, expressing activation markers as CD69 (8, 33, 36), displaying an effector/memory phenotype (8, 33, 36, 47-48) with a large percentage of cells, particularly $CD8^+$ T cells, corresponding to the so-called terminally differentiated T cells (18, 33, 49). Also, about 15% of IHL are homodimeric $CD8^+$ T cells expressing only the α-chain, $CD8\alpha\alpha^+$ T cells, a phenotype that is barely detected in peripheral blood (2, 35).

Box 1| Classification of NKT cells: different cell types in a name

	Type I NKT cells	Type II NKT cells	NK-like T cells
Other names	Classical NKT CD1-d dependent Invariant NKT (iNKT) Vα24 NKT (human) Vα14 NKT (mouse)	Non-classical NKT Diverse NKT	CD1d-independent CD3+CD56+ cells (human) NK1.1+ T cells (mouse)
Restriction	CD1d	CD1d	MHC, other? (CD1d unrestricted)
A-GalCer reactivity	+	-	-
TCR repertoire	Vα24 –Jα18: Vβ11 (human) Vα14 –Jα18: Vβ8.2,7,2 (mouse)	Diverse	Diverse

1.3.1. Intrahepatic lymphocyte population (IHL): origin hypothesis

The unique composition of intrahepatic lymphocytes can only be understood by insights into the origin of the cells and the mechanisms by which they populate the liver. Mainly two hypothesis have emerged in this context: one proposes a “local generation and differentiation” of lymphocytes within the liver and the other “a recruitment and entrapment of specific subsets from the periphery”, to justify the existence of the distinctive intrahepatic pool.

1.3.1.1. Local origin and maturation

This hypothesis suggests that intrahepatic lymphocytes could be **locally generated and maturated within the liver**. According to this, T cells can mature in a thymus-independent manner from precursor cells existent in the liver and remain largely noncirculating. Indeed, the liver is the most important hematopoietic organ during fetal life. Although this function is drastically reduced in adults, early $CD34^{+}CD45^{+}$ lymphoid progenitors can still be found in a normal human adult liver (50). In fact, hematopoietic stem cells $CD34^{+}$ $c\text{-}kit^{+}$, non derived from the bone marrow, can be detected within the human and murine liver (3, 50-53). Furthermore, recombination-activating gene (RAG) -1 and RAG-2 messenger RNAs as well as preTα messenger RNA, all of which are required for T-cell differentiation, can be detected in intrahepatic T cells, supporting the hypothesis that some T cells can differentiate extrathymically (8, 54-55). Intestinal intraepithelial (i-IEL) CD8αα lymphocytes have been proposed to complete their development program in the murine small intestine, being possible to suggest that a similar T-cell selection process may occur in the liver (8).

Although the evidence suggests that intrahepatic differentiation of unconventional T cells can occur in the adult, it does not imply that this is the major pathway. Indeed, other authors have observed that NKT cells are hardly detectable in younger nude and neonatally thymectomized mice (39, 56). Also, in contrast to thymic precursors, the intrahepatic precursor cells did not show ability to origin conventional T-cells or αβ TCR NKT cells after adoptive transfer in the absence of the thymus (57), probably due to the absence of Notch ligand in the liver, known to be required for maturation in the thymus (58).

Therefore, although all the requirements for extrathymic differentiation of lymphocytes within the liver appear to be met, it remains to be established whether it occurs physiologically and what T-cell subsets are involved. In any case, it could account for the contribution of some subtypes or represent a backup system (53).

1.3.1.2. Specific recruitment and entrapment of lymphocytes in the liver (dynamics of entry and exit)

This model proposes that the unique composition of intrahepatic lymphocytes is determined by a **dynamic equilibrium of immigration, retention within the liver and emigration or death** (48, 53). T cells and other lymphocytes are highly mobile cells, as demonstrated by the intense recirculation of naïve T cells through lymphoid tissues and the ability of effector/memory cells to enter and leave a variety of tissues during physiological immune surveillance (53). The strongest support for the dynamic model comes from data on the differential trafficking of lymphocyte subsets into the liver. Klugewitz and colleagues have predicted that a high frequency of a given subset in the liver would be reflected by a higher entry rate into the liver compared with the spleen, indicating that cell subsets with a 'liver-seeking' behavior are found at increased frequencies among intrahepatic lymphocytes. This was true for most of the subsets found to be enriched in the liver, including $DX5^+$NK cells, invariant NKT cells and memory $CD4^+$ T cells. By contrast, populations, such as naïve $CD4^+$ T cells, resting $CD8^+$ T cells or B cells that are under-represented in the liver, behave as classical recirculating cells with a preferred immigration into lymphoid organs (48). These findings confirm observations made in other studies. Conventional T cells, such as short-term activated $CD4^+$ and activated $CD8^+$ T cells, $CD62L^{low}$ or $CD25^{high}$ can be selectively retained within the liver. This also applies to Th1 and Th2 effector cells and $CD4^+$ memory cells ($CD45RB^{low}$) (18, 59-61). Furthermore, pre-apoptotic T cells that express altered surface molecules, such as B220, can be trapped within the liver by interactions with the asialo-glycoprotein receptor on hepatocytes, arguing for passive trapping (53, 62). Also it was found that αβ TCR NKT cells as well as γδ TCR NKT cells can be recruited into the liver through an LFA-1-dependent mechanism involving binding to neighboring liver-resident NK cells (63-64). Furthermore, circulating NK and NKT cells express patterns of chemokine receptors, including CXCR4, CXCR6, CXCR3 and CCR5, which would enable them to be recruited by chemokines expressed in the liver (65-67). In parallel, binding between liver parenchyma and activated T cells has been shown to be also promoted by ICAM-1/LFA-1 (18) and it was recently found that TLR-4, in the absence of Ag on hepatocytes, plays the

more significant role (68). Furthermore, a distinct nonlymphoid recirculation pathway has also been demonstrated for γδ T cells from the gut, suggesting that γδ T cells can recirculate with a high preference for re-entry into the gut (69).

These findings indicate that distinct recirculation specificities and retention of certain lymphocyte subsets within the liver can significantly contribute for their enriched representation amongst the intrahepatic lymphocyte population (53). The shape of population is result of a dynamic equilibrium of immigration, retention and emigration of distinct T cell populations.

However, it is important to note that the two theories could be not mutually exclusive. Indeed, neither could *per se* explain the peculiar composition of intrahepatic lymphocyte population and, at the same time, both can contribute to the explanation. Importantly, independently of which hypothesis could be more correct or account more accurately for the presence of such unique population, the hepatic microenvironment should allow and favor the presence of the particular subsets of intrahepatic lymphocytes. Indeed, local survival and expansion/proliferation might be relevant for the existence and enrichment of the specific subsets, probably having an important role in shaping the intrahepatic lymphocyte pool, both in case of local origin or specific recruitment.

In this context, it is plausible to think that the hepatic microenvironment, namely the cytokine milieu, could influence the distribution of intrahepatic lymphocytes: by (i) directing hematopoiesis within the liver; (ii) driving a selective expansion of particular lymphocyte subpopulations; (iii) influencing the recruitment of specific circulating lymphocytes; (iv) promoting a local differentiation, leading to possible phenotypic changes to infiltrating cells.

Thus, it can be hypothesized that the hepatic cytokine milieu could have a role in driving a selective accumulation of lymphocyte subsets that are derived from either liver-resident or circulating lymphocytes, and should be favorable to the homeostasis of the lymphocyte populations mainly enriched in the liver.

2. Interleukin-15 (IL-15)

Interleukin-15 (IL-15) is a pleiotropic cytokine of the 4-α-helix bundle cytokine family that includes not only cytokines such as IL-2, IL-3, IL-4, IL-6 and IL-21, but also growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin and classical hormones, including human growth hormone and prolactin (70-72). A special feature of IL-15 is that it shares with IL-2 the IL-2 receptor beta (IL-2Rβ) and also the γc receptor chain that, besides IL-2, also shares with several other cytokines such as IL-4, IL-7, IL-9 and IL-21 *(see below)*, all of which use additional private receptor subunit(s) responsible for the specificity of binding and/or downstream signaling (7, 73).

IL-15 was independently discovered in 1994 by two groups, based upon its ability to mimic IL-2-dependent T cell proliferation (70-72). Burton *et al* (1994) demonstrated that the human T-cell leukemia virus-1 (HTLV-1) cell line HuT-102 secreted a 14 kDa lymphokine capable of stimulating T cell proliferation and large granular lymphocytes activation, which was provisionally designated as IL-T (70). Simultaneously, Grabstein *et al* (1994) reported the isolation of a 14-15 kDa cytokine termed IL-15, which shared many biological properties with IL-2, from the supernatants of the simian kidney epithelial cell line CV-1/EBNA (72). Given that cytokines often share receptor subunits and can therefore display a high degree of redundancy, the existing similarities in the action between IL-2 and IL-15 on cells of the same type were not surprising (70, 72, 74). However, a series of later studies have shown that, despite their many overlapping functional properties, IL-2 and IL-15 are, in fact, quite distinct players in the immune system (75-78). Namely, IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ knockout mice display profound lymphadenopaty, splenomegaly, and T cell-dependent autoimmunity, which is associated with a reduced ability in T cells to undergo activation induced cell death (AICD) in these mice (76, 79). By contrast, IL-15 has been shown to inhibit IL-2-mediated AICD and IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice show, instead, lymphopenia characterized by a reduction in innate immune cell lineages, including NK cells, NKT cells and γδ T cells, as well as $CD8^{+}$ T cells. Thus, IL-15 appears to provide essential signals for multiple lymphoid cell development and/or homeostasis (75, 78).

2.1. IL-15 expression

Contrary to IL-2, which is produced mainly by activated T cells, IL-15 mRNA is constitutively expressed by a large variety of cell types, including monocytes,

macrophages, dendritic cells, epithelial cells, fibroblasts, keratinocytes, neural cells; and tissues including kidney, lung, heart, skeletal muscle, placenta, brain, intestine and liver (72, 80-90). Freshly isolated peripheral blood mononuclear cells (PBMCs), normal T cells and T cell lines express IL-15 mRNA at very low levels (72, 91), whereas detection of IL-15 protein appears to be mostly restricted to monocytes, DCs, fibroblasts and epithelial cells (80-81, 83). Despite such widespread distribution, however, it is extremely difficult to detect IL-15 in cell culture supernatants (92-94).

It has been reported that IL-15 protein expression is widely regulated, both at post-trancriptional and post-translational mechanisms. Specifically, multiple start sites in the 5´-UTR of the IL-15 mRNA may regulate translational efficiency of IL-15 production (72, 95). A negative regulatory sequence in the 3´-UTR of IL-15 mRNA may also regulate the efficiency of IL-15 secretion (96). Moreover, alternative splicing of the IL-15 mRNA leads to the existence of two distinct IL-15 isoforms, which differ only in the length of their signal peptide: one with a 48-aa long signal peptide (**IL-15LSP**) and another with a 21-aa short signal peptide (**IL-15SSP**); and exhibit differential patterns of intracellular distribution, trafficking, secretion and endosomal localization (7, 91-94, 97-98). IL-15SSP is not secreted, but rather stored intracellularly in the cytoplasm, while IL-15LSP is found in the Golgi, early endosomes and the ER, a pathway possibly leading to cytokine secretion (7, 93-94, 98). Both IL-15 isoforms may also exhibit nuclear localization (98).

This way, the discrepancy between IL-15 transcription and protein secretion could be explained by the fact that IL-15 has a complex, multifaceted control of expression with regulation at the levels of transcription, translation and intracellular trafficking and translocation (7, 93-94, 98-100). But also, and importantly, another characteristic that account for the main absence of soluble IL-15 is the striking fact that the main biological form of IL-15 is rather bound to the high-affinity receptor unit, IL15Rα (101), as will be further discussed.

2.2. IL-15 receptor complex and signaling

2.2.1. IL-15 receptor complex

The IL-15 receptor complex is constituted by a combination of three receptor subunits that include the common **gamma chain** (γ_c) (shared by IL-2, IL-4, IL-7, IL-9, IL-21, as already mentioned), the **beta chain** shared with IL-2 (IL-2/IL-15Rβ) and a private high-affinity

subunit **IL-15Rα** (Figure 4). IL-15 shares, this way, the β and γ_c subunits with IL-2 (IL-2R/IL-15$\beta\gamma_c$), which can explain in part the existence of some functional similarities between IL-2 and IL-15 (7, 9, 102-103). However, those two cytokines have different private receptors: while IL-2 binds to its private receptor unit, IL-2Rα, IL-15 binds to its own private receptor unit, IL-15Rα. Besides, IL-2Rα binds IL-2 with low affinity, while IL-15Rα alone has high affinity to IL-15 and together with the other subunits forms a heterotrimeric high-affinity receptor complex IL-15R$\alpha\beta\gamma_c$, whereas the IL-15R$\beta\gamma_c$ in the absence of IL-15Rα only binds IL-15 with intermediate affinity (9). Some evidence suggests that IL-15Rα itself can mediate certain intracellular signals (104-105). Due to the high-affinity of IL-15Rα for IL-15, it has been hypothesized that the IL-15Rα may act as a molecular sink for excess IL-15, or possibly associate with other yet to be identified receptor components (106). Interestingly, IL-15Rα not only exists as a membrane-bound receptor but also in soluble form (sIL-15Rα) generated by specific proteolytic cleavage (107-110). Shedding of the soluble form of this high-affinity receptor may reduce the availability of free IL-15 and compete with the membrane-bound receptor and therefore reduce the biological activity of IL-15 (111). This is supported by *in vivo* evidence which suggests that administration of sIL-15Rα results in the inhibition of NK cell proliferation (112) and antigen-specific T-cell responses (113). However, conversely, it has been recently reported that sIL-15Rα can convert IL-15 to a superagonist that enhances the IL-15 responses in $CD8^+$ and NK cells both *in vivo* and *in vitro*. It is speculated that upon sIL-15Rα binding to IL-15 a conformational change occurs in the cytokine, which enhances its interaction with the $\beta\gamma_c$ receptor (109, 111).

2.2.2. Juxtacrine signaling: IL-15 *trans*-presentation

As mentioned above, although IL-15 was initially identified as a soluble factor, there is increasing evidence that IL-15 mediates its biological functions mainly in a cell membrane-associated form (80, 83, 101, 114-122). In fact, the accumulated evidence indicates that under physiological conditions, the biologically active form of IL-15 is actually **surface-bound IL-15** (80, 83, 101, 110, 115, 118-119, 121), which has been shown to reduce the amount necessary to exert its biological effects (7, 101). It has been shown that IL-15 can be presented at the cell surface of cells such as monocytes and fibroblasts bound to IL-15Rα. This stable IL-15/IL-15Rα complex is then capable of activating in *trans* neighbour cells bearing IL-15R$\beta\gamma_c$ or the trimeric receptor (7, 101, 115, 120), mediating a **juxtacrine signalling**, which seems unique among cytokines. These studies revealed a novel mechanism by which cells expressing the IL-15Rα chain can form stable complexes IL-

15/IL-15Rα at the cell surface and ***trans*-present** IL-15 to neighbouring T and NK cells. Moreover, this binding reportedly enables IL-15Rα to recycle between endosomes and the cell membrane to *trans*-present IL-15 again, leading to the persistence of IL-15 after withdrawal of the cytokine from the culture medium (101). However, other data indicates that the cell surface IL-15 is not necessarily linked to the IL-15Rα but instead could be anchored to the plasma membrane as a transmembrane-IL-15 (80, 83, 110, 115, 122). Budagian *et al* (2004) suggest that membrane-bound IL-15 could actually exist in activated monocytes in the two ways: **bound to IL-15Rα and in a transmembrane form** (110), but it remains controversial. Yet, the accumulated evidence points more to a surface bound IL-15 in the context of a IL-15/IL-15Rα complex, since it has been demonstrated that IL-15Rα, expressed in opposing cells, has a crucial role in $CD8^+$ T cell proliferation and NK cell homeostasis, while IL-15Rα expression by lymphocytes is dispensable for IL-15 action *in vivo* (75, 77, 114-116, 120, 123-124); supporting thus the requirement of IL-15Rα for the *trans*-presentation.

2.2.3. Reverse signaling

Another important aspect of membrane-associated IL-15 is its reported ability to mediate **reverse signaling** events. The existence of reverse signaling is now well established, and a broad array of experimental data from diverse sources and systems convincingly demonstrate its biological significance (7). It has been demonstrated that membrane-bound IL-15 is implicated in reverse signaling in human monocytes and PC-3 prostate carcinoma cells (110, 122, 125). Besides inducing signaling pathways upon stimulation with recombinant sIL-15Rα or anti-IL-15 Abs (110), the reverse signaling through membrane IL-15 considerably increased the production of several proinflammatory cytokines by monocytes including IL-6, IL-8 and TNF-α, thereby indicating the relevance of this process to the complex immunomodulatory function of these cells (110, 122). The stimulation of transmembrane IL-15 also enhanced the transcription of IL-6 and IL-8 in the PC-3 cell line, and promoted migration of PC-3 cells as well as LnCap human prostate carcinoma cells stably expressing IL-15 upon the cell surface (110). These data are in agreement with a report of Neely *et al* (2004), showing the ability of monocyte surface-bound IL-15 to function as an activating receptor and participate in reverse signaling events (122). The ligation of surface IL-15 by *cross*-linked anti-IL-15 Abs was found to activate a number of signaling pathways, including ERK1/2 and p38, as well as the small Rho-family GTPase Rac3. In addition, it resulted in the MAPK dependent IL-8 secretion, and induced monocyte adhesion (110, 122).

The fact that IL-15 can exist as a biologically active transmembrane molecule that possesses dual ligand/receptor qualities with a potential to induce **bi-directional signaling** provides another striking example of a unique role of this pleiotropic cytokine in immune homeostasis (7).

2.2.4. Intracellular signaling: JAK/STAT

IL-15-mediated signaling in T lymphocytes results in the activation of Janus kinase (JAK) (7, 126). JAKs play critical signaling roles for a number of members of the cytokine receptor superfamily by activating signal transducer and activator of transcription (STAT) proteins (7, 127). After receptor–ligand interaction, associated JAKs are brought into close apposition, which allows their *cross*-phosphorylation and activation: the IL-15Rβ chain recruits **JAK1**, whereas IL-15Rγ_c activates **JAK3**, which in turn results in the phosphorylation and activation of **STAT3** and **STAT5**, respectively (7, 126). Specific tyrosine residues in the cytoplasmic domains of the IL-2Rβ and γ_c subunits also become phosphorylated, and serve as docking site(s) for STAT via the STAT Src homology 2 (SH2) domain (128). Subsequently, recruited STATs are phosphorylated by activated JAKs (7, 127). The tyrosine phosphorylated STATs transcription factors form either homo- and/or heterodimers are translocate to the nucleus to bind to their target DNA regulatory elements and participate in the activation of gene expression (7, 127). However, additional signaling pathways through the IL-2/IL-15R complexes could occur, namely in other cell types (*see below*), and include the ***src*-related tyrosine kinases** (Lck, Fyn and Lyn), **Syk kinase**, **PLCγ**, **PI3-kinase (PI3K) and Akt,** induction of **Bcl-2** and stimulation of the **Ras/Raf/MEK/MAPK** pathway that ultimately results in **fos/jun** activation (129-132). IL-15 has also been shown to activate **NF-kB** and **AP-1** transcription factors (129, 133), and control **c-myc** (134) (Figure 4).

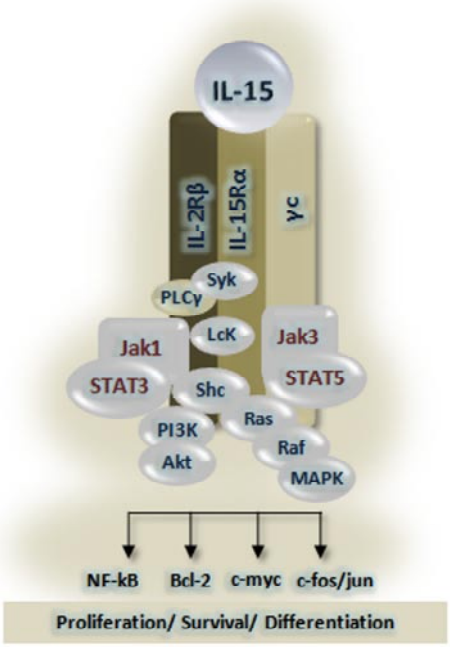


Figure 4. IL-15 receptor complex units and intracellular signaling

Representation of IL-15 three subunits (IL-15Rα/ IL-2Rβ/ γ chains) and intracellular signaling in lymphocytes. IL-15 activates Jak1/Jak3 and STAT3/STAT5. Additional pathways involve Syk kinase and PLCγ, Lck kinase, Shc, resulting in the activation of PI3K/Akt and Ras/Raf/MAPK cascades, that could lead to the subsequent expression of bcl-2, c-myc and c-fos/jun genes and NF-kB activation. Adapted from (7).

2.3. Biological functions of IL-15

IL-15 plays a multifaceted role in the development and control of the immune system, affecting a wide range of target cell population, including **NK cells**, **NKT cells**, certain subsets of **intestinal epithelial lymphocytes (IEL)**, and **memory CD8$^+$ T cells** (Figure 5). Indeed, IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice both exhibit selective losses of memory CD8$^+$ T cells, NK cells, NKT cells, and subsets of IEL, indicating that IL-15 provide essential positive homeostatic signals for these subsets of cells (75, 77). The phenotypes of these two strains of mice are also very similar, supporting that IL-15Rα is required for physiologically relevant IL-15 signals. In fact it has been described that NK cells, NKT cells, CD8αα IELs, and memory CD8$^+$ T cells depend on the availability of IL-15 "pools" (75, 77, 135-137) (Table I).

Table I. Summary of major mice phenotypes with targeted disruption of IL-15 or IL-15R components (adapted from (9))

Mutation	Major phenotypes	References
IL-15$^{-/-}$	NK cells absent	(77)
	NKT cell deficiency (thymic and pheripheral)	
	Memory CD8$^+$ T cell deficiency	
	IEL γδTCR decrease	
	IEL CD8αα decrease	
	Decreased weight and cellularity of peripheral LN	
IL-15Rα$^{-/-}$	NK cells absent	(75)
	NKT cell deficiency (thymic and peripheral)	
	Single-positive CD8$^+$ T cell deficiency (thymus)	
	Memory CD8+ T cell deficiency	
	IEL γδTCR decrease	
IL-2/IL-15Rβ$^{-/-}$	NK cells absent	(138-140)
	NKT cell deficiency (thymic and pheripheral	
	IEL γδTCR decrease (TCRαβ, CD8αα, TCRγδ)	
	Dendritic epidermal γδ T cells (DETC) absent	

2.3.1. An essential role for IL-15 in NK development

2.3.1.1. NK cell development

Receptors for IL-2, IL-15, and IL-7 are expressed in various stages of immature NK cells, and *in vitro* studies indicate that IL-2, IL-15, and IL-7 can all support NK cell differentiation (141-145). However, studies of IL-2$^{-/-}$, IL-2Rα$^{-/-}$, IL-7$^{-/-}$, and IL-7Rα$^{-/-}$ knockout mice fail

to exhibit significant defects in NK cell development (145-149). By contrast, IL-15$^{-/-}$, IL-15Rα$^{-/-}$, IL-2Rβ$^{-/-}$, and $\gamma_c^{-/-}$ knockout mice all contain dramatically reduced numbers of mature NK cells, suggesting that IL-15 signals may be important for the differentiation of NK cells *in vivo* (9, 75, 77, 145, 149-150). Indeed, IL-15 is expressed by bone marrow (BM) stromal cells and studies with a 3-week culture of CD34$^+$ hematopoietic progenitor cells (HPC) supplemented with IL-15 induced the differentiation of functional CD56$^+$ NK cells in the absence of stroma or other cytokines (9, 143). However, two BM stromal cell factors, ligands for the class III receptor tyrosine kinases (RTK): c-kit ligand (KL) and flt3 ligand (FL) are thought to be important in an early phase to develop the initial phenotype CD34$^+$IL-2/IL-15RβCD56$^-$ that then respond to IL-15; suggesting that those signals are also needed in order to develop mature differentiated CD56^{dim} NK cells with proper NK receptor acquisition (9, 151). Studies with murine models (144, 152) strongly support human models, pointing that IL-15 could indeed serve as physiologic NK cell hematopoietic central factor (9).

2.3.1.2. Mature NK cell homeostasis

IL-15 has been shown to play a dominant role in the survival of peripheral NK cells, being proposed that IL-15 may function as an NK cell survival factor *in vivo* (153-154). Indeed, there is a scarcity in mature NK cells in IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice, and adoptive transfer of mature NK cells into either IL-15$^{-/-}$ or IL-15Rα$^{-/-}$ mice results in the abrupt loss of these cells (124, 145, 155), supporting that IL-15 is critical for **NK cell survival** and indicating that IL-15Rα provides critical survival signals possibly in a IL-15Rα trans-presentation manner (124).

2.3.1.3. Mature NK cell activation, proliferation, cytotoxicity and cytokine production

Besides playing a major role in NK cell development and survival, IL-15 also regulates NK cell functions. Namely, it has been shown to increase **proliferation** and selective expansion of resting NK cells (9, 103, 153, 156-158), to increase the levels of **NK cell cytotoxicity** (103, 156-158), stimulating natural killing. Also it has been shown to stimulate and costimulate NK cell **cytokine** and chemokine production and regulate interactions between macrophages and NK cells. Indeed, it has been described that IL-15 acts in concert with IL-12 to induce the macrophage activating factors IFN-γ and tumor necrosis factor (TNF)-α (103, 159-160) and IL-15 alone appears to be a potent stimulus for GM-CSF production by resting CD56$^+$ human (103, 161) NK cells. Thus, it has been

proposed that in both mice and humans, activated macrophages and NK cells could interact through a paracrine feedback loop, in which macrophage-derived IL-15 contributes with other monokines (especially IL-12) to the proinflammatory cascade leading to innate immune production of cytokines as IFN-γ; while TNF-α production seems to mediate an autocrine NK cell induced apoptosis (159) as an autoprotective mechanism (9). NK cells have also been described to produce the CC **chemokines** macrophage inflammatory protein (MIP)-1α and MIP-1β after stimulation with IL-15, which is increased with the addition of IL-12 (160, 162-163), working at the same time as chemoattractants for NK cells (164) and activators of macrophages (165), potentially increasing macrophage/monocyte and NK cell *cross*-talk in inflammation or infection conditions.

2.3.2. IL-15 plays a role in the development, homeostasis and activation of i-IEL, γδ T cells, and NKT cells

In addition to NK cells, IL-15 has been shown to regulate innate immune T cells, including γδ T cells, intestinal intraepithelial lymphocytes (i-IEL) and NKT cells.

The **γδ T cells** are a diverse population of lymphocytes that have been shown to play an important role in immune regulation (166). Studies using IL-15 and IL-15R knockout mice demonstrated that IL-15 has an important role in dendritic epidermal γδ T cells (DETC) and i-IEL γδ T homeostasis and function (75, 77, 114, 167-168). DETC are a skin-specific member of the γδ T-cell population that migrate to the skin during fetal life in mice (169). The DETC population in skin was absent after treatment with anti–IL-2/15Rβ in utero,(170) yet were present in normal numbers in IL-$2^{-/-}$ mice (171). Furthermore, Vγ3 DETCs are absent in IL-2/15R$\beta^{-/-}$ mice (138) and $\gamma_c^{-/-}$ mice (172). Collectively, these studies suggest that IL-15 is critical to DETC **growth and survival** after activation and may be important in selective localization of these cells in the skin. Additionally, besides having a specific role in DETC or i-IEL γδ T cell (*that will be discussed below*), using a model of lymphopenia-induced homeostatic expansion French JD *et al* (2005) have found that IL-15 play also an important role in general lymphoid **γδ T cell homeostasis** (167).

i-IEL consist of both TCRαβ and TCRγδ T cells located at the basolateral surfaces of the intestinal epithelia and are thought to play a key role in mucosal immunity (173). TCRγδ i-IELs express the CD8αα homodimer, whereas TCRαβ i-IELs express CD8αβ, CD8αα, or CD4 (174). Murine **$CD8\alpha\alpha^+$ i-IELs** are thought to develop **extrathymically** and express the IL-2/15R$\beta\gamma_c$ receptor components, while αβ T cells $CD8\alpha\beta^+$ or $CD4^+$ i-IELs are thought to depend on the thymus for development (175-176). Indeed**,** mice with targeted disruption of the IL-2/15Rβ (139) or γ_c (172) have a dramatic reduction of both TCRγδ and

TCRαβ/CD8αα i-IELs, and a definitive demonstration of the requisite IL-15/IL-15R complex participation in the development of CD8αα i-IELs was provided by mice with targeted disruption of the IL-15Rα and IL-15: IL-15Rα$^{-/-}$ mice had a 2-fold reduction in total i-IELs and a 5- to 10-fold reduction in TCRγδCD8αα i-IELs (75). IL-15$^{-/-}$ mice had a 2-fold decrease in total i-IEL numbers, a 2-fold increase in the ratio of TCRαβ to TCRγδ i-IELs, and a dramatic reduction in TCRαβ CD8αα i-IELs (77). In addition, IL-15 has been shown to stimulate **proliferation, cytotoxicity, and IFN-γ production** by human **TCR γδ IELs** (136). Lai *et al* (1999) suggests that IL-15 plays also a role in **TCR αβ i-IEL survival** in the absence of antigen stimulation, as well as in their **expansion** and survival in the presence of antigen (177). Interestingly, it has been described that IL-15 does not affect IEL development in the thymus, but regulates homeostasis of putative precursors and mature CD8αα^{+} IELs in the intestine (177). And, a recent work has shown that *trans*-presentation of IL-15 by intestinal epithelial cells is completely sufficient to direct the IL-15-mediated **development** of **CD8αα^{+}** T cell populations within the IEL (119).
Thus, IL-15 plays a critical role in the expansion and function of i-IEL and DETC cells, thereby contributing to mucosal immune defense.

IL-15 has been shown to be important for the **expansion, survival, homeostasis and functional maturation** of **NKT cells** (77, 135). Indeed, murine Vα14^{+} NKT cells proliferate in response to IL-15, and their numbers are severely reduced in IL-2/15Rβ$^{-/-}$, IL-15Rα$^{-/-}$, and IL-15$^{-/-}$ mice (75, 77, 140) yet are normal in IL-2$^{-/-}$ mice (140, 178). The few NKT cells present in IL-15$^{-/-}$ mice underwent normal thymic selection, suggesting that IL-15 is important for the **expansion, survival, or functional maturation** of committed NKT cells (77). In fact, IL-15, but not IL-7, was found to be required for maintain the **homeostasis** of peripheral Vα14^{+} NKT cells and that is affected by competition for IL-15, and that IL-15−requiring cells such as NK cells and CD8^{+} memory cells (135, 154). Therefore, NKT cells depend upon IL-15/IL-15R complex-mediated signals for expansion and homeostasis, in contrast to NK cells, which appear to have an absolute requirement for IL-15/IL-15R for development.

It is important to note that those studies referring NKT cell numbers correspond to IL-15 and IL15R knockout mice. However, as it was already described, mice bears mainly Vα14^{+} invariant NKT cells (CD1d-restricted), while human livers are enriched in CD1d-unrestricted NK-like T cells, making thus very difficult to ascertain about the importance of IL-15 to the development and homeostasis of those cells.

2.3.3. IL-15 and αβ T cells

Beside the crucial role in development and homeostasis of innate immune cells, IL-15 has been shown to play a major role in T cells, especially **$CD8^+$ T cells**. Indeed, IL-15 was first identified as a T-cell growth factor through its ability to promote the proliferation of CTLL cells and mitogen stimulated T cells in a fashion similar to that of exogenous IL-2 (70, 72).

2.3.3.1. T cell development

IL-15Rα$^{-/-}$ mice have a selective deficit in $CD8^+$ T-cell numbers in both the thymus and periphery (75), and IL-15$^{-/-}$ mice have reduced numbers of memory-phenotype $CD8^+$ T cells in the spleen and lymph nodes that were reversible upon provision of exogenous IL-15 (77). However, follow-up studies have not revealed an obvious defect in thymic selection (145). Thus, although it is possible that IL-15 signaling may support $CD8^+$ T cell differentiation in the thymus, compelling evidence in this regard is currently lacking (145). Thus, IL-15 may not be a major requisite for the development of $CD8^+$ T cells but may be critical for their **expansion** or **survival** in the periphery (77).

2.3.3.2. Naïve and memory T cell homeostasis and activation

Once mature T cells exit the thymus, they depend on signals from MHC molecules as well as cytokines for their continued survival in the periphery (145, 179). Interestingly, IL-15 *per se* has been shown the ability to induce **survival, activation and proliferation** of both **naïve** and **memory $CD8^+$ T cells** (180-182). Expression of both IL-15Rα and IL-2/15Rβ increases after TCR activation, and heterologous IL-15 can support the **survival** and/or **proliferation of activated T cells** (145, 183)). Stimulation of TCR-engaged T cells with IL-15 has been shown to induce various other activation antigens, such as IL-2Rα, FasL, CD30, TNFRII, CD40L, CD69, (9, 180, 184-185). The *in vitro* proliferation of resting T lymphocytes in response to anti-CD3 plus IL-15 has been shown to be greatly reduced in IL-15Rα$^{-/-}$ lymphocytes, supporting the idea that IL-15 requires induction of the IL-15Rα to optimally **activation** of resting T cells (75). In agreement, IL-15 has also been found to regulate T cell activation during some types of immune responses, namely in vesicular stomatitis virus (VSV) (186-187). One of the key features of **memory T cells** is their ability to survive as lineages of expanded cells in the absence of antigen- or TCR-mediated signals. Memory T cells rely predominantly on IL-2, IL-15, and IL-7 cytokines for their homeostasis during these prolonged periods of quiescence**. IL-15 is a key cytokine for**

homeostatic proliferation and maintenance of memory CD8$^+$ T cells (187-190). Multiple lines of evidence suggest that IL-15 signals are important for maintaining memory CD8$^+$ T cells. The **survival effects of IL-15 on CD8$^+$ naïve and memory CD8$^+$ T cells** are mainly associated with the upregulation of Bcl-2 and Bcl-xL (182, 188). Memory phenotype CD8$^+$ T cells are selectively expanded by heterologous IL-15, consistent with the higher expression levels of IL-2/15Rβ on these cells compared with naïve CD8$^+$ T cells or CD4$^+$ T cells (188); which is in agreement with the fact that **memory phenotype CD8$^+$ T cells are depleted in IL-15^{-}/$^{-}$ mice** and **IL-15Rα^{-}/$^{-}$ mice** (75, 77). In parallel, several studies suggest that, in addition of having an important role in survival, IL-15 appears to play a greater role in supporting **CD8$^+$ T cell proliferation** *in vivo* (123, 186-187, 191). Memory phenotype **CD4$^+$ T cells** express lower levels of IL-2/15Rβ than do memory phenotype CD8$^+$ T cells, and memory phenotype CD4$^+$ T cells are present in normal numbers in IL-15Rα^{-}/$^{-}$ mice, suggesting that IL-15 signals may be less important for the homeostasis of CD4$^+$ T cells than for CD8$^+$ T cells (75, 188). Although, there are studies showing enhanced *in vitro* priming of CD4$^+$ T cells with IL-15 (192-193) and memory CD4$^+$ responsiveness to endogenous IL-15 (194), being suggested that under normal physiological conditions, IL-15 also have a role in homeostasis of CD4+ memory cells, however at a lower level, due to the fewer expression of receptor (194).

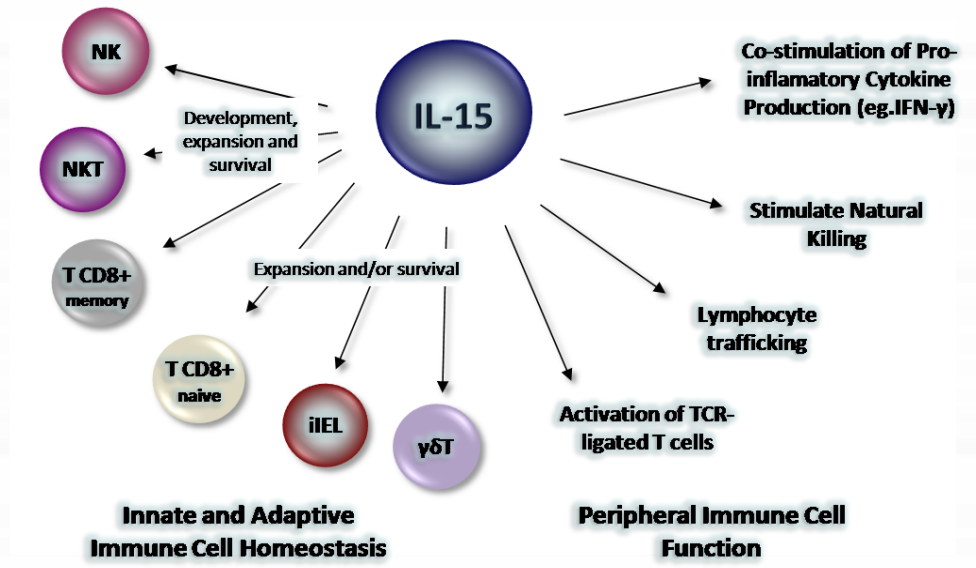


Figure 5. IL-15 is a pleiotropic cytokine that functions in NK cell development, lymphocyte homeostasis, and peripheral immune functions.

IL-15 has a critical role in the development of the NK lineage, as well as in its survival, expansion, and function. Other innate lymphocytes (NKT, CD8αα, i-IEL, TCRγδ DETCs) and memory-phenotype CD8+ T cells depend upon IL-15 for survival or expansion. IL-15 also plays multiple roles in peripheral innate and adaptive immune cell functions. Adapted from (9)

2.3.4. IL-15 and other immune cells

In parallel with the key functions described, it is now recognized that signaling via the IL-15/IL-15R system can also modulate other immune cells.

IL-15 controls secretory activities and **survival** of ***neutrophils*** by inducing IL-1R antagonist, preventing the loss of the anti-apoptotic myeloid cell differentiation factor-1 (Mcl-1) and decreasing activity of caspases- 3 and -8) and ***eosinophils*** by upregulating GM-CSF production and NK-kB activation, and was also shown to enhance **phagocytosis** (195-202).

IL-15 can also serve as a growth factor for **mast cells**, supporting the **proliferation** (203) and **preventing apoptosis** of bone-marrow-derived mast cells and mast cells (204). In addition, IL-15 also induces a STAT-6 dependent mast cell production of IL-4 (205). Interestingly, mast cells reportedly use a novel distinct high affinity receptor (provisionally designated as IL-15RX) and a different signal transduction pathway and do not require IL-2/IL-15Rβγc (203-205).

Monocyte and ***macrophage*** cell lineages have long been known to express and produce IL-15 that can be up-regulated by infection-associated key stimuli like IFN-γ and LPS as well by bacterial, protozoan and viral **infections** (80, 121, 206-208). They also respond to IL-15 stimulation by increasing anti-infection immune responses, namely by increasing IL-12, IL-8 and monocyte chemotactic protein 1 (MCP-1) (209-210). As already described, membrane-bound IL-15 in monocytes is capable of stimulating *in trans* T cells via **juxtacrine signaling** (80, 121) while also capable of inducing **reverse signaling** events in the monocytes (expressing cells), resulting in activation of FAK, Rho, p38, ERK1/2 and expression of proinflammatory cytokines such as TNF-α, IL-6 and IL-8 (110, 122).

Dendritic cells are also known to express both IL-15 and IL-15Rα that can be further upregulated, for example, by IFN-α/-β, double-stranded RNA and LPS (206, 211-212). Moreover, stimulation of DC with IL-15 leads to the **upregulation of co-stimulatory molecules**, MHC class II and MHC class I related chains A or B on the cell surface, **increases IFN-γ release** (81, 211-212) and enhances the ability of DCs to stimulate $CD8^+$ T cell proliferation and differentiation (213-214) and to activate NK cells (215). And it has been found that IL-15 *trans*-presentation by dendritic cells contributed to the development of memory-phenotype $CD8^+$ T cells, supporting a role for DCs in memory $CD8^+$ T-cell homeostasis (118).

Also, the IL-15/IL-15R system seems to be critical for the functional maturation of ***DCs*** and ***macrophages*** in response to microbial infections, namely by controlling IL-12 production (216), and expression of IL-2 by myeloid DCs (217). Furthermore, IL-15 has

been shown to trigger **survival** signals for murine DCs, since they show signals of increased apoptosis in IL-15$^{-/-}$ mice, and both IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice have reduced amounts of DCs in peripheral blood and spleen (101).

Since IL-15 or IL-15Rα knockout mice do not reveal significant abnormalities in ***B cell*** responses (75, 77), it has long been thought that IL-15 plays an irrelevant role in B cell biology. However, this concept is changing and several lines of evidence indicate an essential role of IL-15 in modulating B cells. While exerting no activity on resting B cells, IL-15 was found to **stimulate proliferation of activated B cells** and, in combination with recombinant CD40L, the production and secretion of IgA, IgG1 and IgM is increased (218). In addition, IL-15 induces proliferation of lymphoblasts from patients with B-cell chronic lymphocytic leukemia (219), supports HIV-1-driven polyclonal B cell responses (220) and contributes to tumor propagation in multiple myeloma (221). Moreover, IL-15 greatly **inhibits apoptosis** of activated human B lymphocytes *in vitro* and mouse B lymphocytes *in vivo* (74, 132). Finally, IL-15 has been shown to be biologically active in the membrane-bound form in DCs in **germinal centers** and is thought to **support B cell proliferation and survival** via a cell-to-cell contact dependent mechanism (117).

2.3.5. IL-15 and non-immune cells

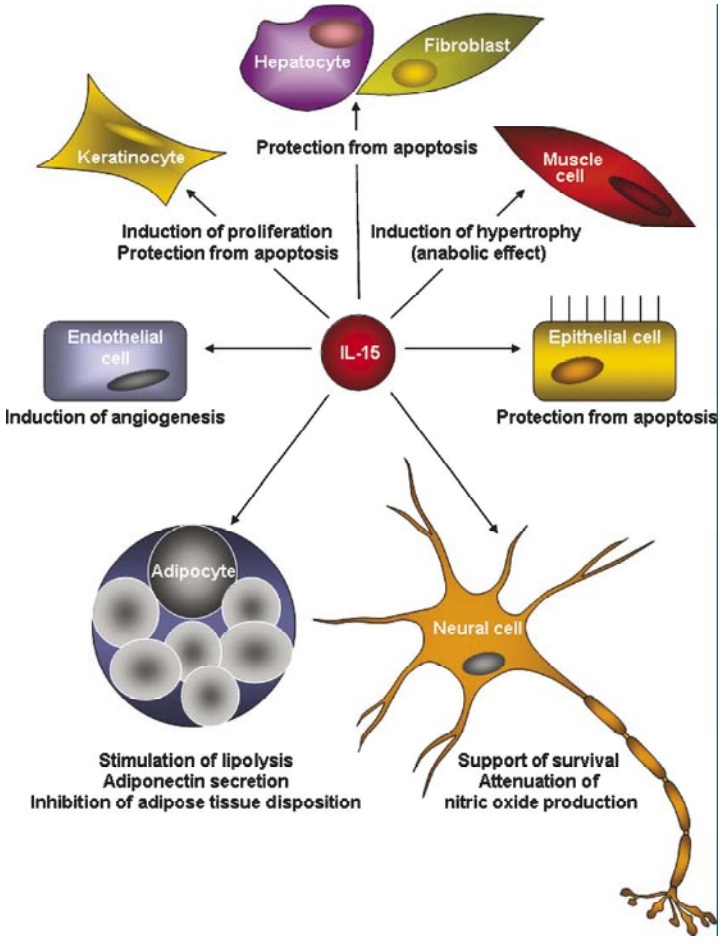
In addition to the broad range of functions that IL-15 exerts towards immune cells, perhaps, one of the most defining functional differences between IL-2 and IL-15 is that IL-15 is increasingly being recognized as a major modulator of many different types of non-immune cells, while the characteristic functions of IL-2 are almost exclusively restricted to T and NK cells (Figure 6).

Fibroblasts from human spleen, as well as gingival and skin fibroblasts constitutively express IL-15 (83, 222-223). In patients with rheumatoid arthritis, fibroblasts expressing elevated levels of IL-15 are considered as a main source of the increased levels of IL-15 in the synovium (224). Interestingly, IL-15 also **inhibits apoptosis** of synovial fibroblasts (225). Spleen derived fibroblasts expressing IL-15 were found to induce NK cell differentiation from CD34^{+} progenitors (222), while dermal fibroblasts expressing membrane-bound IL-15 support proliferation of activated T cells (83). Besides serving a significant source of IL-15, fibroblasts can also be themselves targets of IL-15 that, after binding IL-15Rα, triggers intracellular signaling pathways. Indeed, stimulation of IL-15Rα by IL-15 has shown to induce recruitment of TRAF2 to IL-15Rα, activation of NF-kB (104), and transactivation of Axl receptor tyrosine kinase pathway, which results in the

phosphorylation of PI3K/Akt and upregulation of Bcl-x_L and Bcl-2 (226), leading namely in **apoptosis inhibition** (226).

Endothelial cells, namely human umbilical cord blood endothelial cells and human intestinal microvascular endothelial cells, are capable to express IL-15 and IL-15Rα mRNA (227-228). IL-15 was found to **stimulate angiogenesis** *in vivo* and *in vitro* (225, 228). IL-15 induces hyaluronan expression by endothelial cells, important for T-cell adhesion, recruitment and promotion of extravasion of activated T cells (229). Transendothelial migration of T cells is also increased by IL-15 induced binding capacity of LFA-1 integrin (230). Therefore, IL-15 has both direct effects on endothelial cell biology and angiogenesis and promotes lymphocyte extravasion.

IL-15 is highly expressed in **skeletal muscle** at mRNA level (72). Nieman *et al* (2003) have further shown that IL-15 is one of the most abundantly expressed cytokines at the mRNA level in human muscle (231). Adding to this, several studies have shown that IL-15 has an **anabolic effect** on muscle cell culture and decreases the muscle degradation rate in a cachexia model, suggesting that IL-15 might be of importance in muscle growth (86, 232-234).



Interestingly, IL-15 also seems to play a role in reducing adipose tissue (235). This anti-adipose effect was found to depend on the expression of IL-15Rα on **adipocytes** (236). A role for IL-15 in muscle-fat *cross*-talk has been hypothesized and some evidences suggest that IL-15 functions in modulating fat through a muscle-to-fat endocrine axis (237).

Figure 6. IL-15 functions in non immune cells

IL-15-mediated effects in different non-immune cell types (7).

IL-15 and all three receptor subunits of the IL-15R are also widely expressed in the central nervous system (e.g., neurons, microglia, astrocytes) and by **neuronal** cell lines (84, 238-239). It has shown a role in supporting microglial cell **growth** and attenuation of their nitric oxide production (238). Also, recently, IL-15 was shown to play a role in neural cell differentiation from neuronal stem cells through a signal transduction pathway involving IL-15Rα and STAT3 (240).

IL-15 can also be expressed by **epithelial cells**, namely from intestine, kidney, liver and keratinocytes. Human **keratinocytes** were found to express both IL-15 and IL-15Rα protein on the cell surface. IL-15 was found to inhibit both anti-Fas and methylcellulose-induced keratinocyte apoptosis *in vitro* (82, 241) and also to induce their proliferation (241). **Renal tubular epithelial cells** (TECs) were also found to express IL-15 that seems to protect them from apoptosis (85) and there is a report showing the ability for TEC derived IL-15 to stimulate intratubular $CD8^+$ T cells (242). Also, it was shown that membrane-bound IL-15 on renal tumor cells is capable of rescuing NK cells from IL-2 starvation-induced apoptosis in a juxtacrine manner (243). **Intestinal epithelial cells** also express IL-15 that was shown to induce their proliferation (87) and protect them from apoptosis (244). As well, as above described, a recent work has show IL-15 *trans*-presentation by intestinal epithelial cells being capable of mediating **development of $CD8\alpha\alpha^+$** T cell populations within the IEL (119). Interestingly, IL-15 expression by **hepatocytes** was also described at mRNA level (245).

All the aforementioned data strongly points to IL-15 as a strong candidate playing a role in tissue-associated T lymphocyte physiology. Interestingly, as described, IL-15 has been shown to be constitutively expressed in the liver, namely by hepatocytes. Taking into consideration the distinctive lymphocyte composition of the human liver, together with the described biological functions of IL-15 (see Figure 7), it is tempting to speculate that liver IL-15 could play a key role in the development, differentiation and homeostasis of some of the intrahepatic lymphocyte populations described, namely $CD8^+$ T cells.

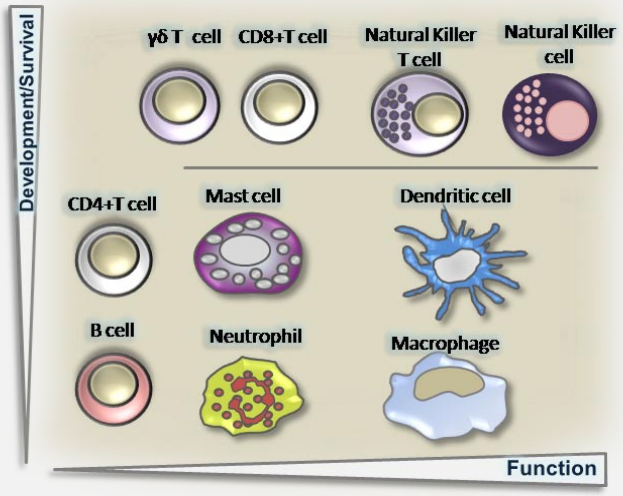


Figure 7. IL-15 effect in the development/ survival and function of the different leukocyte subsets

Schematic representation of IL-15 effects in the development/ survival and functions of the different leukocyte cell populations. The most favored populations correspond to the lymphocyte subsets mainly enriched in human livers: NK, NKT, memory CD8+ T cells and γδ T cells.

In this regard, one major feature of the impact that IL-15 has on human $CD8^+$ T cells is the loss of CD28 expression (181, 246-248). $CD8^+CD28^-$ T cells are a prevalent T cell subset that often co-expresses HLA class I-specific inhibitory receptors such as CD94/NKG2A (249-250). Despite their presence in circulation and epithelial tissues such as the intestine (251-252) and liver (31, 35, 253), the origin of $CD8^+$ T cells expressing NK receptors still remains uncertain. In the next section, an overview of the structure, expression and function of NK receptors will be presented.

3. NK receptors

For many years, since the early 1990s, it was thought that NK function was exclusively controlled by inhibitory mechanisms and killing was considered as a default result of the absence of inhibitory signals: “missing self hypothesis” (254-264). This initial view that MHC plays a decisive role in NK–target cell interactions cannot however explain why NK cells spare autologous cells lacking MHC class I expression (such as human erythrocytes) or kill certain MHC class I-sufficient tumor cells (265). The subsequent identification of various activating (or stimulatory) NK cell receptors, that are able to directly induce cytotoxicity and cytokine production upon engagement with ligands in target cells, has led to modification of the missing-self recognition model. It is now clear that NK cell function is strictly **regulated by a balance between positive and negative signals provided by the triggering of a diverse array of cell-surface activating or inhibitory receptors expressed at NK cell surface**. Thus, normal cells express NK-inhibiting MHC class I molecules and no or few activating NK cell receptor ligands, leaving NK cells quiescent. In contrast, transformed or infected cells stimulate NK cell responses by increased expression of activating NK cell receptor ligands and a reduced MHC class I outfit (265).

3.1. NK receptors function: inhibitory and activating

As referred above, NK receptors can be divided in inhibitory or activating accordingly to their function (Figure 8). And is the balance between negative and positive signals resultant from their binding to the correspondent ligands present in the target cell that will dictate NK cell function.

Although the extracellular domains of NK cell **inhibitory receptors** are diverse, the intracytoplasmic signaling motifs of these transmembrane receptors are remarkably similar. The cytoplasmic tails have a conserved sequence of amino acids (S/I/V/LxYxxI/V/L), known as immunoreceptor tyrosine-based inhibitory motifs (**ITIM**). (266-270). Upon ligand binding to inhibitory receptors, the ITIM become tyrosine phosphorylated (the binding activates Src family kinases that phosphorylate tyrosine residues) leading to the recruitment of intracellular phosphatases such as the tyrosine phosphatases SHP-1 and SHP-2, or the inositolphosphatase known as SHIP (268). **These tyrosine phosphatases are able to dephosphorylate protein substrates of tyrosine kinase linked to activating NK cell receptors** (271). This would imply that there is coaggregation of activating and inhibitory receptors (270, 272-273). This way,

ITIMs provide docking sites for phosphatases that oppose the activity of tyrosine kinases, enzymes that are essential for NK cell activation, leading to NK cell inhibition (274).

Activating, or stimulatory, receptors are characterized by short cytoplasmic domains lacking signal transduction elements. Instead, and although there is a large range of activating receptors, several of them share signaling pathways through their association with common transmembrane adaptor proteins such as **CD3ζ**, **FcεRγ** and **DAP12** (Figure 8). These adaptor proteins contain cytoplasmic tails with immunoreceptor tyrosine-based activation motifs (**ITAMs**). This motif contains a tyrosine separated from a leucine by any two other amino acids, giving the signature **YxxL**. Receptor-ligand binding leads to phosphorylation of tyrosine residues within these motifs. This in turn leads to recruitment of protein tyrosine kinases of the **Syk** family, such as Syk and ZAP70, and subsequent initiation of downstream signalling pathways through Vav, Rac, PAK1, MEK and ERK (275). **DAP10** is a different transmembrane adaptor molecule, which lacks ITAMs. Instead its intracellular signalling domain contains a **YXXM** motif, which may lead to the associations of DAP10 with phosphatidylinositol 3 kinase (PI3K) or the adaptor molecule grb2 as opposed to Syk or ZAP-70 (276).

3.2. Inhibitory and activating NK receptors: structure, ligands and function

3.2.1. Inhibitory receptors: KIR and C-type lectin-like CD94/NKG2A heterodimer

As described above, NK cells express a repertoire of inhibitory receptors that regulate their activation (Figure 8). The ligands for inhibitory NK receptors mainly belong to the MHC class I family. Several inhibitory receptors for MHC class I have been identified. These receptors include principally (i) the inhibitory **killer cell immunoglobulin (Ig)-like receptors** (KIR) that generally bind to classical MHC class Ia molecules (human leukocyte antigen (HLA)-A, -B, and –C), with the exception to 2DL4 that bind the non-classical HLA-G; and (ii) the inhibitory **CD94/NKG2A** (Natural killer 2A) heterodimeric C-type lectin-like receptors that bind to the nonclassical MHC class Ib (HLA-E) (264, 274). In addition to these key receptors, NK cells can also be inhibited by members of the leukocyte immunoglobulin (Ig)-like receptor/ Ig-like transcript (**LIR/ILT**) family, specifically, LILRB1 (LIR-1/ILT2), which binds a broad range of MHC class I allotypes, LAIR-1, Siglec 7 and KLRG1/MAFA. In the unusual situations of X-linked lymphoproliferative disease and TAP deficiency, NK cells can be inhibited by CD244 (2B4) and the homoypic interaction of

carcinoembryonic antigen-related cell adhesion molecule (CEACAM), respectively (Table II) (270).

Table II. Inhibitory receptors and their ligands (adapted from (270))

	Receptor	Ligand
KIR family	2DL1	Group 2 HLA-C
	2DL2/3	Group 1 HLA-C
	2DL5	Unknown
	3DL1	$Bw4^+$ HLA-B
	3DL2	HLA-A3/A11
C-type lectin-like receptors	CD94/NKG2A	HLA-E
	NKR-P1A	LLT1
LIR/ILT family	LILRB1/ILT2/LIR1	HLA-A, -B, -C
Others	LAIR1	Collagen
	Siglec-7	Sialic acid
	KLRG-1/MAFA	Cadherins
	CEACAM1	CEACAM1

3.2.1.1. Killer cell immunoglobulin-like receptors (KIR)

KIR comprise a group of polymorphic molecules that consist of **both inhibitory and activating receptors** (*that will be discussed further on*) (270, 277) (see table II and III and Figure 8).

The KIR receptors were originally defined serologically in the 1990s, and functional specificity preceded molecular characterization of the receptors (256). KIR are type I trasmembrane glycoproteins with two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like domains in the extracellular portion of the molecule. In general, the inhibitory KIR have a long (L) cytoplasmic tail (KIR2DL and KIR3DL) that contain ITIM sequences, while the activating KIR (KIR-S) contain a short (S) cytoplasmic tail (KIR2DS and KIR3DS) that lack ITIMs but contain a charged lysine residue in the transmembrane portion. The positively charged amino acid is required for pairing with the ITAM containing adaptor molecule DAP12, which is required for signaling (270, 277-278). KIR2DL4 is an exception that will be further described.

Individual KIR bind to classical MHC class I molecules, recognizing different allelic groups of **HLA-A, -B or -C molecules** (279-280). In a very general way, the KIR members with two extracellular Ig-like domains (i.e., KIR2D) recognize HLA-C, while the ones with three extracellular Ig-like domais (i.e., KIR3D) binds to HLA-B and HLA-A (see Table II and III).

The KIR show extensive genetic, expression and functional diversity that can impact NK cells at many different levels .The KIR gene cluster on chromosome 19 contains up to **17 KIR genes or pseudogenes**. There is substantial diversity of KIR at both locus and allelic levels (281-283). Furthermore, the KIR are clonally expressed in a stochastic fashion to generate a repertoire of NK cells expressing different combinations of KIR in the same individual (283-285). Thus, there is a varied pattern of KIR expression that allows a diverse NK cell repertoire capable of sensing minute changes in MHC class I expression (286-287).

An exception to this general classification is **KIR2DL4**. KIR2DL4 is an evolutionarily conserved, framework member of the KIR gene family that is unique among KIR in its genomic organization, protein structure, and function. (i) Unlike other KIR family members, the KIR2DL4 gene is found in almost all KIR haplotypes. The mRNA of KIR2DL4 is expressed in every NK cell, in contrast to all other KIR that are expressed in overlapping subsets of NK cells (283-284, 288-289); (ii) KIR2DL4 binds to **HLA-G** (288, 290), a nonclassical HLA class I molecule expressed by fetal-derived trophoblast cells that invade the maternal decidua (291-292), by activated monocytes (293-294), by thymic epithelial cells (295), and by certain tumor cells (296); (iii) KIR2DL4 carries a single ITIM (the tyrosine of the second ITIM is substituted by a cysteine residue); and (iv) in addition to the presence of an inhibitory motif, KIR2DL4 contains a charged residue in its transmembrane region (297) (see Figure 8). These two properties may explain earlier observations on the function of KIR2DL4. Indeed, both inhibitory (291) and activating (298) functions have been described for KIR2DL4. (v) The activating functions of KIR2DL4 depend on the transmembrane association with the adaptor molecule **FcεRγ** instead of DAP12, contrarily to other activating receptors (299); however, it has also been shown that some KIR2DL4-mediated activation signals are independent of the association with FcεRγ (300). Besides having inhibitory and activation potential, engagement of KIR2DL4 has been pointed to result in activation despite the inhibitory potential conferred by the presence of an ITIM in its cytoplasmic tail (289, 298, 301). This is manifested by the potent production of IFN-γ, as well as of other cytokines, indicating it mostly as an activating receptor with inhibitory potential than the opposite, leading however to weak or absent cytotoxic activity (298, 300-302). However, the very low cell surface expression of KIR2DL4 (301, 303) has been difficult to reconcile with the functional outcome associated with this receptor (302).

3.2.1.2. C-type lectin-like receptor CD94/NKG2A

CD94/NKG2A is a heterodimeric inhibitory receptor consisting of two type II transmembrane glycoprotein C-type lectin subunits, CD94 and NKG2A, linked by disulphide bonds (304-306). The CD94 glycoprotein also forms heterodimers with other distinct members of the NKG2 family *(as will be discussed below)*.

In contrast to KIR, **CD94/NKG2A** is a broad detector of MHC class I expression, as CD94/NKG2A binds to the nonclassical MHC class Ib molecules **HLA-E**. The groove within the HLA-E molecule actually holds leader peptides that are derived from both classical and nonclassical HLA types (306-307). In this way, NK cells survey HLA-E-expressing target cells using their inhibitory CD94-NKG2A as sensor to assess the net overall target cell expression of total MHC class I (307). Unlike HLA-A, -B and -C, HLA-E is relatively non-polymorphic, as is its receptor CD94/NKG2A. Overall the binding of CD94/NKG2A to HLA-E is dominated by the CD94 moiety (308).

The C-type lectin-like receptor NKR-P1A (CD161) is another inhibitory NK receptor. However it does not interacts with MHC class I molecules but with non-MHC-encoded self-surface molecules, lectin-like transcript-1 (LLT1) (309-310) (see table II).

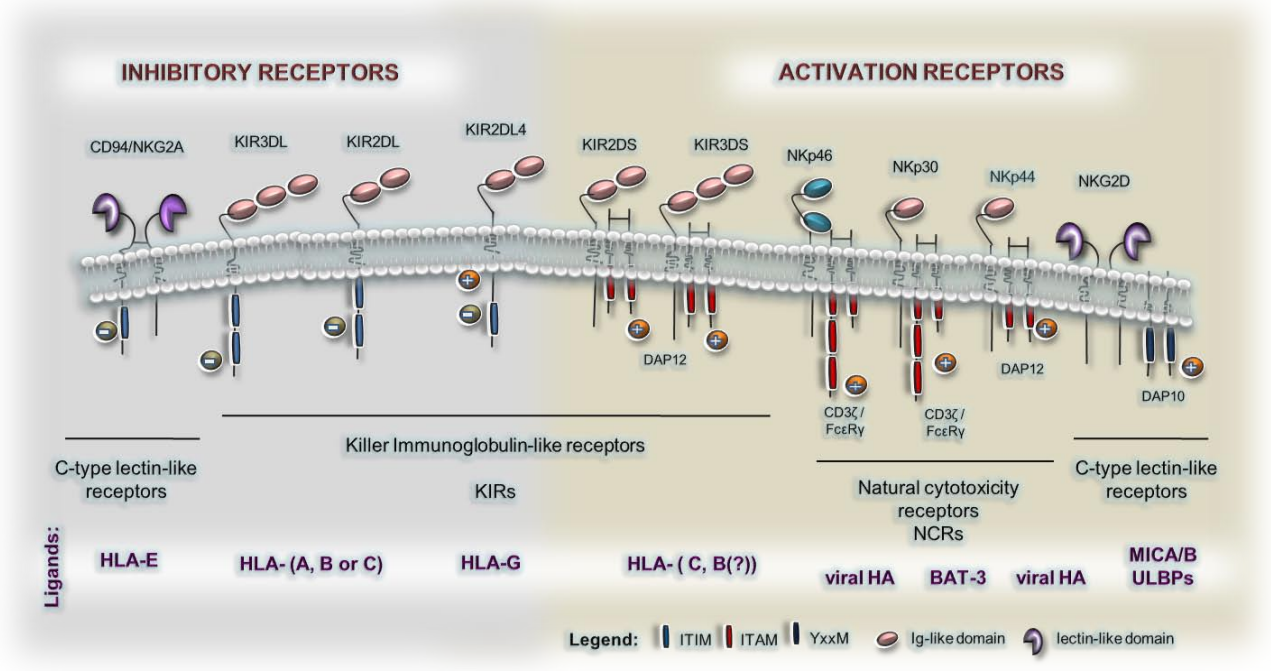


Figure 8. NK receptors

Schematic representation of different inhibitory and activating NK receptors: KIR, C-type lectin-like, NCR; with KIR2DL4 settling in the interface between inhibitory and activating. The different known cell ligands are also shown in the bottom.

3.2.2. Activating receptors: NKG2D, NCR, activating KIR and CD94/ NKG2C/E

In comparison to the inhibitory receptors there are many more activating receptors expressed by each NK cell. When a critical threshold of activating signaling exceeds the counterbalancing influence of the inhibitory receptors, NK cells will mount an effector response (270, 274).

While inhibitory KIR represent the large majority within inhibitory NKR, it appears that the activating KIR (KIR2DS and KIR3DS) represent a relatively minor component of the activating NK cell repertoire. Instead there are a number of different receptors. These receptors are listed in Table III. In humans, major activating NK receptors are the C-type lectin-like receptor NKG2D and the natural cytotoxicity receptors (NCR), which include NKp30, NKp44 and NKp46. Besides (i) NKG2D, (ii) NCR and (iii) activating KIR there are also other activating receptors that includes C-type lectin-like receptors that form heterodimers with CD94 (CD94/NKG2C, CD94/NKG2E), the low-affinity immunoglobulin G receptor CD16 and CD244, which may have both activating and inhibitory functions (270, 311) (see table III and Figure 8).

Table III. Activating receptors and their ligands (adapted from (270))

	Receptor	Ligand
Natural cytotoxicity receptors	NKp30	BAT-3
	NKp44	Viral haemagglutinin
	NKp46	Viral haemagglutinin
C-type lectin-like receptors	NKG2D	MIC-A/B, ULBPs
	CD94/NKG2C	HLA-E
	CD94/NKG2E	HLA-E
KIR family	2DS1	Group 2 HLA-C (?)
	2DS2	Group 1 HLA-C (?)
	3DS1	$Bw4^+$ HLA-B?
	2DS3	Unknown
	2DS4	HLA-Cw4
	2DS5	Unknown
	2DL4	HLA-G
Others	CD244 (2B4)	CD28
	CD16	IgG
	CD266 (DNAM-1)	CD112, CD155
	CRACC	CRACC
	NTB-A	NTB-A

3.2.2.1. NKG2D

The NKG2D receptor belongs to the C-type lectin-like family of receptors and is one of the best characterized stimulatory receptors expressed on both human and mouse NK cells. NKG2D differs from the other NKG2 proteins because it (i) is present as a homodimeric receptor (312), (ii) shares only approximately 20% amino acid identity with the other NKG2 proteins (313) and (iii) does not bind to Qa1b/HLA-E (314-315). Instead, it recognizes the MHC class I-like stress-induced self ligands: MICA and MICB, and UL16 binding proteins (ULBP) in humans (314, 316-320). **MICA/ B and ULBP** molecules share structural similarities to MHC class I molecules. In particular, MICA/ B alleles are highly polymorphic and may vary in their affinity for NKG2D (270). These ligands have highly restricted expression on healthy cells (known only to be expressed in intestinal epithelial cells and thymic epithelium Instead, they are only up-regulated by cellular stress, tumour transformation and viral infection (317, 321-323).

In contrast to other stimulatory receptors, NKG2D is expressed on all NK cells. **In addition, and of note, it is also expressed on the majority of $CD8^+$ T cells and γδ T cells, contrarily to other NKR that can only be expressed in a very small percentage of those cells** (314, 319, 324). NKG2D has two charged amino acids in its transmembrane domain that allows association with the signaling adaptors, DAP10 or DAP12 (314, 325-326). In mouse, alternative splicing of NKG2D NK cells results in two distinct isoforms: NKG2D-short (S) and -long (L). The NKG2D-S can associate with both DAP10 and DAP12, whereas NKG2D-L associates only with DAP10 (314). Freshly isolated naïve NK cells express only the NKG2D-L isoform that associates with DAP10. Activated NK cells express both NKG2D isoforms and can therefore associate with both adapters (314). However, in human NK cells, there are no evidence for physical association of NKG2D and DAP12; accordingly, DAP12 is not tyrosine phosphorylated upon NKG2D *cross*linking (325, 327-328). Although activated $CD8^+$ T cells express both NKG2D isoforms, they lack expression of DAP12; consequently, NKG2D in $CD8^+$ T cells can mediate costimulation only via DAP10 (314, 326). Thus, in humans **DAP10** is thought to be the adaptor molecule for NKG2D signaling.

3.2.2.2. Natural cytotoxic receptors (NCR)

The natural cytotoxic receptors (NCR) belong to the Ig-superfamily and contain a charged aminoacid in their transmembrane domain, which associates with ITAM-bearing adaptor

molecules (329). Because NK cells have long been known to readily lyse MHC-deficient tumor cells, the existence of NCR that must recognize non-MHC molecules was suspected (330). Indeed, NCR show a non MHC-restricted natural cytotoxicity, recognizing non-MHC molecules, fact that was in the origin of their name. Both NKp46 and NKp30 are described to be exclusively expressed **only on NK cells**, activated and resting, while NKp44 was shown to be upregulated after IL-2 activation and also reported on plasmocytoid dendritic cells (331). Thus, recently NKp46 and NKp30 were described as the only NK specific markers known so far (329). A most distinctive role of the NCR in NK cell activity has been attributed to their involvement in recognition and killing of tumor cells. This has become evident by the ability to NCR monoclonal antibodies to block NK-mediated killing of many tumor cell lines (332-336) and by the strict correlation that exists between the density of NCR expression on NK cells and their ability to kill tumor targets (336).

NKp46 was the first NCR to be identified (333, 335, 337). Analysis of the tissue distribution of NKp46 revealed that it is expressed on both activated and resting NK cells but it is was not found on other cell types tested (329). The activation of NKp46 is mediated through association with the adaptor molecules CD3ζ and FcεRγ that contain the activation ITAM motif (333-334, 338). In humans, NKp46 is a 46kDa glycoprotein that contains two Ig-like extracellular domains (333) (see Figure 8). While the cellular ligands of NKp46 remain unknown, a recent report indicates that heparin sulfate might be involved in the recognition of tumor cells by NKp46 and NKp30 (339). However, since heparin sulfate is also found on normal cells, it is not likely to represent a specific activating ligand of NKp46 or NKp30, but may act as a co-receptor that enhance the cytotoxic activities of these receptors (329). NKp46 is also involved in the killing of virally infected cells, and it was shown that the **hemagglutinin (HA) molecules** of different influenza strains serve as a specific functional ligand for NKp46 (340-341) and NKp44 (342). Since many viral families (at least seven) express HA, this mechanism may explain how NK cell can kill a broad range of viruses using a limited repertoire of lysis receptors (329).

NKp44 was the second identified NCR on human NK cells. It encodes a 44kDa surface glycoprotein. In NK cells, both expression and activation of NKp44 depends on the adaptor molecule **DAP12** (334, 343) (see Figure 8). Unlike other NCR, NKp44 is not expressed on resting NK cells, but requires (at least *in vitro*) IL-2 activation in order to be upregulated. After IL-2 stimulation, all NK clones were reported to express the NKp44 protein (332, 334). As described above, like NKp46 very little information is available

regarding the cellular ligands of NKp44, being the **hemagglutinin (HA)** the only known ligand identified so far (329, 342).

NKp30 receptor, the third identified NCR is, like NKp46, expressed on all resting as well as activated NK cells. The activation of this receptor implies association with the adaptor molecule **CD3ζ** (332). As mentioned above, similar to NKp46, NKp30 was suggested to interact with heparin sulfate as a co-receptor (339). However, NKp30 and NKp46 are most likely to recognize different cellular ligands, based on the differential susceptibility of targets to these two receptors (344). While the cellular ligands of NKp30 remain unknown, it has been shown that the HCMV tegument protein, pp65, functionally interacts with NKp30 (345) and BAT3 has been pointed as a potential soluble ligand (346).

Interestingly, it has been suggested that a functional *cross*-talk exists between the three NCR since engagement of a single NCR leads to the activation of the signaling cascade associated with the others (347), and simultaneous blocking of both NKp44 and NKp46 led to significantly increased inhibition (332, 334).

3.2.2.3. Activating KIR (KIR-S)

As previously described, the KIR family also has activating members (KIR2DS and KIR3DS) that have a short cytoplasmic tail lacking an ITIM motif, and a charged lysine residue in the transmembrane domain conferring association with the immunoreceptor tyrosine based activation motif-containing **DAP12** signaling adaptor (287, 348-350). They are listed in table III and include KIR2DL4, since it have been described to have a preferential activation function as aforementioned (although does not trigger cytotoxicity). And, contrarily to their inhibitory counterpart, the activating KIR (KIR-S) represent a relatively minor component of the activating NK cell repertoire and the biological purpose of activating KIR is not completely understood.

Despite their homology to inhibitory HLA class I-binding KIR, and although some studies point to HLA-A, B and C as ligands of activating KIR (351), very few studies have been able to document binding of activating KIR to HLA class I molecules. Because of very weak, or sometimes possibly nonexistent, binding of activating KIR receptors to MHC class I molecules, a number of roles have been suggested involving MHC or non MHC ligands (352). As such, activating KIR could recognize alternative forms of classical HLA class I, differing from their normal counterparts by alternative folding or in the peptides

presented (353). A high level of HLA class I expression may be necessary for KIR-S function, occurring under conditions of IFN-γ production associated with infections. It is also possible that KIR-S bind non-HLA class I molecules either expressed by stressed self cells or encoded by pathogens. Roles for KIR-S involving alternative-MHC or non- MHC ligands are supported by some examples. A non-MHC ligand for one KIR-S molecule has been reported on melanoma cells (354). The roles and functions of activating KIR receptors still seem to pose a perplexing question in the understanding of NK cell biology.

3.2.2.4. Stimulatory CD94/NKG2 receptors

The stimulatory isoforms of the CD94/NKG2 receptor family are the **CD94/NKG2C** and **E** receptors (355). Both receptors recognize and bind to the nonclassical MHC class I molecules **HLA-E** in humans (356). The concept that both stimulatory and inhibitory NK cell receptors bind to the same ligand may be confusing; however, **the inhibitory CD94/NKG2A receptor was shown to have higher binding affinity for HLA-E than the stimulatory CD94/NKG2C** receptor, thereby favoring inhibition. Moreover, there seems to be a direct correlation between the binding affinity of the peptide–HLA-E complexes for CD94/NKG2 receptors and the triggering of a response by the NK cell (324, 357).

4. NK receptors and CD8+ T cells: how and why

Evidence accumulated during the last two decades has shown that NK receptors can also be expressed by T cells. In the 90´s it was initially found that minor subsets of T cells could express KIR (358-359), shown to be expressed mostly by $CD8^+$ T cells (249) either TCRαβ or TCRγδ. Together with KIR, CD94/NKG2A was also found to be expressed in $CD8^+$ T cells in early studies (359) both conferring T cells the ability to recognize HLA class I molecules on target cells leading to a decrease in CTL cytotoxicity (249, 359). In the last years, also activating receptors have been described to be expressed in T cells. Within them, NKG2D receptor has been found to be normally expressed in almost all $CD8^+$ T cells, contrarily to the other NKR (319, 360). Indeed, it is now emerging the concept that NKR triggering in $CD8^+$ T cells could modulate T cell function, by increasing or decreasing TRC threshold accordingly to the triggering of activating or inhibitory receptors present on the cell surface. Interestingly, some studies suggest that NK receptors can be expressed during the differentiation of $CD8^+TCR\alpha\beta^+$ cells into effector and memory cells (361-363). However, although the knowledge of NK receptor expression by CD8+ T cells has been described in the literature, the factors necessary for their

induction remain largely unknown. Interestingly, the analysis of T cell clones sharing identical TCR gene rearrangements and diverse KIR phenotype unambiguously demonstrated that the acquisition of KIR expression occurs after rearrangement at the TCR locus (364-366). Indeed, NKR induction in T cells has been mainly associated with antigen-specific expansion and, remarkably, it has been suggested that IL-15 could have a role in the induction of CD94/NKG2A in $CD8^+$ T cells, in the context of antigen stimulation (367-368).

5. AIMS OF THE THESIS

Thus, the particular characteristics of the liver provide a distinctive capacity for hepatocytes to interact strongly with T cells, which is unique between solid organs. However, a possible role of hepatocytes in interacting with lymphocytes in the absence of antigen presentation has never been dissected. Moreover, the lymphocyte composition of normal adult human liver is dominated by rapid-acting innate cells that are characterized by the expression of natural killer (NK) cell receptors, containing the richest population of CD8+ T cells expressing CD56 marker and other NK receptors – NK-like T cells. Thus, it is plausible to think that the hepatic microenvironment, namely the cytokine milieu, should be favorable to the homeostasis of intrahepatic lymphocyte population. IL-15 has been shown to be constitutively expressed in the liver; is known to play a crucial role in the development and/or homeostasis of the cells that are mainly enriched in the liver; and, as aforesaid, pointed to have a possible role in regulating NKR in CD8+ T cells, making interesting the possibility that this cytokine play a role in this context.

Based on the integration of the aforementioned data, the aims of this thesis were:

Aim#1. To delineate the role that hepatocytes and IL-15 might have in T cell homeostasis, by the study of T cell survival, activation and proliferation parameters after *in vitro* interaction with hepatocytes and/or IL-15.

Aim#2. To examine the impact that IL-15 could have on CD8+ T cell differentiation, examining a putative NK receptor expression on purified CD8+CD56- T cells after *in vitro* cultures with IL-15.

Aim#3. To perform *in vitro* phenotypic and functional studies of the IL-15-cultured CD8+ T cells, namely dissecting cytotoxicity and cytokine secretion after NK receptor crosslinking.

6. References

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II – RESEARCH WORK

1. Hepatocytes and IL-15: a favorable microenvironment for T cell survival and CD8+ T cell differentiation

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1.1. Abstract

Human intrahepatic lymphocytes (IHL) are enriched in CD1d-unrestricted T cells co-expressing NK receptors (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. Interleukin (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. On the other hand, IL-15 was capable of inducing antigen (Ag)-independent upregulation of NKR, including NKG2A, Ig-like receptors (KIR), 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 hypothesized that the increased percentage of intrahepatic “non-classical” NKT cells could be in part due to a local $CD8^{+}$ T cell differentiation.

1.2. Introduction

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 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 (IHL) have a distinctive phenotypic composition compared to blood and other organs. They are enriched in NK and NKT cells and the CD4/CD8 ratio shows a skewing towards 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 mouse, human livers contain few CD1d-restricted NKT cells; instead they are enriched for $CD3^+CD56^+$ NKT cells co-expressing an oligoclonal TCR and NK receptors (NKR) such as CD94/NKG2 and KIR (16, 17), making even more intriguing the origin of this "non-classical" NKT cell population. It has been proposed that the unique hepatic microenvironment, either through interactions with epithelial cells or local cytokines, may shape the development of this distinctive intrahepatic population (11, 13, 18).

Interleukin-15 (IL-15) is a gamma-common cytokine that has been reported to be present constitutively in the hepatic microenvironment (13, 19). Upregulation 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 promote activation, survival and differentiation of both memory and naïve $CD8^+$ T cells (21, 23-25). Interestingly, some studies suggest that NK receptors can be expressed during the differentiation of $CD8^+TCR\alpha\beta^+$ cells into effector and memory cells (26, 27). Although the factors necessary for their induction remain uncertain, some studies suggest that IL-15, in the context of antigen stimulation, could upregulate NKG2A expression in $CD8^+$ T cells (28), 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 non-tumoral) 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 cells, both through the induction of cell survival (IL-15 and hepatocytes) and NK receptors (IL-15). The likelihood that local differentiation of $CD8^+$ T cells takes place within the liver microenvironment deserves further investigations.

1.3. Material and Methods

1.3.1. Reagents and monoclonal antibodies

RPMI-1640 GlutaMAX®, MEM GlutaMAX®, DMEM-F12 (w/ L-Glutamine+15mM HEPES), bovine insulin, dexamethasone, trypsin/EDTA, Fetal Bovine Serum (FBS) and antibiotic/antimycotic (100x) solution (Amphotericin B/Penicillin/Streptomycin, thereafter APS) were from Gibco BRL (Paisley, Scotland). 5-(and –6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Annexin V - Alexa488 were purchased from Molecular Probes (Amsterdam, The Netherlands). Human serum was obtained from Cambrex (New Jersey, USA). Recombinant human IL-15 was obtained from R&D Systems (Minneapolis, USA). Propidium iodide (PI) was obtained from Sigma-Aldrich (Madrid, Spain). The used monoclonal antibodies (mAb) are listed in Table I.

1.3.2. Cell lines

The HepG2 and HeLa cell lines were obtained from European Collection of Cell Cultures (ECACC, Wiltshire, UK) 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 (ATCC, LGC Standards, Middlesex, UK) and maintained in RPMI-1640 Glutamax® supplemented with 10% FBSi and 1% APS solution. Immortalized Human Hepatocytes (IHH, clone 10.3) were kindly provided by Dr. Nguyen and maintained in DMEM-F12 as described (29). Cells (0.5×10^6) were placed in 75 cm^2 flasks (Nunc, Roskilde, Denmark) and incubated at 37 ºC, 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 37ºC. After harvesting, cells were washed with PBS 1x and resuspended in the corresponding medium. For use in co-cultures, hepatocyte cell lines were trypsinized as described and placed at $0.5x10^6$ cells/well in 24 well plates overnight to recover. Afterward, cells were harvested, irradiated (10000 rad) and washed before co-culture.

1.3.3. Isolation of Peripheral Blood Lymphocytes and T cell subsets

Fresh peripheral blood mononuclear cells (PBMC) or cord blood were obtained from buffy coats after centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Contaminating red blood cells were lysed in lysis solution (10 mM Tris, 150 mM NH4Cl, pH 7.4), 10 min at 37

ºC. Partially purified peripheral blood lymphocytes (PBL) were obtained by routine overnight culture. T cells were enriched by rosetting with sheep red blood cells (ProBiologica, Lisboa, Portugal) 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, Bergisch-Gladbach, Germany). 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.

1.3.4. Culture conditions

For co-culture 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. Co-cultures 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, Roskilde, Denmark) in a final volume 1ml of RPMI supplemented with 3% human serum and 1% APS solution, at 37ºC, 5% CO_2, 95% humidity. IL-15 was added at a final concentration of 10ng/ml. In some experiments, co-cultures were performed in the absence of cell contact using either cell culture inserts (0.2 µm of pore diameter, Nunc, Roskilde, Denmark) or conditioned media (CM) from overnight cultures of HepG2 or IHH. Supernatants were centrifuged twice prior to addition to PBL cultures. For blocking experiments, HepG2 cells were first pre-incubated for 1 hour at 4 ºC with saturating amounts of anti-IL-15 (clone MAB2471) or the respective mouse IgG1 isotype control, both from R&D. For NKR expression studies, 10^6 $CD8^+CD56^-$ T cells/ml were labeled with CFSE as described below and cultured in RPMI supplemented with 10% human serum and 1% APS solution, at 37ºC, 5% CO_2, 95% humidity, during 6 and 12 days in the absence or presence of IL-15 or a combination of CD3/CD28 cross-linking antibodies. For CD3/CD28 stimulation pure $CD8^+CD56^-$ T cells were pre-incubated with 10µg of anti-CD3 (clone OKT3) and 4µg of anti-CD28 (ImmunoTools, Friesoythe, Germany) for 30 min at 4ºC 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) antibodies (DakoCytomation, Glostrup, Denmark) overnight at 4ºC in PBS 1x.

1.3.5. Flow cytometry determinations

Cell stainings were normally performed at 4 ºC for 30 min in PBS 1x or staining buffer (PBS, 0.2% BSA, 0.1% NaN_3) in 96-well round-bottom plates (Greiner, Nürtingen, Germany). Irrelevant mouse mAb were used as negative controls to define background staining. In second-step stainings, rabbit anti-mouse FITC conjugated antibodies were used. T cell death was determined by two methods: (i) a decrease in cell size according to FSC/SSC parameters; (ii) double Annexin V and PI staining by using Ca^{++}-based staining buffer (10 mM Hepes/ 140 mM NaCl/ 2.5 mM $CaCl_2$). T cell activation and division were studied by three methods: (i) determination of cell size and complexity according to FSC/SSC parameters (blasts); (ii) CD69 expression; and (iii) 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 ºC. Then, cells were washed twice with PBS/20% FBSi and resuspended in RPMI. 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 Becton Dickinson, Mountain View, CA). For each sample, 50000 events were acquired using FSC/SSC characteristics and analyzed using CellQuest or FlowJo softwares.

1.3.6. 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, Minneapolis, MN) according to manufacturer's instructions. Specific primers for IL-15 (sense: 5´-CCGTGGCTTTGAGTAATGAG-3´, anti-sense: 5´-CAGATTCTGTTACATTCCC-3´) and IL-15Rα (sense: 5´-GGCGACGCGGGGCATCAC-3´, anti-sense: 5´-TCGCTGTGGCCCTGTGGATA-3´) were used and RNA amplified as previously described (19), (30). For NK receptors mRNA expression, total RNA was isolated from $CD8^+CD56^-$ T cells prior and after culture with IL-15 using a RNeasy Mini Kit from Qiagen (Hilden, Germany) according to manufacture's instructions. Primers used for NKR and amplification conditions have been previously described (31, 32) (Table II).

1.3.7. Statistical analysis

Statistical analyses were performed using Excel or GraphPad Prism 5 software. Student *t*-test was used to test the significance of the differences between group means. Statistical significance was defined as $P<0.05$.

Table I. List of antibodies used

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

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

	Sense Primer	Anti-Sense 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

1.4. Results

1.4.1. HepG2 cells and immortalized human hepatocytes induce T cell activation and proliferation and increase survival

Previous studies have suggested that primary mouse hepatocytes could deliver antigen-dependent activation signals to T cells (1-3). However, studies involving an antigen-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 co-culture, showed an increase in the percentage of blast cells when compared to PBL alone. Analysis of the phenotype of the blast cells showed that they were mainly $CD3^+$ T cells (Figure 1A, upper histogram inset). All blast cells analyzed were viable based on lack of propidium iodide labeling (Figure 1A, middle histogram inset). The increase in the percentage of T cell blasts was observed for each single experiment performed and was statistically significant (Figure 1B, graph). The activated phenotype was also confirmed by an increase in CD69 expression in PBL after co-culture with HepG2 cells (Figure 1B, 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 Figure 1C about 15% of $CD3^+$ T cells present in the PBL preparations proliferated after the co-culture. Noteworthy, non-tumoral human hepatocytes (IHH) were also able to drive T cells into proliferation, though to a lesser extent, ruling out a possible unspecific effect resulting from the use of a tumoral cell line (Figure 1C, histograms). Accordingly, both HepG2 and IHH were capable to induce T cell proliferation in a statistical significant way when compared with PBL alone (Figure 1C, graph). In addition, both hepatocyte cell lines were able to reduce markedly the percentage of dead PBL in culture as determined by propidium iodide labeling (see Figure 1A, lower histogram inset). As summarized in the graph of Figure 1D, the percentage of dead PBL was significantly reduced by interaction with HepG2 cells (~2-fold) and with non-tumoral IHH cells (~4-fold). Further analysis by double labeling with Annexin V and propidium iodide clearly showed the capacity of HepG2 and IHH cells to inhibit apoptosis (Figure 1D, histograms).

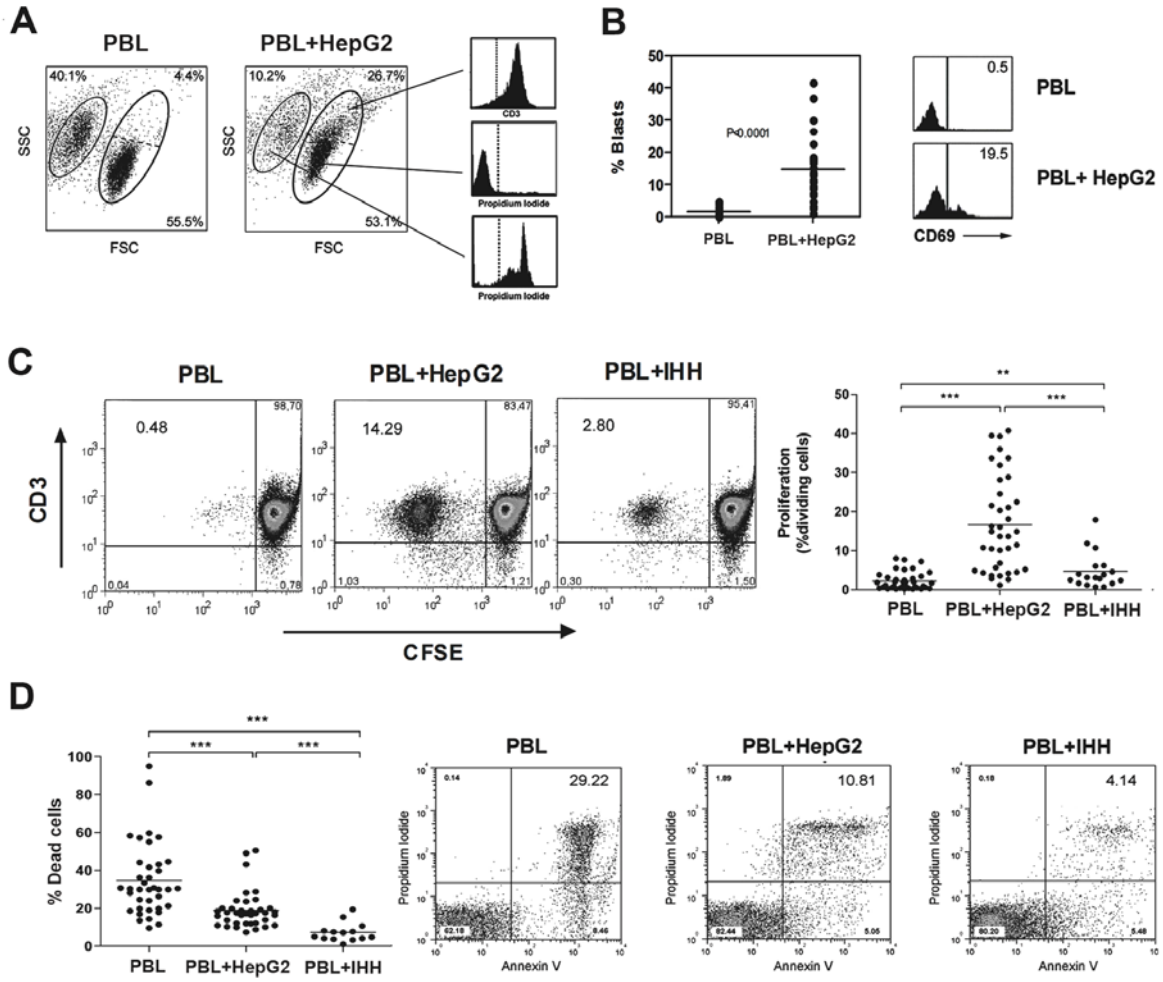


Figure 1. Hepatocytes promote T cell growth and survival. PBL ($1x10^6$) labeled with CFSE were co-cultured either alone or with hepatocyte cell lines (HepG2 or IHH) ($0.25x10^6$) for 7 days and then harvested, stained and acquired in a FACScalibur. (A) Dot plots (FSC vs. SSC) of PBL culture alone (left) 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 corresponds mainly to $CD3^+$ T cells; middle histogram illustrates that the gated population of lymphocytes are viable as determined by negativity to propidium iodide (PI) labeling; lower histogram illustrates that the gated population of lymphocytes are 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 upregulation in PBL CD69 expression after co-culture with HepG2 cells. (C) Left dot plots illustrating the proliferation of $CD3^+$ T cells after PBL co-culture with HepG2 or IHH cells, by CFSE quenching. On the 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) On the 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. On the 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. P values are shown (*** P<0.0001; ** P<0.01; * P<0.05).

1.4.2. HepG2-induced T cell proliferation, but not survival, is cell-contact dependent and requires the presence of accessory cells

In order to ascertain whether the effect observed on T cell proliferation involved or not direct cell contact with HepG2 cells, cultures using cell culture inserts were performed. As shown in Figure 2A, T cell proliferation in cultures with inserts was markedly reduced when compared to 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 conditioned medium were performed. Interestingly, HepG2 conditioned medium was able of inducing T cell proliferation and survival above background levels (Figure 2B). In contrast, conditioned medium from IHH cells showed no effect in driving T proliferation, but still was capable of decreasing T cell death (Figure 2B). Finally, in order to find out whether the HepG2-induced T cell proliferation and survival was 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 (Figure 2C, left graph). Addition of the removed accessory cells to the co-cultures of HepG2 cells, namely monocytes, partially restored cell proliferation but never to the levels seen in co-cultures of HepG2 cells and PBL (see addendum, Figure 1). Although accessory cells appear to be required for the observed HepG2-induced T cell proliferation, pre-incubation of PBL preparations with antibodies against MHC-class II molecules in order to block any possible presentation of hepatocyte-derived antigens did not abolished proliferation (see addendum, Figure 2). Also, blocking of typical T cell ligands in HepG2 cells (MHC class I, MHC class II, gp180 and CD1d) did not abolished PBL activation/ proliferation (addendum, Figure 3). Additionally, the percentage of dendritic cells in PBL preparations was shown to be very low (see addendum, Figure 4), indicating, together with the other findings, as very unlikely the possibility of cross-presentation of HepG2 antigens. Importantly, even though HepG2 cells were incapable of driving pure T cells into proliferation they still retained their capacity to induce survival (Figure 2C, right graph). Unlike HepG2 cells, two other epithelial-like cell lines (HeLa and AGS) did not exert either proliferation or survival activities towards $CD3^+$ T cells present in PBL preparations (see below).

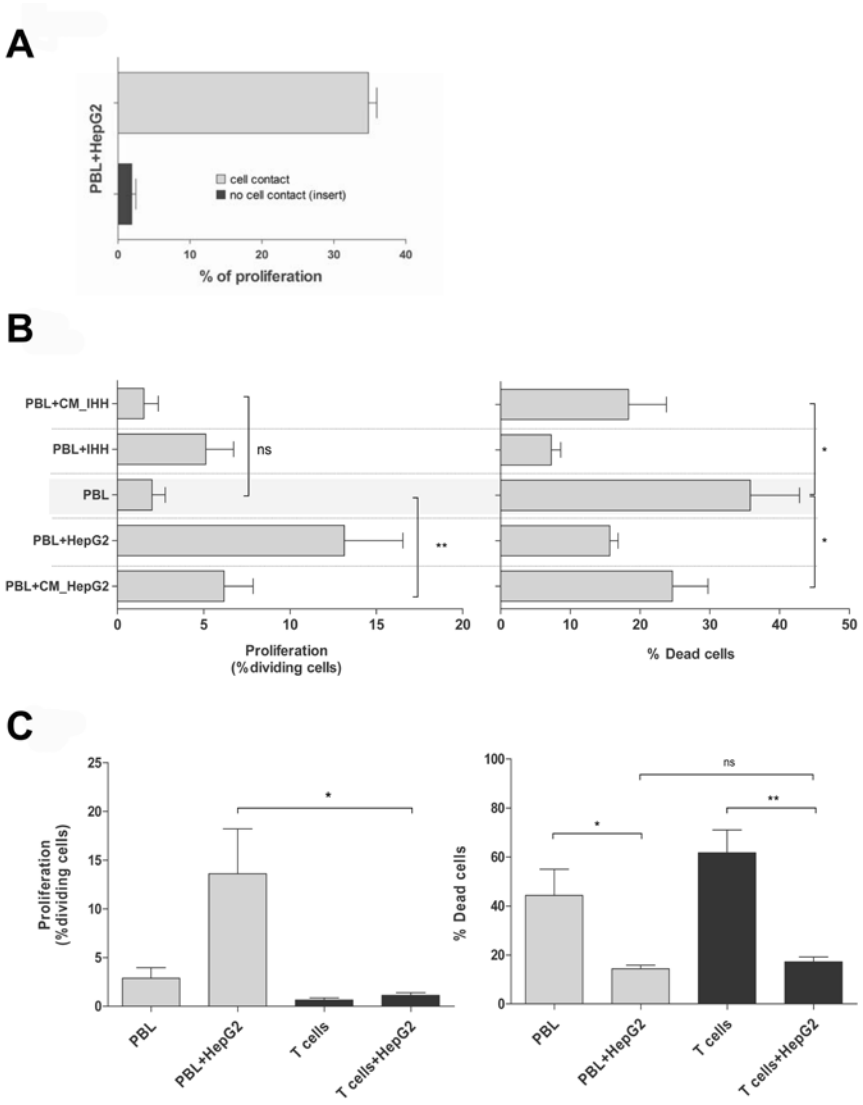


Figure 2. Hepatocyte-driven T cell proliferation is mainly cell contact dependent and, contrarily to cell survival, requires accessory cells. PBL were labeled with CFSE and cultured for 7 days in the following conditions: alone, with HepG2 (or IHH) cells, with HepG2 cells separated by a cell culture insert, with conditioned media (CM) from cultures of HepG2 (or IHH). Afterwards, cells were harvested and acquired in a FACScalibur. (A) The graph shows the percentage of proliferating PBL (CFSE) in co-cultures with HepG2 cells without (grey bar) or with (black bar) cell inserts (PBL+HepG2: 34.80±1.16 vs. PBL+HepG2_insert: 4.43±1.04). (B) Left graph: percentage of proliferating PBL in the presence of hepatocyte cell lines (HepG2 or IHH) or the corresponding conditioned media (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 conditioned media (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 t tests were performed accordingly. Results are presented as mean±SEM and represent from 2-18 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. P values are shown (*** P<0.0001; ** P<0.01; * P<0.05; ns=non significant).

1.4.3. HepG2 cells and immortalized human hepatocytes 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 Figure 3A, 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 (Figure 3A). RT-PCR of IL-15Rα originated two products of 531 and 432 bp (Figure 3A), corresponding to two mRNA isoforms (including or lacking the 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. Since expression of IL-15 has been shown to be highly controlled both at transcriptional and post-transcriptional 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 (Figure 3B).

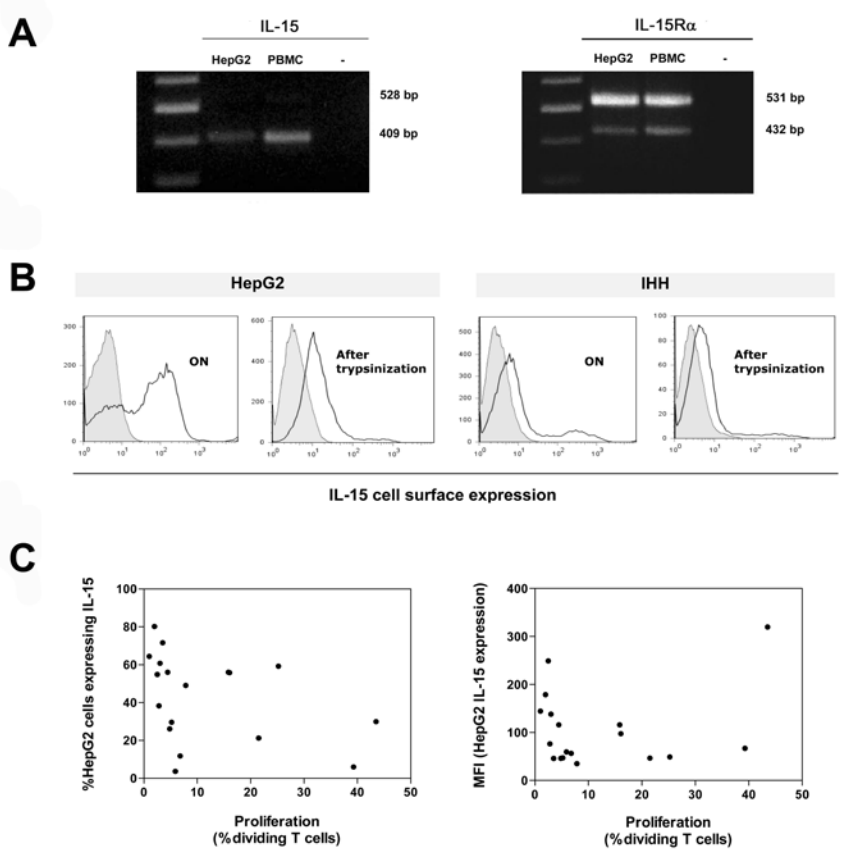


Figure 3. Expression of IL-15 and IL-15Rα by hepatocyte cells and lack of correlation with T cell proliferation. (A) Total RNA was isolated from HepG2 cells and expression of IL-15 and IL-15Rα analyzed by RT-PCR as described in *Material and Methods*. Graph shows mRNA expression for IL-15 (band of 409 bp and

a faint band of 528 bp) and IL-15Rα (bands of 531 bp and 432 bp), both in HepG2 cells and PBMC. (B) HepG2 or IHH cells were cell surface stained with mouse anti-human IL-15 monoclonal antibodies (clone MAB2471), or with the corresponding isotype control, followed by a rabbit anti-mouse-FITC at 4ºC, 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 antibodies were used as control (solid grey). (C) Co-cultures 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. Prior to the co-culture IL-15 cell surface expression levels by HepG2 cells were measured by flow cytometry. Each dot corresponds to a single experiment.

Analysis of IL-15Rα expression also revealed the presence of this receptor at the plasma membrane (addendum, Figure 5). Of note, IL-15 cell surface expression was also observed in IHH cells (Figure 3B). 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 (33-36), we examined whether membrane-bound IL-15 could play a role in the HepG2-induced T cell proliferation and/or survival observed in the co-culture experiments. Despite cell surface expression of IL-15, pre-incubation of HepG2 cells with anti-IL-15 antibodies prior to the co-culture with PBL did not abrogate the HepG2-induced T cell proliferation or T cell 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 (Figure 3C, left and right graphs, respectively).

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

Exps #	Percentage of dividing cells		Percentage of dead cells	
	+ isotype control	+ anti-IL-15	+ isotype control	+ 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

1.4.4. 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 (Figure 4A, left graph). As illustrated in Figure 4B, the IL-15 effect was observed prominently on $CD8^+$ T cells. In this particular experiment, the presence of HepG2 cells together with IL-15 lead 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 to IL-15 alone (Figure 4A, left graph). IL-15 also induced a marked decrease in cell death which was not influenced by the presence of HepG2 (Figure 4A, right graph).

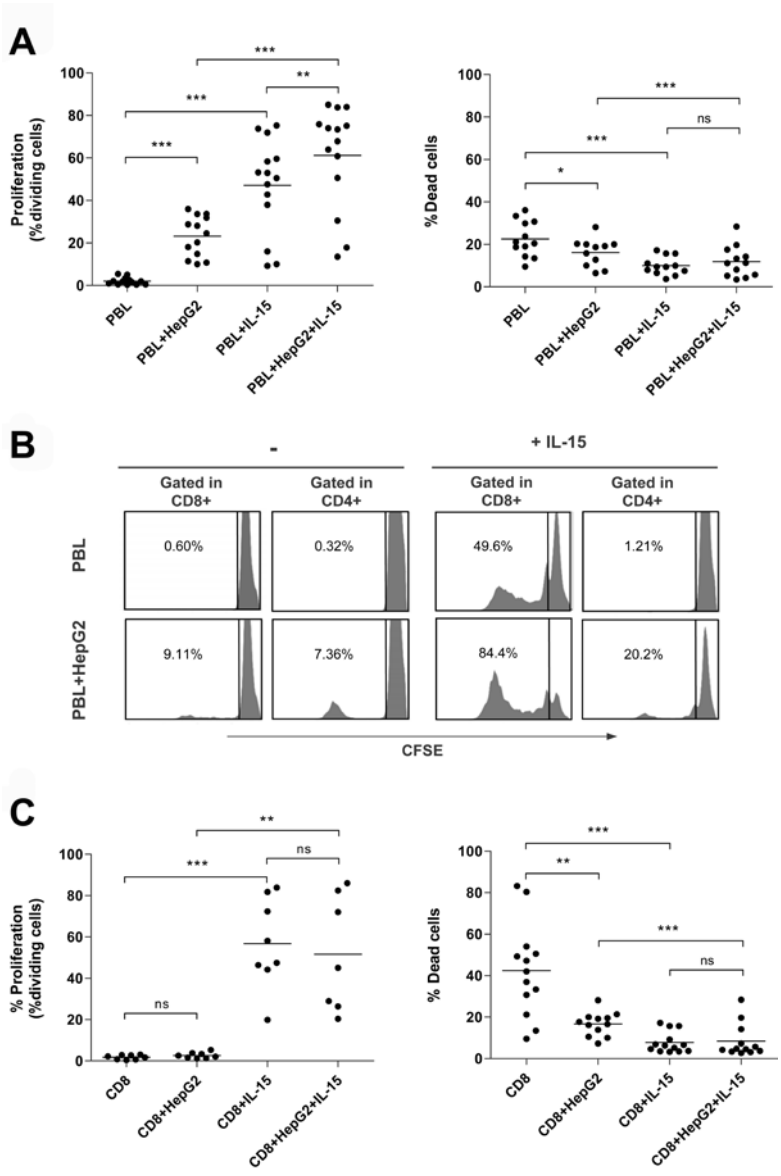


Figure 4. IL-15 and HepG2 effect on proliferation and survival of PBL and $CD8^+$ T cells. PBL and pure $CD8^+$ T cells were labeled with CFSE and cultured either alone, with HepG2 cells, with 10 ng of IL-15 or with both HepG2 and IL-15 for 7 days. Then, cells were harvested, acquired in a FACScalibur 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 8 different paired experiments. P values are shown (*** P<0.0001; ** P<0.01; * P<0.05; ns=non significant).

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 (Figure 5). Also, as referred above, and unlike HepG2 cells, these cell lines *per se* did not induce T cell proliferation (Figure 5). Finally, we examined the effect of IL-15 and HepG2 cells on $CD8^+$ T cells. As previously shown with pure $CD3^+$ T cells (Figure 2C), 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 (Figure 4C). When the combined effect of IL-15 and HepG2 cells was examined, no significant differences were observed comparing to IL-15 alone (Figure 4C), 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.

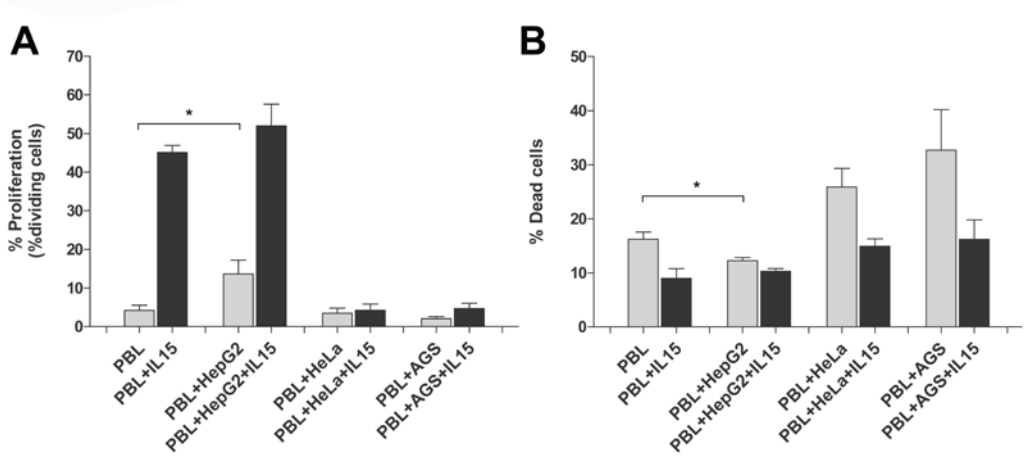
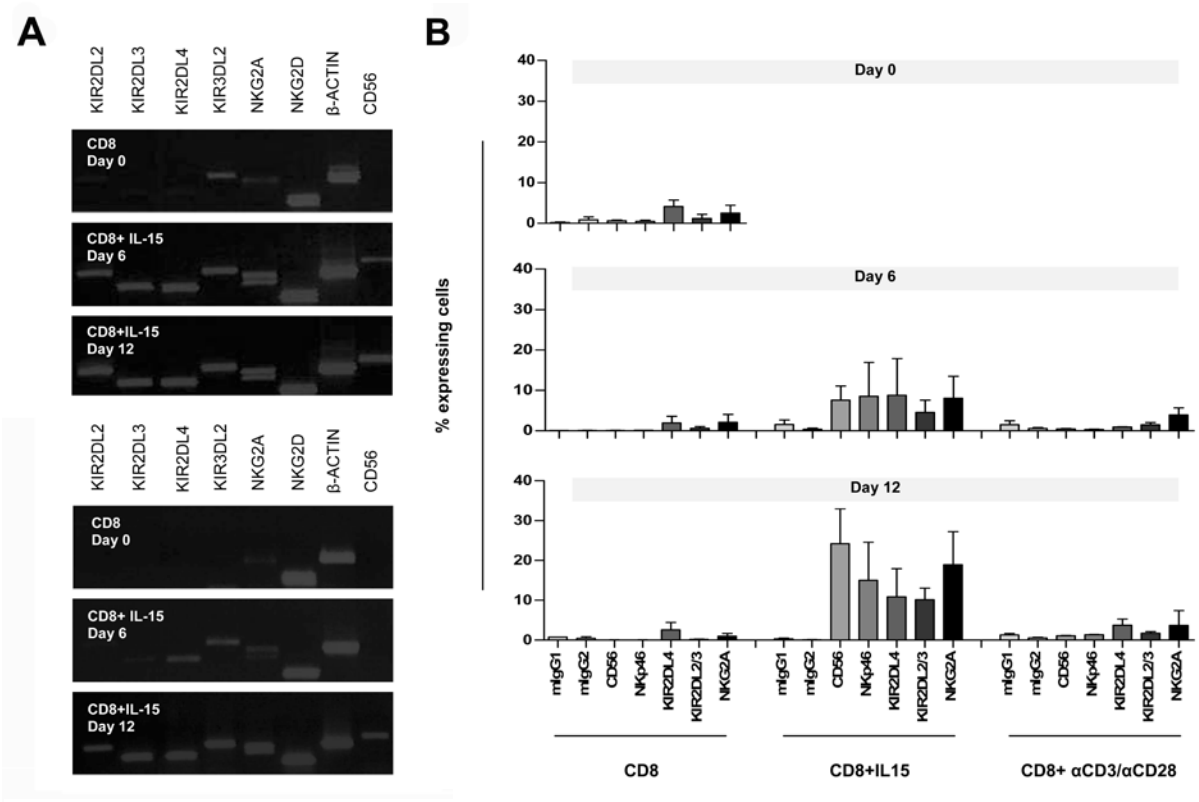


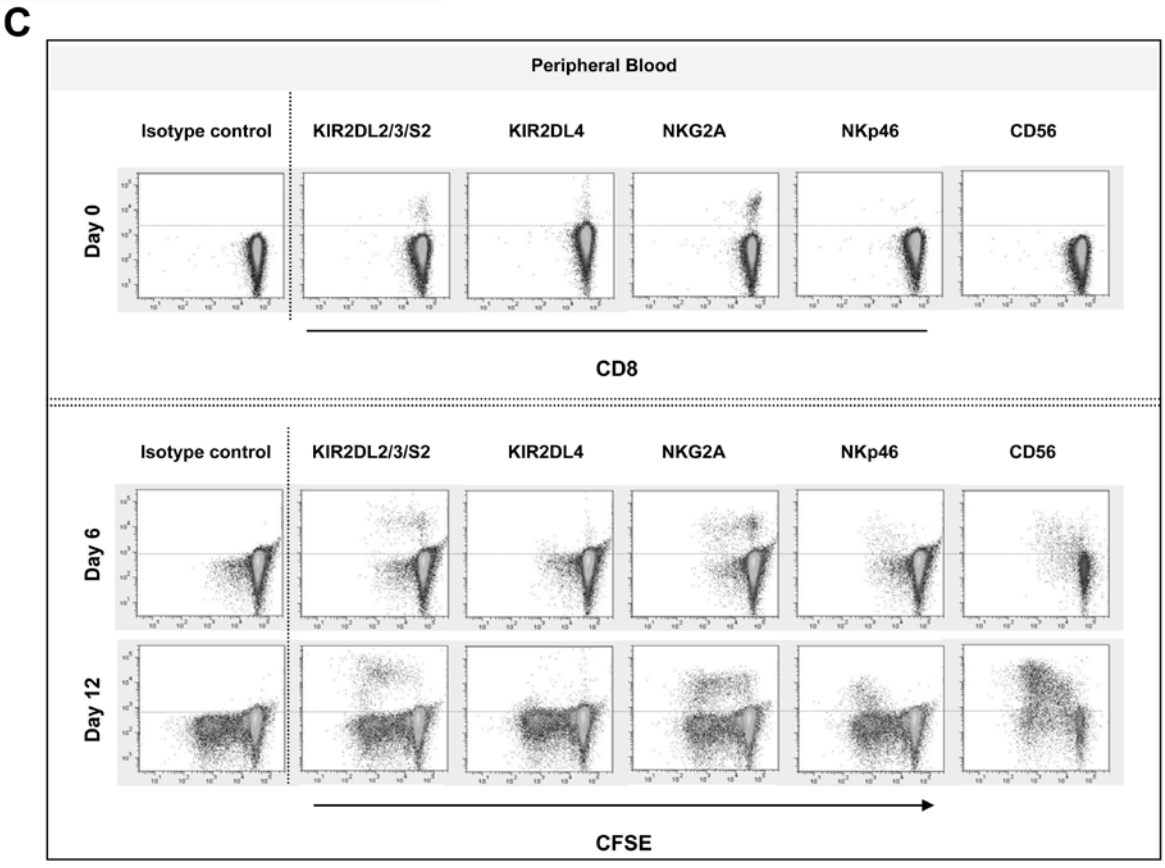
Figure 5. HeLa cells and AGS cells do not induce T cell proliferation and survival. PBL ($1x10^6$) labeled with CFSE were co-cultured either alone or with HepG2, HeLa or AGS cell lines ($0.25x10^6$) for 7 days in the presence or absence of IL-15 (10ng), and then harvested, stained and acquired in a FACScalibur. 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 4 different experiments. P values are shown (*** P<0.0001; ** P<0.01; * P<0.05).

1.4.5. Soluble IL-15 induces NK receptor expression in $CD8^+$ T cells

Even though $CD8^+$ T cells present in the human liver are characterized by the presence of a number of NK receptors (NKR), little is known about the factors that originate or regulate their expression. Thus, we 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, cultured in the presence and absence of IL-15, and NKR (KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL2, NKG2A, NKG2D and CD56) expression analysed by RT-PCR. As shown in Figure 6A, 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 six days of culture with IL-15, being more evident after twelve days (Figure 6A). Noteworthy, IL-15 induced $CD8^+CD56^-$ T cells to express CD56 mRNA, suggesting a *de novo* expression of this typical NK receptor. 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 set of experiments, NKp46 expression was also studied since it is considered to be a bona-fide marker for NK cells. As summarized in Figure 6B and 6C, $CD8^+CD56^-$ T cells expressed barely detectable levels of some NKR at the cell surface, but culture in the presence of IL-15 induced upregulation of all NKR screened. Noteworthy, IL-15 induced *de novo* cell surface expression of CD56, which is also suggested for NKp46 (Figure 6B/C). 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 upregulation of some NKR (e.g. KI2DL2/3 and NKG2A) could be due to expansion of preexisting 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 (Figure 6C). Indeed, if we consider that NKR-expressing cells did not show a proliferative advantage over non NKR-expressing cells (Figure 6C) and that an increase in mRNA expression for those NKR was observed (Figure 6A), it is likely that the upregulation 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 NK receptor expression on proliferating T cells (Figure 6C). Additional experiments with pure $CD8^+$ T cells from cord blood samples, known to have very low numbers of NKR expressing $CD8^+$ T cells (37), were performed. Double labeling

(CFSE *versus* NKR) flow cytometry results shown in Figure 6D strongly suggest that IL-15 induce *de novo* expression of CD56, KIR2DL4 and NKp46 in cord blood $CD8^+$ T cells, reinforcing the results obtained with peripheral blood $CD8^+$ T cells.





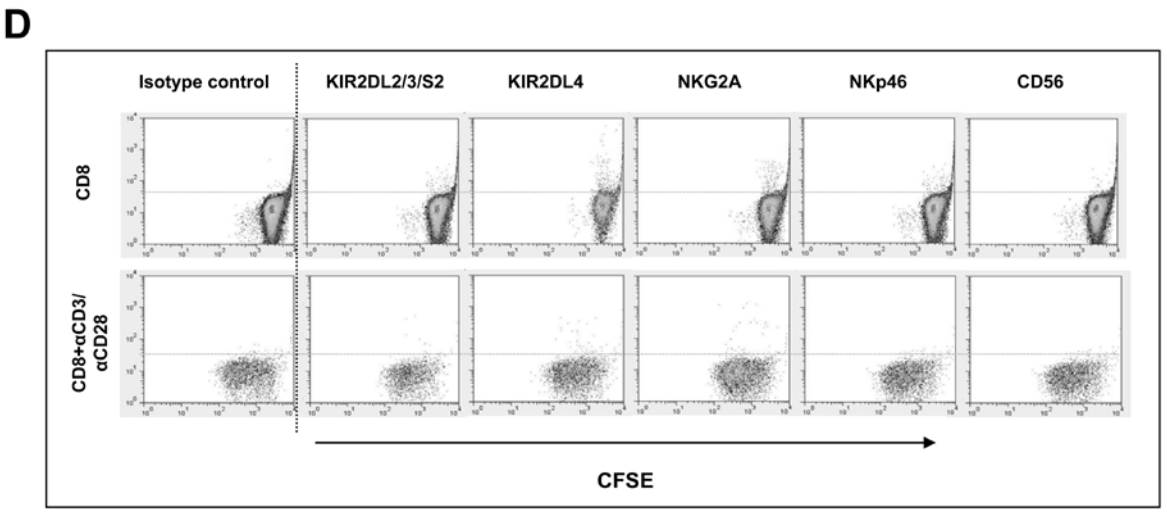


Figure 6. IL-15 induces NK-receptor expression in pure CD8$^+$CD56$^-$ T cells. Pure CD8$^+$CD56$^-$ T cells were obtained as indicated in *material and methods* and cultured with 10ng of IL-15. Analysis of NK receptor expression by RT-PCR and flow cytometry was performed as indicated. (A) RT-PCR gels from two different healthy donors showing mRNA expression of different NK receptors 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±SEM) of cells expressing NK receptors 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+αCD3/αCD28). NKR expression by CD8$^+$CD56$^-$ T cells at day 0 is included. (C) Flow cytometry data showing CD8 versus NKR expression in CD8$^+$CD56- T cells at day 0 (upper dot-plots row), and CFSE versus NKR expression in CD8$^+$CD56$^-$ T cells at days 6 and 12 after culture with IL-15 (lower dot-plots rows). (D) Dot-plots showing CFSE versus NKR expression at day 6 in CD8$^+$CD56$^-$ T cells either unstimulated (CD8) or stimulated with CD3/CD28 cross-linking (CD8+αCD3/αCD28). (E) Upper dot-plots: expression of NK receptors by cord blood CD8$^+$ T cells at day 0; lower dot-plots: CFSE versus NKR expression in cord-blood CD8$^+$ T cells at day 6 after culture with IL-15.

1.4.6. 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 (Figure 6A). 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 (Figure 7A). 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 (Figure 7B).

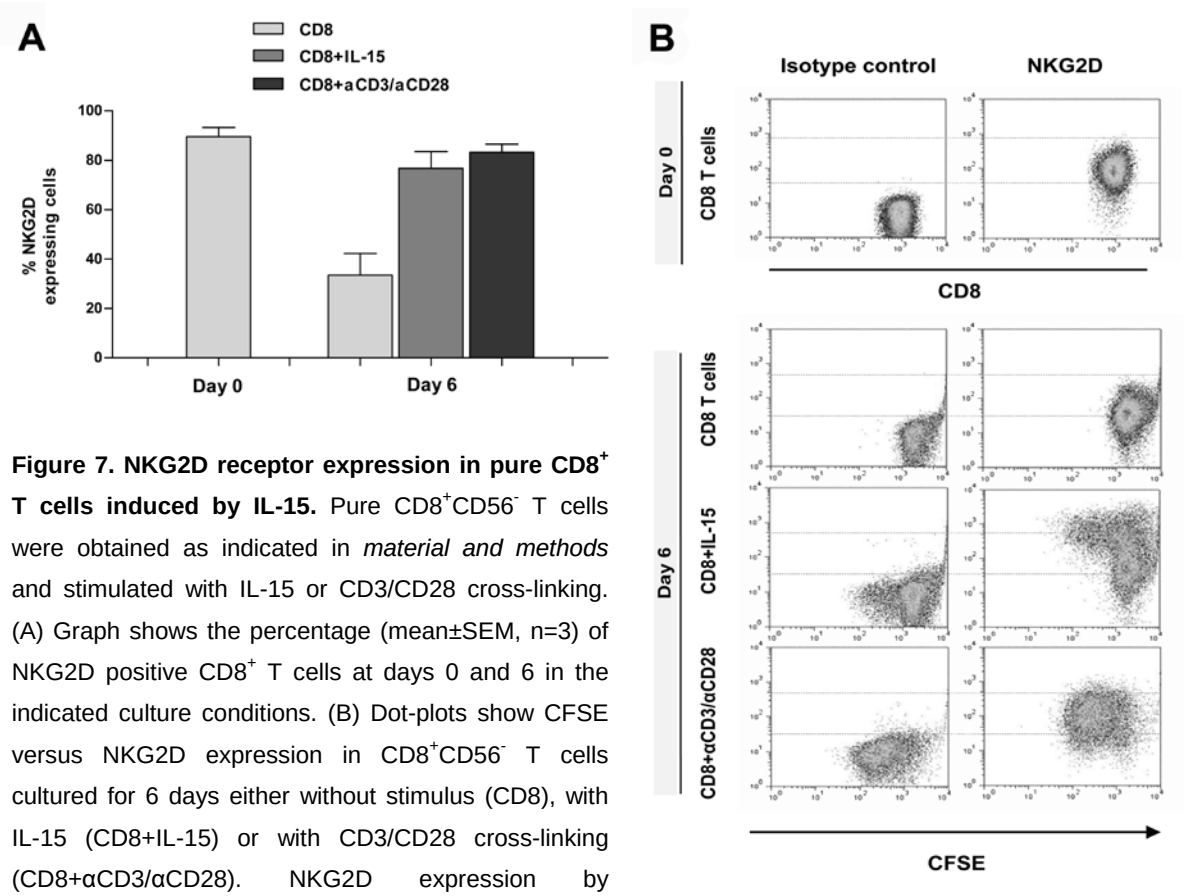


Figure 7. NKG2D receptor expression in pure CD8$^+$ T cells induced by IL-15. Pure CD8$^+$CD56$^-$ T cells were obtained as indicated in *material and methods* and stimulated with IL-15 or CD3/CD28 cross-linking. (A) Graph shows the percentage (mean±SEM, n=3) of NKG2D positive CD8$^+$ T cells at days 0 and 6 in the indicated culture conditions. (B) Dot-plots show CFSE versus 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.

1.5. Discussion

In this study we have found that the hepatoma cell line HepG2 is able to deliver antigen-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 antigen presentation via cell surface MHC class II is not involved (see addendum). 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 antigens as the cause of T cell proliferation. Also, the lack of expression of MHC class II molecules by HepG2 cells (38), 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 non-tumor immortalized human hepatocytes (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-conditioned media was 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 antigen-activated lymphocytes (39). Nevertheless, we do not exclude the possibility that other non-hematopoietic cells, such as enterocytes and fibroblasts, could have the ability of inducing survival and proliferation of T cells (18, 40).

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 (41, 42). 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 non-tumoral 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 (33), 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 on hepatocyte physiology through reverse signaling (43). 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. While 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.

To investigate further the possible role of IL-15 in $CD8^+$ T cell differentiation, we studied the effect of this cytokine on NK receptor 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 (44). Thus, while CD94/NKG2A cell surface expression on T cells is modulated by exposure to antigen, the external signals controling expression of inhibitory KIR have been to date unknown (45). In the present study, we have shown that IL-15 is capable of inducing Ag-independent upregulation of NKR in $CD8^+$ T cells, including Ig-like receptors (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 upregulation 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 (46). We have shown that IL-15 induces up-regulation of the level of NKG2D in a fraction $CD8^+$ T cells without increasing the percentage of NKG2D positive cells. Upregulation of NKG2D has previously been shown in effector intestinal cytotoxic T cells by IL-15 (47).

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 (48). 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 “non-classical” $CD3^+CD56^+$ NKT cells with an oligoclonal TCR, not restricted by CD1d, that express other NKR (16, 17). 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 (49, 50). This does not rule out the possibility that liver IL-15 could also mediate development of NKT cells from hepatic haematopoietic stem cells, as suggested by others (13). Since 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: (i) the presence of physiological levels of bioactive IL-15; (ii) the presence of resident T cells in continuous contact with IL-15; and (iii) 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 (18,40) and a recent study has shown a link between the presence of IL-15 in the small intestine and the expression of NK receptors by intraepithelial human cytotoxic lymphocytes (42).

Collectively, our results have shown that hepatocytes contribute in a decisive manner to maintain T cell survival, while IL-15 is involved in proliferation, survival and differentiation, namely at the level of expression of bona-fide NK cell receptors 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 NK receptor diversity on selected subsets of T cells and may contribute to the high levels of “non-classical” NKT cells in the human liver.

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Addendum

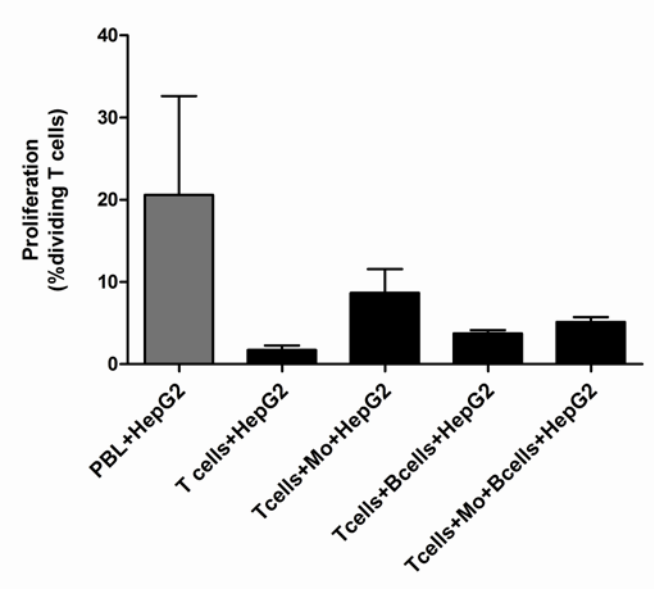
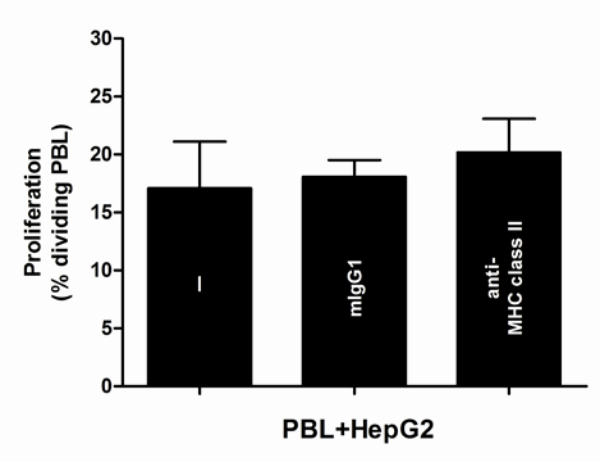


Figure 1. Effect of monocytes and B cells on the proliferation of T cells induced by HepG2. T cells (obtained from PBL) were cultured with or without monocytes and B cells (from the same PBL preparation) in a ratio 5:1 in the presence of HepG2 cells for 7 days. The graph shows the percentage of proliferating T cells in each culture condition. Proliferation seen in cultures of the original PBL preparations with HepG2 is also shown (grey bar).

Figure 2. Blocking of MHC class II in PBL does not affect the proliferation induced by HepG2 cells. PBL were either untreated or pre-incubated with anti-HLA-DR -DP –DQ antibodies (clone CR3/43, DakoCytomation) or with the corresponding isotype control (mouse IgG1) for 30 min at 4ºC. Then cells were washed twice and added to culture with HepG2 cells for 7 days. The graph shows the percentage (mean±SEM) of PBL proliferation for each condition. The results are representative of 3 separate experiments.



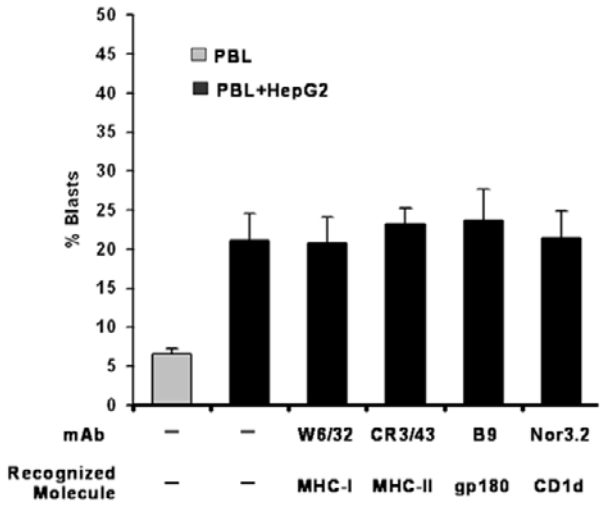


Figure 3. PBL blast transformation induced by HepG2 cells is not mediated by typical T cell ligands. HepG2 cells were pre-incubated for 1 hour at 4 ºC with monoclonal antibodies against the molecules indicated, and cultured with PBL for 7 days. The graph shows the percentage of blasts based on FSC/SSC parameters in each culture condition. Values represent the mean±SD from at least 3 different experiments.

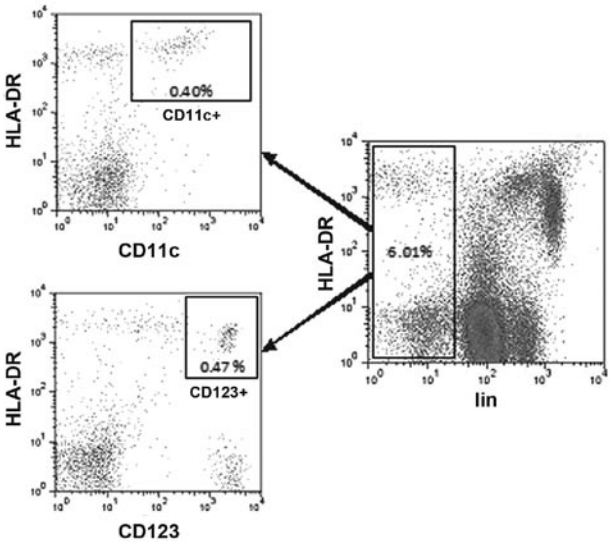


Figure 4. Frequency of dendritic cells in PBL preparations. PBL were labeled with a combination of antibodies: lineage-specific (anti- CD3, CD14, CD16, CD19, CD20, CD56)-FITC and anti-HLA-DR-PE, together with anti-CD123 PECy5 and anti-CD11c, or the correspondent isotype controls, for 30 min at 4ºC and acquired in a FACSCalibur. The percentage of myeloid CD11c+ (upper left dot-plot) and plasmacytoid CD123+ (lower left dot-plot) dendritic cells obtained after gating on lin- cells (right dot-plot) are shown. The results are representative of 4 separate experiments.

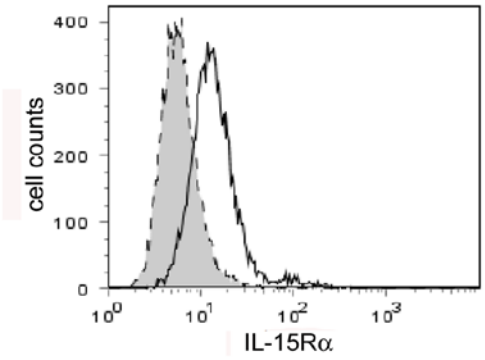


Figure 5. HepG2 cells express IL-15Rα at the cell surface. HepG2 cells were stained for 30 minutes at 4ºC with IL-15Rα Abs (R&D). The cells were then incubated with the appropriate secondary antibody for another 30 minutes at 4ºC, washed and acquired in a FACScalibur. Mouse IgG was used as isotype control (grey dashed line).

2. IL-15 induces $CD8^+$ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion

Adapted from Correia MP, Costa AV, Uhrberg M, Cardoso EM, Arosa FA.
Submitted

2.1. Abstract

During the last years it has been shown that a small percentage of $CD8^+$ T cells are capable of expressing NK receptors (NKR), though their origin remains largely unknown. In a previous study we have found that IL-15 was capable of inducing NKR expression in highly purified human $CD8^+CD56^-$ T cells, including CD56, NKp46, KIR2DL2/3, KIR2DL4, NKG2A and NKG2D. Here, we have shown that IL-15-driven NKR induction in $CD8^+$ T cells was linked with CD56 *de novo* acquisition, consistent with an effector-memory phenotype, and paralleled with increased anti-apoptotic (Bcl-2) levels, high granzyme B/perforin expression and with the ability of displaying *in vitro* NK-like cytotoxicity. Noteworthy, dissection of NK receptor functional outcome in IL-15 cultured $CD8^+$ T cells revealed: (i) that NKG2D cross-linking was able *per se* to upregulate degranulation levels and (ii) that KIR and NKG2A cross-linking upregulated secretion of different cytokines such as IFN-γ, TNF-α, IL-1β and IL-10. These results suggest that IL-15 is capable of differentiating $CD8^+$ T cells into NK-like T cells by inducing functional NK receptor acquisition, which ultimately modulate their function by controlling cytotoxicity and also cytokine secretion, suggesting that these NK-like T cells could have a regulatory role.

2.2. Introduction

In the last years, it has been shown that NK receptors could be expressed, not only by NK cells, but also by T cells. In early studies, Killer Immunoglobulin-like Receptors (KIR) were found to be expressed in a small fraction of T cells from normal donors expressing either TCRαβ or TCRγδ, mostly $CD8^+$ T cells (1-2). Together with KIR, CD94/NKG2A was also found to be expressed in $CD8^+$ T cells (1). These receptors were shown to provide T cells with the ability to recognize HLA class I molecules on target cells, leading to a decrease in CTL cytotoxicity (1-3). It is now known that besides inhibitory receptor, $CD8^+$T cells also express activating receptors, (4-5) and the notion that NK receptor expression in $CD8^+$ T cells could modulate T cell function, by increasing or decreasing TCR threshold accordingly to the triggering of activating or inhibitory NKR, is now emerging. Despite this knowledge, the environmental signals that trigger NK receptor acquisition by $CD8^+$ T cells remain largely unknown. In a previous study, we have found that the γ-common cytokine IL-15, known to be crucial to NK and invariant NKT cell development and homeostasis, as well as for the activation, survival and differentiation of both memory and naïve $CD8^+$ T cells (6-7), was capable of inducing the expression of NKR in purified $CD8^+CD56^-$ T cells after a prolonged exposure (8). Between those NKR there were both inhibitory and

activation and from different structural families: KIR (KIR2DL2/3, KIR2DL4), C-type lectin-like receptors (NKG2A, NKG2D), the most common NK marker, CD56, and the recently considered the truly bona-fide marker of NK cells, the natural cytotoxicity receptor (NCR) NKp46. (8).

Although the term “NKT cell” is mainly used to describe CD1d-restricted NKT cells expressing an invariant TCR chain, it also includes CD1d-unrestricted NKT cells with an oligoclonal TCR, known also as NK-like T cells, which are less studied. NK-like T cells have been described to be mostly $CD8^+CD56^+$ T cells and, contrarily to mice, constitute the NKT cell population mostly enriched in human livers (9-10). In this context, we have hypothesized that those NK-like T cells does not necessarily consist in a different lineage, but could in part rather be $CD8^+$ T cells that under certain conditions as under prolonged exposure to IL-15 in a favorable environment such as the liver, differentiate and acquire NK receptors (8). In this study we have characterized further the $CD8^+NKR^+$ phenotype and dissected the functional outcome of the NK receptor triggering. Accordingly, we have found that IL-15-induces the formation of $CD8^+CD56^+$ T cells co-expressing other NKR, displaying an effector memory phenotype with increased survival and cytotoxic potential, obtaining a population endowed with TCR-mediated and NK-like cytotoxicity. Noteworthy, we have observed that NK receptors were functional, capable of modulating cytotoxicity, increased after NKG2D triggering, and also cytokine secretion after KIR and NKG2A cross-linking. To our knowledge, this is the first evidence showing that KIR and NKG2A mAb cross-linking can induce the secretion of a varied pattern of cytokines by $CD8^+$ T cells. These results reinforce the view of IL-15 being one of the environmental factors involved in the generation of NK-like $CD8^+$ T cells with possible a regulatory phenotype, linking between innate and acquired immunity.

2.3. Material and Methods

2.3.1. Reagents and monoclonal antibodies

RPMI-1640 GlutaMAX®, Fetal Bovine Serum (FBS) and Amphotericin B/Penicillin/Streptomycin (APS) were from Gibco BRL (Paisley, Scotland). 5-(and –6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and the LIVE/DEAD® Viability/Cytotoxicity Kit (for mammalian cells) were purchased from Molecular Probes (Amsterdam, The Netherlands). Human serum was obtained from Cambrex (New Jersey, USA). Recombinant human IL-15 was obtained from R&D Systems (Minneapolis, USA).

Permeabilization buffer (Foxp3 Staining Buffer Set) is from eBioscience (San Diego, CA, USA). The monoclonal antibodies (mAb) used are listed in Table I.

Table I. List of antibodies used in the study

Antibody	Conjugate	Company	Isotype	Clone
CD3	APC	Immunotools	mIgG2a	MEM-57
CD8	PE-Dy647	Immunotools	mIgG2a	MEM-31
CD56	PE	Immunotools	mIgG2a	MEM-188
CD56	APC	Immunotools	mIgG2a	MEM-188
KIR2DL2/S2/L3 (NKAT2)	PE	BD	mIgG2a	DX27
KIR2DL4 (CD158d)	PE	R&D Systems	mIgG2a	181703
NKG2A (CD159a)	PE	Coulter	mIgG2b	Z199
NKp46 (CD335)	PE	Coulter	mIgG1	BAB281
NKG2D (CD314)	PE	Coulter	mIgG1	ON72
Granzyme B	PE	Immunotools	mIgG1	HC4
Perforin	PE	Immunotools	mIgG2a	Delta G9
CCR7	APC	eBioscience	mIgG2a	3D12
CD45RA	PE-Dy647	Immunotools	mIgG2b	MEM-56
Bcl-2	PE	Caltag Laboratories	mIgG1	100
CD107a (LAMP-1)	PE	BD		H4A3
Mouse IgG	PE	Immunotools	mIgG1	PPV-06
Mouse IgG	PE	Immunotools	mIgG2a	713
Mouse IgG	PE	Immunotools	mIgG2b	GC198
Rabbit anti-mouse (RAM)	None	Dako Cytomation	-	-
Mouse IgG	None	eBioscience	mIgG1	P3
CD3	None	eBioscience	mIgG2a	OKT3
CD56	None	Biolegend	mIgG1	HCD56
NKp46 (CD335)	None	Biolegend	mIgG1	9E2
KIR2DL2/3/S2 (NKAT2)	None	Biolegend	mIgG2a	DX27
KIR2DL4 (CD158d)	None	R&D Systems	mIgG2a	181703
NKG2A (CD159a)	None	R&D Systems	mIgG2a	131411
NKG2D (CD314)	None	Biolegend	mIgG1	1D11

2.3.2. Cell lines

K562 (human immortalized myeloid leukemia), Jurkat E6.1 (human immortalized T cell leukemia) and P815 (murine mastocytoma) cell lines were obtained from European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cell lines were cultured in 75 cm2 flasks (Nunc, Roskilde, Denmark) in RPMI-1640 Glutamax® supplemented with 10% inactivated FBS and 1% APS solution at 37°C, 5% CO2 and 95% humidity. The medium was regularly changed and cells were always washed twice before use.

2.3.3. Isolation of Peripheral Blood Lymphocytes and T cell subsets

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats after centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Contaminating red blood cells

were lysed in lysis solution (10 mM Tris, 150 mM NH4Cl, pH 7.4), 10 min at 37°C. Peripheral blood lymphocytes (PBL) were obtained by overnight culture in Petri dishes. Purified $CD8^+$ $CD56^-$ T cells were obtained after two-step isolation: First, $CD8^+$ T cells were isolated from PBL using a CD8 negative isolation kit (Miltenyi Biotec). Then, the negatively selected $CD8^+$ T cells were subjected to positive isolation with CD8 beads (Miltenyi Biotec), obtaining a population >99.5% $CD8^+CD3^+CD56^-$.

2.3.4. Culture conditions and CFSE proliferating assay

Purified $CD8^+CD56^-$ T cells were cultured in RPMI-1640 Glutamax® supplemented with 10% human serum and 1% APS solution, at 37°C, 5% CO2, 95% humidity, during 12 days with 10 ng of IL-15 (R&D Systems). For proliferation studies, 10^7 cells/ml cells were labeled with CFSE at a final concentration of 10 μM for 10 min at 37°C with occasional mixing, and then washed twice with PBS/ 20% FBS prior to cell culture. Rounds of cell division were determined by sequential halving of CFSE-fluorescence intensity after the period of culture.

2.3.5. Cell-mediated cytotoxicity and redirected killing assays

The cytotoxicity assays were performed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes). Briefly, target cells were previously labeled with 3,3´-dioctadecyloxacarbocyanine ($DiOC_{18}(3)$) and washed twice with PBS. For redirected killing assays, the FcR positive P815 target cells were pre-incubated with 10 μg of anti-CD3 Ab (OKT3), or the respective mouse isotype control (mIgG), for 20 min at 4ºC and again washed twice. Then, target cells were platted together with the effector cells at a 5:1 E:T ratio in 96-well plates and propidium iodide (PI) was added for counterstaining accordingly to manufacturer instructions. The cells were then incubated for 3 hours at 37°C and immediately acquired in a flow cytometer. The percentage of dead target cells was determined accordingly to $DiOC_{18}(3)^+PI^+$ cells. The specific cytotoxicity was calculated as follows: % specific cytotoxicity= (% of dead target cells alone) – (% of dead target cells in the presence of effector cells).

2.3.6. CD107a degranulation assay after NKR crosslinking

CD107a degranulation studies were performed on $CD8^+$ T cells obtained after 12-day culture with IL-15. The supernatant was collected and re-used as culture media in the

assays. In order to do NKR cross-linking, the $CD8^+$ T cells were pre-incubated separately with 10 μg of the different NKR functional-grade antibodies (see table I) , anti-CD3 antibody (OKT3) or isotype control antibodies for 30 min at 4ºC and washed twice. Then, mAb-labeled cells were ressuspended in the originally collected culture supernatant and added to 96-well culture plates previously coated with rabbit anti-mouse (RAM) antibodies. Then, 10 µl of CD107a-PE antibody or mIgG-PE isotype control were added and cells were incubated during 4h at 37ºC. Afterwards, cells were washed twice with PBS and acquired in the flow cytometer.

2.3.7. Cytokine quantification by fluorescent bead immunoassay (FlowCytomix)

A fluorescent bead immunoassay was used for quantitative detection by flow cytometry of IFNγ, IL-1β, IL-6, IL-10, MIP-1β, and TNF-α (FlowCytomix Assay, Bender MedSystems GmbH, Austria) in culture supernatants, accordingly to the manufacturer instructions. This assay allows the identification of different cytokines based on two different populations (A and B) in the flow cytometer due to the use of two sets of beads with different sizes, and discrimination of the different cytokines within two populations according to variations in the intensities of an internally fluorescent dye detected in the FL-3 channel; while allowing the quantification of each specific cytokine in the FL-2 channel. The supernatants were obtained after performing NKR crosslinking of NKR on IL-15-cultured $CD8^+$ T cells, as described above, and culturing cells for 48h at 37°C. Supernatants were collected, centrifuged at 13000×g for 15 min and stored at -20°C until used. After processing samples according to manufacturer's instructions, they were acquired in a FACSAria flow cytometer (BD, Mountain View, CA) and the amount of cytokine (pg/ml) determined by using a FowCytomix 2.3 Software (Bender MedSystems).

2.3.8. Flow cytometry determinations

Extracellular cell stainings were performed at 4°C for 30 min in PBS in 96-well round-bottom plates. For intracellular stainings, cells were previously fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience), accordingly to the manufacturer instructions. Irrelevant mouse mAbs were always used as negative controls to define background staining. After staining, cells were washed and acquired in a FACSCalibur (BD, Mountain View, CA) and analyzed using CellQuest or FlowJo softwares.

2.3.9. Statistical analysis

Statistical analyses were performed using Excel or GraphPad Prism 5 software. Student t-test was used to test the significance of the differences between group means. Statistical significance was defined as $P<0.05$.

2.4. Results

2.4.1. IL-15 induces de novo formation of $CD8^+CD56^+$ co-expressing other NKR

As we have previously shown (8), after 12-days of culture with IL-15, purified $CD8^+CD56^-$ T cells were capable of up-regulating and/or *de novo* expressing several NK receptors, including KIR (KIR2DL2/3, KIR2DL4), C-lectin-like receptors (NKG2A, NKG2D), Natural Cytotoxic Receptors, NCR (NKp46), and the most NK common marker, CD56 (Figure 1). In Figure 1A, it is possible to see that acquisition of the different NKR by $CD8^+$ T cells after 12 days in culture with IL-15 occurs mainly in cells that underwent several cycles of division, as determined by CFSE loss (see CD56 and NKp46). Indeed, the initial purified $CD8^+$ T cell population was negative for CD56 and NKp46, while the other NKR were only slightly expressed (Figure 1B, Day0). After culture with IL-15 there is a clear CD56 (about 20%) and NKp46 (about 5%) de novo expression in the $CD8^+$ T cell population, as well as the increased expression of the other NKR (ranging between 6-12%) (Figure 1B, Day12), demonstrating that the initial $CD8^+CD56^-$ T cell population was capable of *de novo* expressing and/or up-regulating NKR (Figure 1A and 1B). Noteworthy, the cells after the period of culture remain $CD3^+CD8^+$ (Figure 1C). Although NKG2D was already expressed by almost all $CD8^+CD56^-$ T cells, culture with IL-15 induced an up-regulation in the mean fluorescence intensity (MFI), indicating that this cytokine is able to up-regulate further NKG2D expression levels (Figure 1E). Importantly, simultaneous analysis of CD56 and the other NKR revealed that the majority of $CD8^+$ T cells expressing NKR also co-expressed the CD56 marker (Figure 1D), indicating that NKR expression by $CD8^+$ T cells is closely linked to *de novo* CD56 acquisition.

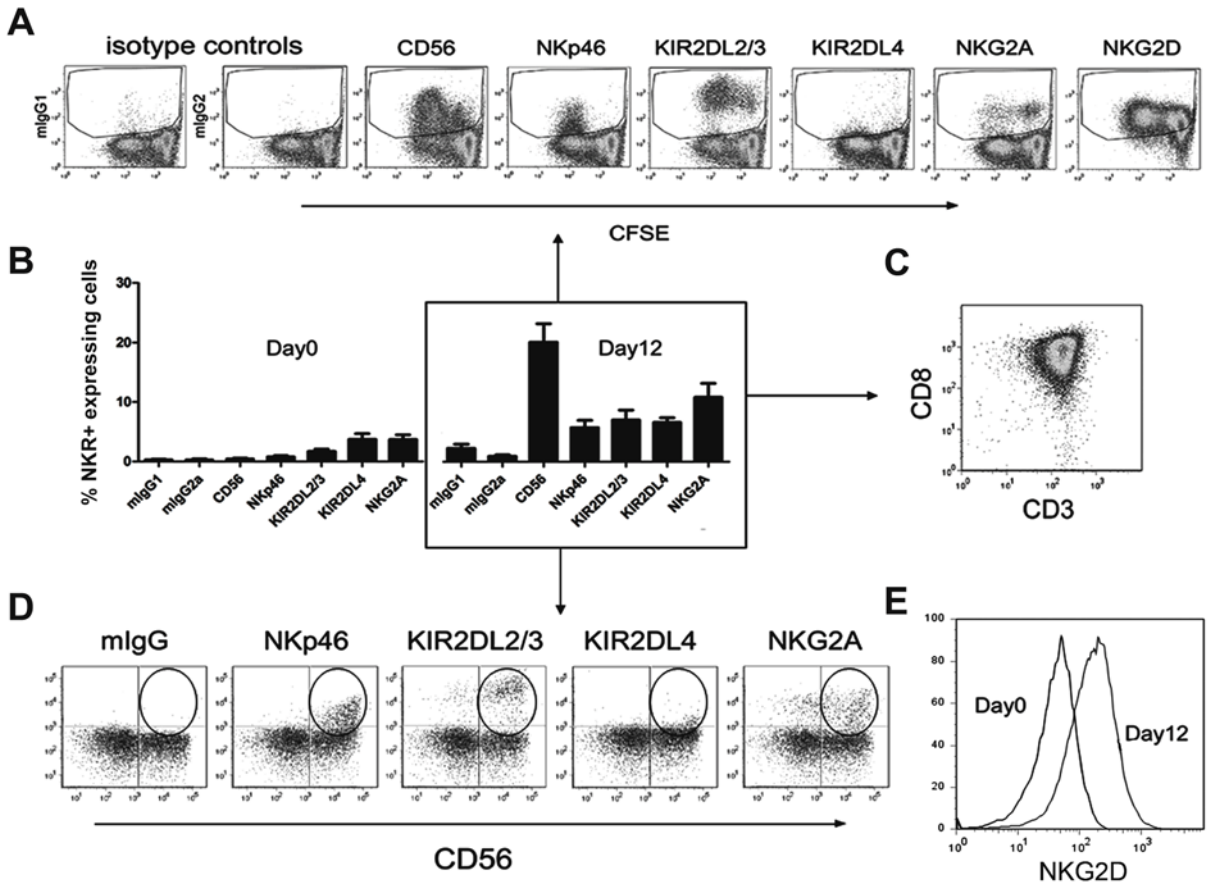


Figure 1. IL-15-driven induction of NK receptors in purified CD8$^+$ CD56$^-$ T cells is paralleled by CD56 acquisition. Purified CD8$^+$CD56$^-$ T cells were obtained as indicated in *material and methods*, labeled with CFSE and cultured with IL-15 (10ng/ml) for 12 days. (A) Dot-plots showing NKR expression in CD8$^+$ T cells after 12 days with IL-15 vs. CFSE halving. Isotype controls (mIgG and mIgG2) are included. (B) Graph showing the percentage (mean±SEM) of NKR$^+$ CD8$^+$ T cells at day 0 (without IL-15) and after 12 days of culture with IL-15 for all NKR, P<0.05. (C) Dot-plot showing that after 12-day culture with IL-15 all cells remain CD8$^+$CD3$^+$. (D) Dot-plots illustrating CD56 vs. NKR expression in IL-15 cultured CD8$^+$ T cells. (E) Histogram showing upregulation of NKG2D expression in CD8$^+$CD56$^-$ T cells after 12 day-culture with IL-15. Data is representative from at least 7 different experiments.

2.4.2. IL-15-induced CD8$^+$NKR$^+$ T cells display an effector memory phenotype and express high levels of Bcl-2

Next, we wanted to determine markers of differentiation and survival in the IL-15-induced CD8$^+$NKR$^+$ T cells. In order to use a representative marker for CD8$^+$NKR$^+$ T cells we used CD56 since we have found it to be the most accurate marker of *de novo* NKR expression and shown to be co-expressed with the other NKR (see Figure 1). Based on the

expression of CD45RA and CCR7 markers, four differentiation phenotypes can be defined: $CD45RA^+CCR7^+$ (naïve, T_N), $CD45RA^-CCR7^+$ (central memory, T_{CM}), $CD45RA^-CCR7^-$ (effector memory, T_{EM}) and the $CD45RA^+CCR7^-$ ($CD45RA^+$ effector memory, T_{EMRA}). As seen in Figure 2A, IL-15-induced $CD8^+CD56^+$ T cells were shown to be mainly $CD45RA^+$ and $CCR7^-$, corresponding to a $CD45RA^+$ effector memory phenotype (T_{EMRA}). In some experiments, a part of $CD8^+CD56^+$ T cells were $CD45RA^-CCR7^-$ effector memory cells (T_{EM}), showing that IL-15 induced $CD8^+CD56^+$ T cell generation is consistent with a differentiation towards an T_{EM}/T_{EMRA} effector memory phenotype.

On the other hand, Bcl-2 has been shown to play a central role in preventing $CD8^+$ T cell apoptosis upon TCR triggering (11). Analysis of intracellular Bcl-2 by flow cytometry revealed that the population obtained after 12 day-culture with IL-15 has higher expression of Bcl-2, as determined by MFI levels, than the initial purified $CD8^+CD56^-$ T cell population (Figure 2B, Day 0 vs. Day 12). Noteworthy, simultaneous analysis of Bcl-2 and CD56 revealed that nearly all $CD8^+CD56^+$ T cells were Bcl-2-positive (Figure 2C, upper dot-plot) and expressed significantly higher levels of Bcl-2 (Figure 2C, lower graph) when compared to $CD8^+CD56^-$ T cells, suggesting that the IL-15 generated $CD8^+CD56^+$ T cells show an increased anti-apoptotic potential.

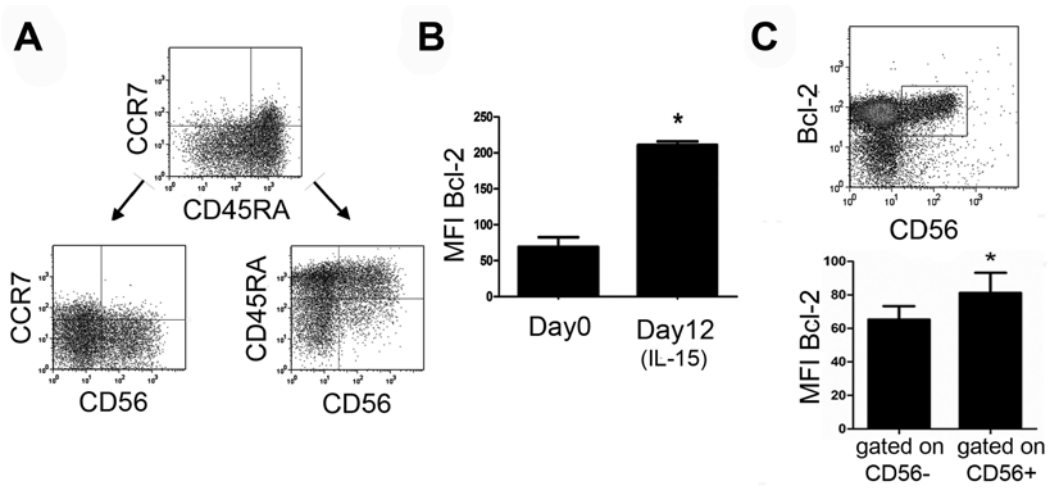


Figure 2. IL-15-induced $CD8^+NKR^+$ T cells display an effector memory phenotype and express high levels of Bcl-2. Purified $CD8^+CD56^-$ T cells were obtained as indicated in *material and methods* and cultured with IL-15 (10ng/ml) for 12 days. (A) Dot-plots showing CD45RA and CCR7 expression (*upper dot-plot*) and CD45RA and CCR7 vs. CD56 co-expression (*lower dot-plots*). One representative experiment out of 6 different experiments performed is shown. (B) Graph showing intracellular MFI of Bcl-2 protein determined by flow cytometry at day 0 and after 12 days in culture with IL-15. (C) Representative dot-plot showing Bcl-2 vs. CD56 expression after 12 days in culture with IL-15 (*upper dot-plot*). The MFI Bcl-2 values (mean±SEM) on gated $CD56^-$ and $CD56^+$ populations are indicated (*lower graph*). Data is representative of 4 separate experiments. * $P<0.05$.

2.4.3. IL-15-cultured CD8$^+$ T cells express high levels of granzyme B and perforin and can display MHC-unrestricted cytotoxicity

In order to assess if the IL-15-differentiated CD8$^+$NKR$^+$ T cells have acquired a cytotoxic phenotype, we went to examine the intracellular expression of granzyme B and perforin. As observed in Figure 3A, purified CD8$^+$CD56$^-$ T cells already expressed some levels of granzyme B and perforin (day 0). However, after 12 days of culture with IL-15 there was a clear increase in the percentage of granzyme B/perforin positive CD8$^+$ T cells (day 12), which was consistently observed in all experiments (Figure 3A). Importantly, simultaneous determination of granzyme B/perforin and CD56 showed that the large majority of generated CD8$^+$CD56$^+$ T cells were granzyme B and perforin positive (Figure 3B), indicating that *de novo* expression of CD56 in IL-15-differentiated CD8$^+$ T cells is paralleled with granzyme B/perforin acquisition.

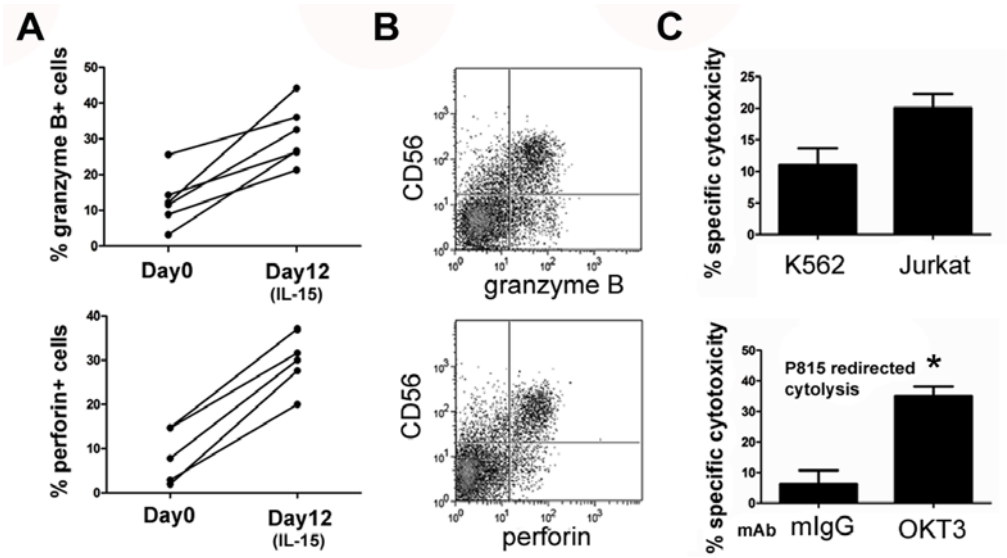


Figure 3. IL-15-generated CD8$^+$CD56$^+$ T cells are granzyme B$^+$/perforin$^+$ and display cytotoxicity towards different targets. Purified CD8$^+$CD56- T cells were obtained as indicated in *material and methods*, and cultured with IL-15 (10ng/ml) for 12 days. (A) Graphs shows a statistically significant increase (P<0.05) in the percentage of granzyme B (*upper graph*) and perforin (*lower graph*) positive CD8$^+$ T cells from day 0 to day 12 after culture with IL-15. (B) Dot-plots showing representative experiments of CD56 vs. granzyme B (*upper dot-plot)* or perforin (*lower dot-plot*) expression after 12 days in culture with IL-15 (n=6). (C-D) IL-15-cultured CD8$^+$ T cells were examined for cytotoxicity using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular probes) as described in *material and methods*: (C) Graph showing the specific cytotoxicity (mean±SEM, n=3) of IL-15 cultured CD8$^+$ T cells towards K562 and Jurkat cells. (D) Graph showing the specific cytotoxicity (mean±SEM, n=3) of IL-15 cultured CD8$^+$ T cells towards P815 cells in redirected assays using OKT3 or an isotype control (mIgG). *P< 0.05.

In the light of these results, we wanted to ascertain if our IL-15-cultured $CD8^+$ T cells could display cytolytic activity. To that purpose, K562 cells (that lack MHC class I molecules) and Jurkat cells were used as targets. The cytotoxicity of freshly purified $CD8^+CD56^-$ T cells prior to culture with IL-15 against either cell line was negligible (data not shown). In contrast, after a 12-day period culture with IL-15, the generated $CD8^+$ T cells were capable of displaying measurable levels of cytotoxicity towards both K562 and Jurkat cells (Figure 3C). In parallel, as illustrated in Figure 3D, redirected cytotoxic assays using P815 cells showed that CD3 cross-linking (OKT3) led to increased cytolysis of P815 cells compared with the control (mIgG). Overall, these results show that a fraction of $CD8^+$ T cells obtained after 12 day-culture with IL-15 were capable of acquiring killer function, displaying both TCR-mediated and MHC-unrestricted (NK-like) cytotoxicity.

2.4.4. IL-15-cultured $CD8^+$ T cells express functional NKR: cytotoxic granule and cytokine release

An important issue arising from these results was to ascertain whether the NKR expressed *de novo*/up-regulated on $CD8^+CD56^-$ T cells upon culture with IL-15 were functional. Thus, we performed analysis of cytotoxic granules release and analysis of cytokine secretion after NK receptor triggering using the bulk IL-15-cultured $CD8^+$ T cells. Controls included mouse IgG (negative) and OKT3 (positive) antibodies. As shown in Figure 4A, control (mIgG-treated) IL-15-cultured $CD8^+$ T cells already expressed low but detectable levels of CD107a, which was not observed in freshly isolated $CD8^+CD56^-$ T cells (data not shown), confirming that after culture with IL-15 $CD8^+$ T cells acquire a cytotoxic potential compared with resting $CD8^+$ T cells. As expected, OKT3 cross-linking induced a marked increase in CD107a levels (Figure 4A) in almost all cells, indicating that IL-15-cultured $CD8^+$ T cells are highly prone to respond to TCR/CD3 stimulation. Interestingly, regarding degranulation after NK receptor specific cross-linking, contrarily to the other NKR studied, NKG2D was *per se* capable of inducing a statistically significant increase in the percentage of CD107a expressing cells when compared to the control (mIgG) (Figure 4A).

Cytokine detection was assessed after 48h of NK receptor cross-linking by using *fluorescent bead immunoassay* for IFN-γ, TNF-α, IL-10, IL-1β and MIP-1β, as described in *Material and Methods*. Figure 4B shows representative dot-plots illustrating cytokine discrimination (according to FSC/SSC parameters and FL-3 fluorescence intensity) and quantification (according to FL-2 fluorescence intensity). As shown in Figure 4C, $CD8^+$ T

cells cultured 12 days with IL-15 (mIgG-treated) produced measurable amounts MIP-1β (4381±2739 pg/ml), TNF-α (1043±212.7 pg/ml) and IFN-γ (1825±1228 pg/ml), and also slight amounts of IL-10 (183±60.21 pg/ml) and IL-1β (202.7± 96.67 pg/ml). IL-6 was not detected in the supernatants. After CD3 cross-linking, there was a marked increase in IFN-γ, IL-10, IL-1β and TNF-α secretion, with IFN-γ being the most abundant cytokine detected in the supernatant (about 30000 pg/ml) (Figure 4B and 4D). Interestingly, cross-linking of the different NKR under study produced contrasting outcomes in cytokine secretion. Thus, while cross-linking of CD56, NKp46 or NKG2D did not result in significant changes in the level of any of the cytokines analyzed when compared to control (mIgG-treated) cells, cross-linking of KIR2DL2/3, KIR2DL4 and NKG2A resulted in a statistically significant increase in the secretion of IFN-γ, TNF-α, IL-10 and IL-1β (Figure 4D).

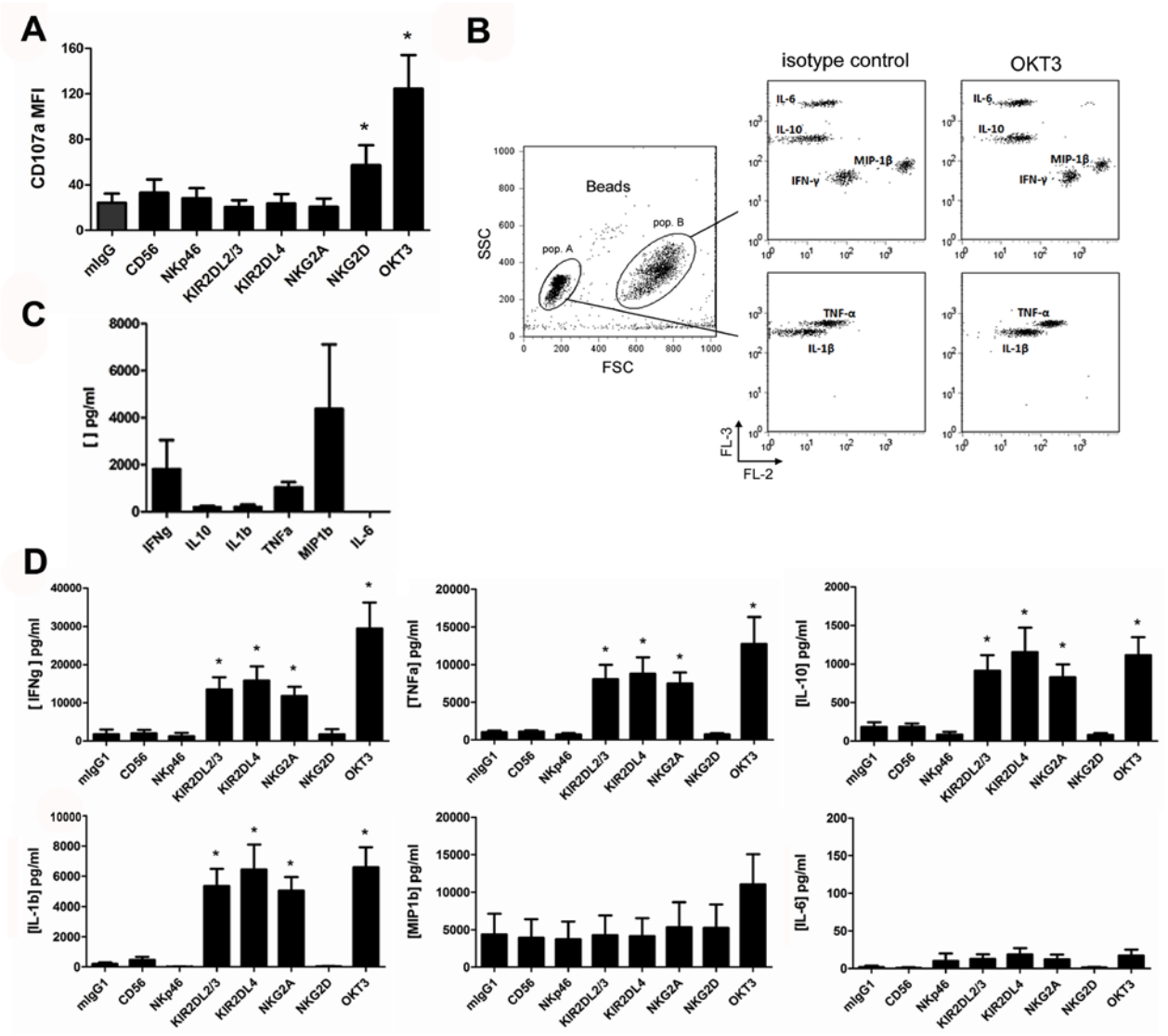


Figure 4. NKR receptors induced by IL-15 are functional and capable of modulating cytokine secretion. Purified $CD8^{+}CD56^{-}$ T cells were obtained, cultured with IL-15 (10ng/ml) for 12 days and analyzed for CD107a expression after mAb crosslinking, as described in *material and methods*. (A) The graph represents CD107a

MFI levels after crosslinking of the different NKR and OKT3. Mouse IgG antibodies were used as control. Data is presented as mean±SEM, n=7, *P< 0.05. (B-D) Cytokine secretion was assessed by fluorescent bead immunoassay (Flowcytomix), as described in the *material and methods*. (B) Dot-plot illustrating a representative experiment of how the different cytokines were quantitated (see *material and methods*) without cross-linking or after OKT3 cross-linking (isotype control). (C) Graph showing the quantification of each cytokine described (pg/ml) in the absence of NKR triggering. (D) Graphs representing the quantification of each described cytokine (pg/ml) after the cross-linking of each NKR (with mIgG and OKT3 included). Data is presented as mean± SEM, n=5, *P< 0.05.

2.5. Discussion

The common γ chain cytokine IL-15 is considered a crucial factor for $CD8^+$ T cell homeostasis and the maintenance and generation of memory $CD8^+$ T cells (12-13). Despite the existence of work documenting the existence of cell surface NKR acquisition by a subset of memory $CD8^+$ T cells, the specific signals that control their expression have, to date, not been clearly defined. In this study, we have unambiguously established by using an *in vitro* model of human $CD8^+$ T cell differentiation that IL-15 is one environmental factor responsible for the acquisition of NKR by $CD8^+$ T cells, closely linked with the acquisition of an effector-memory T_{EM}/T_{EMRA} phenotype. Also, we have found that our *in vitro* IL-15-differentiated $CD8^+CD56^+$ T cells express high levels of the anti-apoptotic protein Bcl-2 and large amounts of intracellular granzyme B and perforin, reinforcing the role of IL-15 as a cytokine responsible for $CD8^+$ T cell survival (12), and suggesting a link between CD56 acquisition by $CD8^+$ T cells and increased resistance to apoptosis concomitantly with increased cytotoxic potential. Interestingly, this phenotype is reminiscent of different “NK-like T cells” described *in vivo* (2, 9, 14-18). In line with these results, IL-15-cultured $CD8^+CD56^-$ T cells have shown TCR-mediated and NK-like cytotoxicity, indicating that IL-15-induced differentiation was associated with acquisition of effector functions.

Importantly, in this study we have shown that IL-15-induced $CD8^+CD56^+$ T cells co-express several activating and inhibitory NKR, including KIR members, NKG2A, NKG2D and the bona-fide natural killer receptor NKp46. Although the consequences of NKR expression for the function of $CD8^+$ T cells after TCR engagement have been described (19), we wanted to ascertain the functional outcome of engagement of the acquired NKR alone. Accordingly, their role in two important $CD8^+$ T cell effector functions, release of cytotoxic granules and cytokine secretion, were dissected. The degranulation assays revealed three important facts. First, that CD3 triggering induced high levels of

degranulation, indicating that IL-15-cultured $CD8^+$ T cells are functional and capable to respond to TCR/CD3-mediated stimuli, as also shown by redirected cytolysis with OKT3. Second, that NKG2D triggering itself was capable of leading to a significant increase in the CD107a levels, which is of note since NKG2D has been shown to function only as a co-stimulatory receptor in human $CD8^+$ T cells, incapable of inducing cytotoxicity by itself (4-5). In agreement with our results, Meresse *et al* have shown there is a the presence of IL-15 (20), $CD8^+$ T cells can acquire the ability to kill NKG2D ligand-bearing targets, which could be related with an increase in NKG2D and DAP10 levels after IL-15 culture and a coordinated IL-15 induction in PI3K and JNK pathways (20). Interestingly, Horng *et al* have found that after activation by IL-15, Janus kinase 3 (Jak3) phosphorylated DAP10, suggesting an association between DAP10 and IL-15R (21). It was also consistent with the fact that, in our study, although almost all purified $CD8^+CD56^-$ T cells expressed NKG2D at day 0, they were not capable of inducing cytotoxicity. Finally, and contrary to NKG2D, any of the other NKR under study were unable to induce a significant effect in degranulation after triggering, which could be due either to the low percentages of $CD8^+$ T cells expressing those NKR and/or to the fact that they are instead involved in cytokine secretion in these conditions (see below).

In the past, $CD8^+$ T cells have been regarded as a homogeneous population of cytotoxic cells producing only a limited number of cytokines, however, it is now becoming clear that $CD8^+$ T cells have the potential to produce a wider array of cytokines. In this study, we have shown that IL-15 cultured $CD8^+$ T cells were capable to secrete IFN-γ, TNF-α, IL-1β and predominantly MIP-1β without further re-stimulation, which agrees with the effector memory phenotype displayed (7, 22-23). Indeed, late memory maturation has been associated with CC chemokine production, particularly by a prominent MIP-1β expression (23). The effect of NK receptor cross-linking had interesting results. Noteworthy, besides having a significant effect in increasing cytotoxicity of IL-15-cultured $CD8^+$ T cells (see above), NKG2D triggering did not lead to an increase in IFN-γ production or any other cytokine studied. These results are in accordance with other studies showing that, contrarily to NK cells, NKG2D in human $CD8^+$ T cells seems to be primarily involved in the regulation of cytotoxic activity and not cytokine secretion (20, 24), which could be probably related with the absence of DAP12 in $CD8^+$ T cells (24-25).

Remarkably, triggering of KIR2DL2/3, KIR2DL4 and NKG2A in IL-15-induced $CD8^+NKR^+$ T cells induced a strong and significant increase in the secretion of IFN-γ, TNF-α, IL-1β and IL-10. KIR2DL4 engagement has been described to induce strong IFN-γ production in

NK cells (26-27), and also induction of TNF-α and MIP-1β have been reported (27-28). In T cells, only a recent study it has been shown modest IFN-γ secretion by *ex vivo* lupus T cells upon KIR2DL4 engagement (29). Similarly to NK cells, our results point to KIR2DL4 as a receptor that fine-tune cytokine secretion by human KIR^+CD8^+ T cells rather than increasing cytotoxicity. Even though KIR2DL4 triggering does not induce cytotoxic activity, it has been pointed as a more activating than inhibitory KIR in NK cells due to its involvement in IFN-γ secretion (26). However, we have shown that similarly to KIR2DL4, triggering of the inhibitory KIR2DL2/3 and NKG2A receptors also lead to the secretion of those cytokines, which represents a novel and intriguing finding. Indeed, to our knowledge, this is the first description that the triggering of inhibitory NK receptors on IL-15-induced $CD8^+NKR^+$ T cells could by themselves trigger such cytokine secretion pattern.

KIR2DL4 exhibits structural features of both activating and inhibitory KIR, and IFN-γ secretion has been shown to be dependent on its charged arginine residue (27). Considering that the other inhibitory NKR lack the charged aminoacid residue, it is possible to speculate that they may use alternative signaling pathways leading to cytokine production. Indeed, it has been shown that tyrosine phosphatase SHP-2 could also function as an SH2-adaptor initiating downstream activating cascades as ERK and PI3K (30-31), pathways reported to be linked with the production of IFN-γ by NK cells (32-34). In fact, PI3K coupling to inhibitory KIR has already been described (35), suggesting that KIR triggering could lead to T cell decreased apoptosis by a PI3K-mediated Akt activation (36). Importantly, IL-15 has been shown to activate the ERK and PI3K-Akt pathways (37), pointing to the possibility that the inhibitory NKR expressed by $CD8^+$ T cell in the presence of IL-15 could make use of alternative pathways that will lead in turn to cytokine secretion.

Noteworthy, the cytokine secretion pattern observed in this study is reminiscent of a Tc1 (IFN-γ and TNF-α) and Tc2 (IL-10) profile (38), suggesting that IL-15-induced $CD8^+NKR^+$ T cells could display an immunoregulatory role. While IL-10 is a cytokine with well known anti-inflammatory and immunoregulatory functions (39), involved in the generation of T regulatory cells (40), IFN-γ is strongly associated with pro-inflammation and immune activation (41). However, IFN-γ has also been reported to be a cytokine capable of suppressing T cell responses and inducing regulatory T cells (41). In addition, a relationship between NKG2A and IFN-γ has been previously described. Indeed, NKG2D has been shown to be synchronously expressed with intracellular IFN-γ in $CD3^+CD8^+$ tumor infiltrating lymphocytes (TILs) (42). Furthermore, exogenously added IFN-γ has

been shown to protect ovarian carcinoma cell lines from CTL lysis via a NKG2A-dependent mechanism, by increasing the expression of HLA-E ligands on the target cells (43), and to facilitate viral evasion from NK killing by a similar mechanism (44). In this context, it is tempting to speculate that triggering of inhibitory NKR on IL-15-induced $CD8^+NKR^+$ T cells could lead to a robust IFN-γ production that in turn would lead to an increase in the amount of their cognate ligands in target cells, pointing to inhibitory NKR themselves as part of an intricate mechanism that could ultimately result in impaired antiviral and anti-tumoral $CD8^+$ T cell function. Intriguingly, a population displaying a memory phenotype characterized by lack of CD28 and expression of CD56 and capable to produce IFN-γ, TGF-β and IL-10, has been described within suppressor $CD8^+$ T cells (45-46). Also, TNF-α and IL-1β have been shown to induce apoptosis and suppression of immune cells, suggesting that they may also display a suppressor activity (47-48). Accordingly, the possibility that IL-15 is generating effector-memory $CD8^+CD56^+$ T cells co-expressing inhibitory NKR and endowed with suppressor activity is a plausible scenario that deserves further investigation. A recent report describing that the IL-10/IFN-γ pathways are essential to the potent immunosuppressive activity of cultured $CD8^+$ NK-like T cells towards naïve responder T cells (49) supports this assumption.

Finally, our results strengthen our previous hypothesis that IL-15-differentiated $CD8^+CD56^+$ T cells could account for the high percentage of NK-like T cells present in human livers. Indeed, we have found that they have phenotypic and functional similarities, namely being effector-memory $CD8^+CD56^+$ T cells expressing other NKR, (9, 50), endowed with capacity to display TCR-mediated and NK-like cytotoxicity (9, 51) and to produce Tc1 and Tc2 cytokines after stimulation (51). In this context, it can be proposed that IL-15 could function as a putative regulator of NKR expression which in turn may shape $CD8^+$ T cell functional phenotype in a tissue-specific manner.

In summary, it is possible to speculate that IL-15 enriched microenvironments could induce $CD8^+$ T cell differentiation into effector NK-like T cells by the acquisition of functional NKR capable of modulating both cytotoxicity and cytokine release. The outcome of cytokine secretion by triggering of inhibitory NKR could suggest that $CD8^+NKR^+$ T cells might also have regulatory functions, bridging innate and acquired immunity through an intricate NK receptor triggering balance.

2.6. References

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III – GENERAL DISCUSSION

Preface

The view of the liver as an organ with immunological importance has increased during the last years. Interestingly, the liver has been pointed as a place where primary activation of $CD8^+$ T cells can occur, namely by hepatocytes. This hepatocyte-driven antigen activation of $CD8^+$ T cells has been associated with "death by neglect" due to the lack of co-stimulation, which was assumed to be important for the immunotolerance needed in the liver (1-2). Noteworthy, both resident and circulating lymphocytes have been found to directly interact with hepatocytes through endothelial fenestrations in normal conditions, which seem to be unique between solid organs (3). However, the role that hepatocytes might have in T cell contact in the absence of antigen presentation was, to date, scarce or even absent, particularly in the human context. Besides, the liver comprises a unique resident intrahepatic lymphocyte population predominantly enriched in NK cells, NKT cells and $CD8^+$ T cells with an activated/memory phenotype (4-8). Interestingly, human livers are mainly enriched in CD1d-unrestricted NKT cells that are $CD8^+CD56^+$ co-expressing other NK receptors, and are more present in the liver than in any other place in the human body (9-11). The origin of intrahepatic lymphocytes is still controversial and could involve specific recruitment and entrapment from the periphery and/or local development from hematopoietic cells reminiscent from fetal liver (12). However, independently of its origin, it is plausible to think that the hepatic microenvironment - namely integrated signals from surrounding cells and the cytokine milieu - should favor the subsets that are mainly enriched in the liver.

IL-15 is a gamma-common cytokine known to have a crucial role in NK and NKT cell development, homeostasis and function, as well as to be of major importance in $CD8^+$ T cell homeostasis. This cytokine has been widely associated with tissue-specific functions (13-15) and, interestingly, has been found to be constitutively expressed in the liver. Noteworthy, observing the lymphocyte subsets enriched in human livers and the populations primarily favored by IL-15, there is a clear correspondence, making thus tempting to speculate that IL-15 could have a role in the shaping of the intrahepatic lymphocyte population. Besides, the increased expression of IL-2/IL-15Rβ on hepatic lymphocytes, even within the conventional $CD3^+CD56^-$ T cells (14), strengthens the suggestion that IL-15 might indeed have an important function in shaping the intrahepatic lymphocyte population.

Hepatocytes and IL-15: a favorable microenvironment for T cell survival and proliferation

◦ *Hepatocytes: a role in T cell maintenance*

In this work, by performing *in vitro* co-cultures with human hepatocyte cell lines (HepG2 cells and immortalized human hepatocytes) and *ex-vivo* isolated human peripheral blood lymphocytes, we have gathered novel data regarding hepatocyte/T cell interaction in the context of T cell survival, activation and proliferation. We have shown that human hepatocytes were capable of delivering strong survival signals to T cells and to sustain basal levels of T cell proliferation. Indeed, although hepatocytes have been described to drive $CD8^+$ T cell activation (1-2, 16), those studies where mainly performed using mice models and merely in the context of antigen presentation. Besides, this $CD8^+$ T cell Ag-driven activation was reported to result in apoptosis due to lack of co-stimulation (1-2, 16). In fact, the liver has been largely associated with activated $CD8^+$ T cell death (see General Introduction), thus the finding that in the absence of Ag-presentation, hepatocytes have instead a direct positive effect on T cell survival, namely in $CD8^+$ T cells, is a novel and interesting finding. Interestingly, soluble factors released by hepatocytes could display some anti-apoptotic effect, suggesting the establishment of a favorable environment for T cell survival even in the absence of T cell contact, broadening the extent of action of the hepatocyte-driven survival effect. Thus, while in antigen-presentation conditions hepatocytes might lead to T cell death, needed for tolerance, in homeostatic conditions they might function as important cells to induce survival. The combination of both increased survival and basal proliferation suggests that hepatocytes might play an **important role in T cell homeostasis**. Indeed, since hepatocytes represent the most abundant cells present in the liver (70-80%) (5) it is plausible to think that they might contribute to create a favorable microenvironment for both peripheral and local lymphocyte homeostasis.

◦ *IL-15: increased T cell survival and sustained $CD8^+$ T cell proliferation*

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 (13-15). Furthermore, IL-15 has been described to be constitutively found in human livers (14, 17-18) and several cell types present in the liver, including dendritic cells (19-20), infiltrating monocytes (14), Kupffer cells (14), and Ito cells (21) have been shown to be capable of expressing IL-15 protein and can thus be pointed to function as truly physiological sources. Besides, although it has been reported that IL-15 exists mainly in a membrane-bound manner, it

has been also found in its soluble form in the liver (14). Indeed, the detection of IL-15 in supernatants of cultured biopsies, in the presence and absence of exogenous stimulation, led Golden-Mason and co-workers to suggest that the IL-15 protein is secreted and available in the liver in adequate amounts to exert its effect on responsive cells (14). In this line, we have performed *in vitro* cultures of human peripheral blood lymphocytes or purified $CD8^+$ T cells with exogenously added IL-15(1). Herein, we have clearly shown that IL-15 is able to **induce considerable levels of proliferation,** particularly driving $CD8^+$ T cell subset into several cycles of division without the need of any additional stimuli. Concomitantly, IL-15 was capable to strongly sustain **T cell survival** (chapter II, 1. and 2.), which was namely associated with an increasing in the levels of the antiapoptotic protein Bcl-2 (chapter II, 2.).

- ***Hepatocytes and IL-15: a role in peripheral and local homeostasis and in the shaping of the intrahepatic lymphocyte population?***

In the context of the initial question of this thesis, we have elucidated two important previously unanswered issues. First, we went to ascertain if the IL-15 biological functions described above are maintained in the perspective of a hepatocyte/T cell interaction by performing *in vitro* co-cultures of hepatocyes with peripheral blood lymphocytes or purified $CD8^+$ T cells in the presence of IL-15. Interestingly, we have found that IL-15 functions were preserved in the presence of hepatocytes, contrarily to what happened when two other epithelial cell lines of different origin (AGS and HeLa) were used, which negatively impacted IL-15-induced $CD8^+$ T cell proliferation and survival (Chapter II, 1.). Of note, in some experiments hepatocytes and IL-15 were even capable of synergistically act in increasing $CD8^+$ T cell proliferation and survival (Chapter II, 1.). Additionally, we have shown for the first time that hepatocytes can express IL-15 protein at cell surface and, although it has apparently no effect in T cell survival and proliferation through *trans*-presentation, the possibility that it might have a role in pathological conditions, in which IL-15 has been described to be increased, and/ or have instead a role for the hepatocyte itself by reverse signaling (see General Introduction), are issues that deserve to be further clarified.

(1) Note: it is crucial to stress that, although IL-15 has been found in a soluble form in the liver, we consider that *in vivo* it is very likely that IL-15 is mainly *trans*presented to $CD8^+$ T cells (see Introduction). Indeed, the aforementioned producers of IL-15 present in the liver, as dendritic cells and macrophages, have been widely described to be capable of express IL-15 at cell surface and to present it in *trans* to $CD8^+$ T cells. Interestingly, Ito cells were shown to promote homeostatic proliferation of liver NKT cells through cell surface Interleukin-15 (21). Nevertheless, while the bioactive form of IL-15 has been found to be mostly cell surface-bound, the biological functions appear to be correspondent to when soluble or plate-bound IL-15 is used; in fact, the major difference seems to be related with a longer half-life and the need of smallest cytokine amounts when IL-15 is in a membrane-bound form.

Thus, hepatocyte and IL-15 delivered signals acting together might contribute to create a favorable microenvironment for **peripheral homeostasis** of circulating T lymphocytes. Indeed, after leaving the thymus, T cells depend on the integration of a multitude of signals given by the different microenvironments they found while in circulation, such as cytokines and epithelial cells (22). Among cytokines, an especial interest has been given to gamma-common cytokines, and particularly to IL-15, in the maintenance of peripheral $CD8^+$ T cell homeostasis (23-26), thus supporting our hypothesis. The novel finding that hepatocytes also deliver positive signals for $CD8^+$ T cell survival is of note, placing hepatocytes as likely players in peripheral lymphocyte homeostasis. These data points to the liver as a place where positive homeostatic signals can be received during T cell circulation, giving a novel perspective that contrasts with the view of the liver as a mere "graveyard" for antigen-activated lymphocytes (27) (see General Introduction).

The fact that the liver might provide a favorable microenvironment for lymphocyte is not surprising if we remember that the liver includes a resident lymphocyte population (IHL). Thus, it makes sense that the integrated signals from IL-15 and hepatocytes might also be crucial to contribute for **local homeostasis** by the maintenance of the intrahepatic lymphocyte population.

Interestingly, since IHL are likely to be constitutively in close contact with both factors, receiving their influence, it is possible to speculate that they also might have a role in **shaping of the lymphocyte population composition**. Indeed, it could be suggested that the presence of IL-15 in the hepatic microenvironment might be an important factor contributing for the maintenance and enrichment of the specific subsets mainly enriched in human livers as NK cells, NKT cells, γδ T cells and $CD8^+$ T cell, namely resulting in the increased percentage of $CD8^+$ T cells found in the liver. Interestingly, we have also found that HepG2 cells and IL-15 were capable of increasing the percentage of activated lymphocytes, namely by an increase in CD69 molecule and in the percentage of blasts cells, making thus possible to consider that they might contribute to the increased number of T cells with an activated phenotype found in the liver. This is quite important since Tu and co-workers (2007) have described increased CD69 expression to be one of the most striking constitutively difference found between peripheral blood and intrahepatic lymphocytes (7).

Based on the results of this thesis, it can be proposed that **hepatocytes play an important role in delivering survival signals to CD8 T cells, while IL-15 drives both survival and activation signals leading mainly $CD8^+$ T cells into cycles of**

proliferation, which could have further implications for CD8$^+$ T cell differentiation (Figure1).

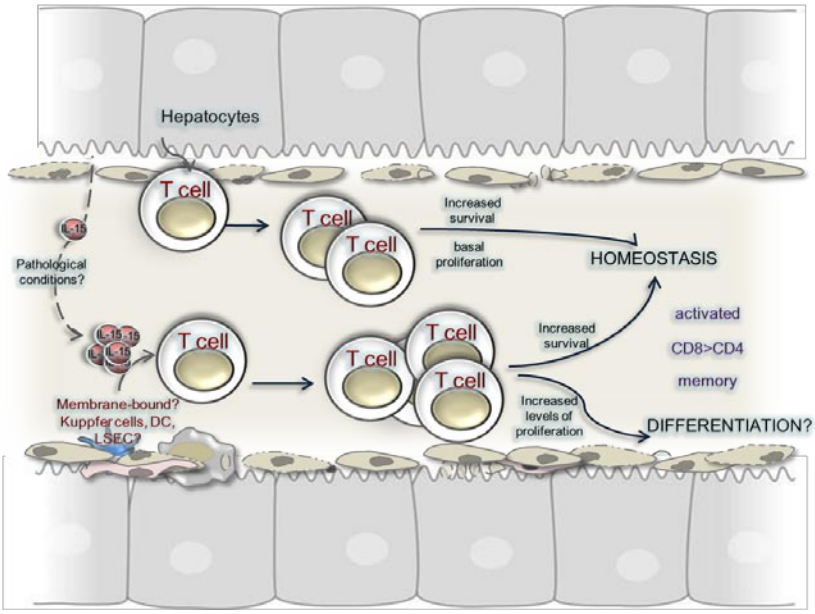

Figure 1. IL-15 and hepatocytes create a favorable microenvironment for local T cell homeostasis

Schematic representation of IL-15 and hepatocytes integrated signals in sustaining T cell survival and proliferation, contributing both for local T cell homeostasis. IL-15-driven signals induce substantial levels of CD8+ T cell proliferation, suggesting a possible additional role in driving CD8+ T cells into a differentiation program.

IL-15 and CD8$^+$ T cell differentiation: reprogramming into an NK-like phenotype

° ***NK receptor acquisition/ upregulation***

NK receptors (NKR) are classically associated with NK cells, being the balance between the triggering of inhibitory and activating receptors that will dictate their function. However, in the last years, accumulating evidence has shown that different NK receptors could also be expressed by minor subsets of αβ a γδ T cells in peripheral blood, mostly CD8$^+$ T cells (28-33). Interestingly, most of these NK receptors are not expressed in naïve TCRαβ T cells and have been related with T cell activation (34-35). However, the signals that leads to NKR acquisition remains largely unknown. As mentioned above, we and others have found that IL-15 is capable to directly drive increased levels of CD8$^+$ T cell activation and proliferation, alongside with sustained survival, creating thus favorable conditions for CD8$^+$ T cell differentiation to occur. Hence, since IL-15 is a cytokine bridging innate and acquired immunity by exerting important functions in NK and NKT cells as well as in CD8$^+$ T cells, we have questioned whether this cytokine could be involved in CD8$^+$ T cell differentiation towards the acquisition of NK receptors.

In this thesis, we have shown that IL-15 was capable of inducing antigen-independent upregulation of several NK receptors in purified $CD8^+CD56^-$ T, both at mRNA (Chapter II, 1.) and protein level (Chapter II, 1. and 2.). Within the studied receptors there were functionally distinct NKR (both inhibitory and activating) and belonging to different NKR families, including KIR (KIR2DL2/3, KIR2DL4), lectin-like (NKG2A, NKG2D), NCR (NKp46) and the NK cell marker CD56. Since the initial $CD8^+$ T cell population was completely CD56-negative, the acquisition of CD56 was clearly correspondent to *de novo* expression, which was also observed for NKp46, both in peripheral blood and umbilical cord blood. Interestingly, NKR acquisition/upregulation was shown to be intrinsically related with CD56 acquisition (Chapter II, 2.), which may have implications to understand NKR expression regulation and $CD8^+CD56^+$ T cell biology, as discussed later on.

Earlier studies have already described that $CD56^+$ T cells can be expanded from fresh PBMC by culturing with IL-15 (36-37). However, in this thesis, we have clearly **demonstrated that IL-15 was capable of inducing *de novo* CD56 expression in purified $CD8^+CD56^-$ T cells**, which is a novel and interesting finding, supporting and extending previous studies performed with bulk CD56-depleted peripheral blood mononuclear cells (PBMC) (38) or in umbilical cord blood (UCB) (39). The fact that a similar IL-15-driven *de novo* expression could occur for the natural cytotoxicity receptor (NCR) NKp46, is noteworthy. Indeed, initial reports showed that unlike other NK cell-associated receptors, NCR were restricted to NK cells and not expressed on T cells (40-44), pointing NKp46 as the most bona-fide NK cell marker. A similar IL-15-induced NCR acquisition was described in umbilical cord blood (39). Also, Jabri and co-workers have demonstrated that intestinal epithelial lymphocytes express functional NKp44 and NKp46 in patients suffering from celiac disease, a condition known to be intrinsically related with IL-15 upregulation (45). In this context, together with those studies, our work **challenges the dogma that NCR are uniquely expressed by NK cells, pointing to IL-15 as a crucial factor involved in NKp46 acquisition by $CD8^+$ T cells**.

Remarkably, in addition to CD56 and NKp46 *de novo* acquisition, we have shown here that IL-15 was also capable to coordinately induce upregulation of Killer Ig-like (KIR2DL2/3, KIR2DL4) and also lectin-like (NKG2A, NKG2D) receptors in $CD8^+$ T cells. NKR induction in T cells has been mainly associated with antigen-specific expansion and, although a few studies pointed to IL-15 as having a possible role in the induction of CD94/NKG2A, this was only reported in TCR-activated T cells (46-47), with no effect on KIR expression (46-47). Thus, it has been widely described that CD94/NKG2A receptor

cell-surface expression on T cells is modulated by exposure to antigen, whereas signals controlling the expression of KIR are to date unknown (48). Though, the work presented in this thesis suggests that IL-15 is capable to induce both NKG2A and KIR (KIR2DL2/3, KIR2DL4, KIR3DL2) upregulation in $CD8^+$ T cells in the absence of antigen activation (Chapter II, 1. and 2.). Since the initial purified population of $CD8^+CD56^-$ T cells could already express minor levels of KIR and NKG2A, one could question whether the increased percentage of NKR expressing $CD8^+$ T cells could result from proliferation of these pre-existent NKR^+CD8^+ T cells. However, several facts support a putative *de novo* acquisition of those receptors. First, we have shown that the combination of α-CD3/ α-CD28 triggering, although inducing even higher levels of proliferation, was incapable of increasing the percentage of NKR^+ expressing $CD8^+$ T cells (Chapter II, 1.). Second, it was seen an mRNA increase for the NKR, without showing any proliferative advantage of NKR^+ $CD8^+$ T cells (Chapter II, 1.). Third, NKR expression was shown to be closely linked with CD56 *de novo* acquisition (Chapter II, 2.), further suggesting that NKR can be specifically induced by IL-15 in purified $CD8^+$ T cells. Indeed, a careful observation of NKR expression in parallel with CFSE halving suggests that *de novo* NKR expression occurs in cells that have entered into several cycles of proliferation, while NKR expression in cells that did not proliferated is probably related with the pre-existent NKR^+ cells (Chapter II, 1. and 2.). Thus, the work presented in this thesis represents the first **evidence pointing to IL-15 as a possible factor involved in KIR and NKG2A upregulation in the absence of TCR activation**.

On the other hand, concordantly with previous reports (49-51), NKG2D was the only NKR constitutively expressed by almost all $CD8^+CD56^-$ T cells. Interestingly, after culture with IL-15, there was an obvious increase in the NKG2D levels of expression (Chapter II, 1. and 2.). Previous reports showing NKG2D increased expression in intraepithelial CTL after culture with IL-15 and in the absence of any additional stimuli (52-54), further supports our results.

- ***IL-15 generated $CD8^+NKR^+$ T cells: effector memory T cells resistant to apoptosis with increased cytotoxic potential***

Herein, we have found that IL-15-induced $CD8^+CD56^+$ T cells display a **phenotype consistent with a progressive differentiation status toward an effector memory (T_{EM})/$CD45RA^+$ effector memory (T_{EMRA}) phenotype**, which is in agreement with previous studies showing a role of IL-15 in the generation and maintenance of memory $CD8^+$ T cells (55-57). Interestingly, this phenotype is reminiscent of the described NK-like

T cell phenotype ($CD56^+$ T cells, KIR^+ T cells and hepatic NKT cells) found *in vivo* (30, 58-62). Indeed, KIR expression has been correlated with T cell differentiation, being proposed that KIR expression and diversification may occur after TCR rearrangement (59-60, 63-64) and at the transition from effector to memory T cells in $CD8^+$ T cells *in vivo* (58). However, the finding that KIR expression could be associated with an effector-memory phenotype has been interpreted to reflect a prior history of chronic antigen or autoantigen exposure (65). In this context, our work presents new evidences showing that **acquisition of a memory phenotype and acquisition of NK receptors in $CD8^+$ T cells could be a coordinated event resulting from IL-15-mediated signals.** Furthermore, we have also found that $CD8^+CD56^+$ T cells express higher levels of Bcl-2 compared with $CD8^+CD56^-$ T cells, suggesting that $CD8^+NKR^+$ T cells are **more resistant to apoptosis**. This is in agreement with clonal analysis studies of human T cells indicating that the KIR- population is more susceptible to activation-induced cell death (AICD), while the KIR^+ population is most resistant and has higher levels of Bcl-2 survival protein (58). Indeed, Young and Uhrberg (2002) proposed that KIR^+ T cells were AICD resistant cells designated to become a memory population initiating the expression of genes encoding KIR and acquiring resistance to apoptosis through increased levels of Bcl-2 expression (66). Thus, our present results strongly support and extend this supposition by placing **IL-15 as a factor involved in the generation and maintenance of memory $CD8^+$ NKR^+ T cells endowed with higher survival capacity.** In parallel, we have shown that those $CD8^+CD56^+$ T were mainly granzyme B^+/$perforin^+$, indicating that **CD56 acquisition was paralleled by increased cytotoxic potential**. In agreement, previous studies have shown $CD8^+CD56^+$ T cells (67-69) and $CD8^+KIR^+$ T cells (61, 70) to express increased levels of granzyme B and perforin.

In summary, the results shown in this thesis strongly suggests IL-15 as a microenvironmental factor capable of driving $CD8^+CD56^-$ T cells into several cycles of division that could lead them to enter into a differentiation program towards the acquisition of NK receptors. This will ultimately result in the generation of a pool of effector-memory $CD8^+CD56^+$ T cells expressing other NK receptors and endowed with improved survival and increased cytotoxic potential (Figure 2).

- ***IL-15 cultured $CD8^+$ T cell population is capable of displaying both TCR-mediated and NK-like cytotoxicity and to secrete Tc1/Tc2 cytokines***

Interestingly, we have found that IL-15 was capable to generate an effector population of CD8+ T cells capable of displaying both TCR-mediated and also non-MHC restricted or

NK-like cytotoxicity. CD8+CD56+ T cells have been pointed to be the functional population endowed with the ability to display **NK-like cytotoxicity** (67-68), being CD56 considered a marker of cytotoxicity in T cells (69). Also, we have demonstrated that IL-15-cultured CD8+ T cells were endowed with the ability to **secrete a Tc1/Tc2 pattern** (IFN-γ, TNF-α, IL-1β, MIP-1β and IL-10) (Figure 2). The increased expression of MIP-1β without further re-stimulation is concordant with the memory phenotype displayed by these cells, being this chemokine considered an important marker of this maturation stage (71). Also, a cytokine polarization towards a Tc1 pattern (IFN-γ and TNF-α) is consistent with IL-15 culture conditions and with the effector-memory phenotype (24, 72-74). **Noteworthy, the capacity to display a Tc1/Tc2 regulatory cytokine secretion pattern was clearly evident after TCR stimulation, a described property of innate lymphocytes, such as invariant NKT cells and γδ T cells** (75-79). Interestingly, the ability to express a Tc1/Tc2 cytokine pattern has been reported by *ex vivo* hepatic T and $CD56^+$ T cells from human livers (4). Of note, in agreement with our results, Kelly-Rogers *et al* (2006) have found that PHA/IL-2 expanded PBMC were likely to produce cytokines consistent with a Tc1 (IFN-γ) and Tc2 (IL-4 and IL-13) pattern and displayed both MHC-restricted and unrestricted cytotoxicity (80). Also, importantly, they have found that within this population, $CD56^+$ T cells where the responsible for the capacity to display NK-like cytotoxicity and shown to express higher levels of IFN-γ, IL-4 and IL-13 cytokines compared with $CD56^-$ T cells (80). This strongly suggests that within our IL-15-cultured $CD8^+$ T cell population, $CD8^+CD56^+$ T cells should be the main responsible for NK-like cytotoxicity and increased cytokine release.

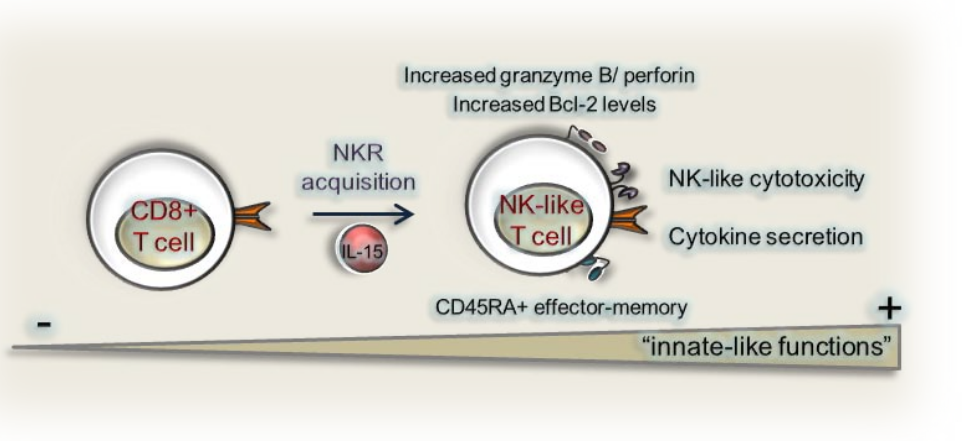

Figure 2. IL-15-driven CD8+ T cell reprogramming into NK-like T cells

IL-15 is capable of driven CD8+ T cells into a differentiation program towards the acquisition of NK receptors, resulting in the generation of a pool of CD45RA+ effector-memory phenotype cells with increased survival (Bcl-2) and endowed with increased cytotoxic potential (granzyme B/perforin). The phenotypic reprogramming into NK-like T cells was shown to be paralleled with the acquisition of "innate-like" functions, capable of displaying NK-like cytotoxicity and cytokine secretion.

In summary, IL-15-induced differentiation was found to be associated with acquisition of effector functions, supporting the view that IL-15 can initiate a $CD8^+$ T cell reprogramming process into NK-like T cells and that it occurs, not only at the phenotypic level, but concurrently at the functional level, displaying functional properties similar to innate lymphocytes as γδ T cells and invariant NKT cells (4, 29, 36, 67-69, 81-83) (Figure2).

◦ ***IL-15-cultured $CD8^+$ T cells and NKR crosslinking: cytotoxicity and cytokine secretion***

Increasing evidence indicates NK receptor expression in $CD8^+$ T cells could modulate T cell function, by increasing or decreasing TCR threshold accordingly to the triggering of activating or inhibitory NK receptors. However, the impact that NK receptors themselves might have on $CD8^+$ T cell function has been scarcely explored. Thus, we went to ascertain the functional outcome of NKR triggering on the $CD8^+NKR^+$ population, which gave some interesting and intriguing results.

◦ *NKG2D was capable to induce per se increased levels of degranulation after mAb crosslinking but not IFN-γ secretion*

NKG2D expression in $CD8^+$ T cells has been mainly pointed to serve as a co-stimulatory receptor (33, 50). Interestingly, we have shown here that NKG2D was capable by itself to increase cytotoxicity after mAb crosslinking in IL-15-cultured $CD8^+$ T cells. As already discussed (see Chapter II, 2.), it has been suggested a link between IL-15 and NKG2D. Thus, besides increasing NKG2D and DAP10 levels (54, 84), IL-15 could be involved in inducing DAP10 phosphorylation (85) and in interacting with downstream signaling pathways as JNK and PI3K (54). Accordingly, in the context of IL-15, NKG2D could function as a functional receptor in $CD8^+$ T cells capable of displaying cytotoxicity independently of TCR activation (see Figure3). Importantly, NKG2D function was shown to be restricted to

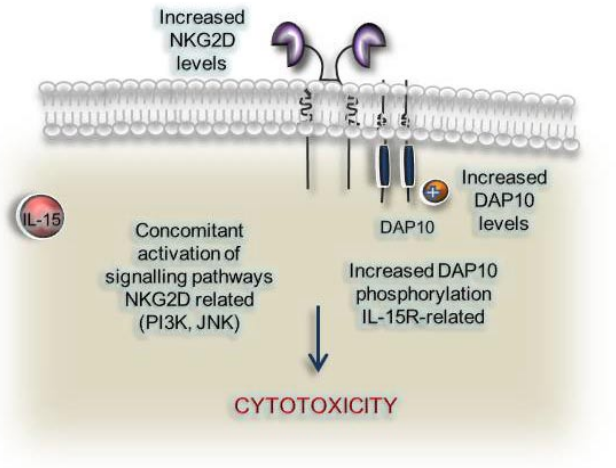

Figure 3. NKG2D is capable *per se* of triggering cytotoxicity in CD8+ T cells in the presence of IL-15

In CD8+ T cells, NKG2D has been described to function only as a co-receptor. However, in presence of IL-15 NKG2D engagement in CD8+ T cells was shown to be capable of induce cytotoxicity by itself, which could be related with increased NKG2D levels, increased DAP10 and/or activation of downstream signaling pathways induced by IL-15.

cytolytic function, since NKG2D crosslinking did not induced $CD8^+$ T cells to secrete cytokines, even in the context of IL-15 stimulation, which is concordant with previous studies (33, 50, 53-54) and might be related with the absence of DAP12 in $CD8^+$ T cells (86-87).

- *NKG2A and inhibitory KIR crosslinking lead to increased Tc1/Tc2 cytokines production: regulatory role?*

Herein, we have found that KIR2DL2/3, KIR2DL4 and NKG2A crosslinking in IL-15-induced $CD8^+NKR^+$ T cells induced a strong and significant increase in the secretion of IFN-γ, TNF-α, IL-1β and IL-10. As discussed (see Chapter II, 2 and also General Introduction), KIR2DL4 engagement has been described to induce strong IFN-γ production and other cytokines in NK cells (88-91) and, although novel for T cells, this result was not completely surprising. However, the fact that triggering of the inhibitory **KIR2DL2/3 and NKG2A receptors also lead to the secretion of such a Tc1/Tc2 pattern of cytokines represents a novel finding**. IL-10 is a cytokine with well known anti-inflammatory and immuregulatory functions (92-93), although, the expression of the other cytokines was rather unexpected at a first sight. However, as described in Chapter II, 2., IFN-γ besides being considered a marker of effector function and the hallmark of Th1/Tc1 pattern (94), showing a strong antitumor effect and an important anti-viral function (94-95), it has also been reported to be a suppressive cytokine secreted by T cells (94, 96). Indeed IFN-γ is now considered one of the most important endogenous regulators of the immune responses (94). Also, TNF-α and IL-1β have been shown to be capable of inducing apoptosis and suppression of immune cells, suggesting that they may also display a suppressor activity by suppressing antiself responses or prevent hypersensitivity to foreign antigen by inducing apoptosis in activated CD4+ cells (97-99).

Most interestingly, it has been shown that IFN-γ protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism, by increasing the expression of HLA- E ligand (100). This is also supported by evidence that IFN-γ can facilitate viral evasion of NK killing. The HCMV UL40 glycoprotein triggers CD94/NKG2A receptors on NK cells only in combination with IFN-γ- induced upregulation of HLA-E on APC (101). Based on these findings, Lukacher has proposed that IFN-γ could make use of inhibitory NK receptors to negatively modulate virus- and tumor- specific $CD8^+$ T cell response in vivo (102). Importantly, our present work is, to our knowledge, the first evidence showing that the **triggering of inhibitory NK receptors could lead to IFN-γ production, suggesting an intriguing regulatory mechanism by which inhibitory NK**

receptors themselves could increase the amount of ligands in target cells, ultimately resulting in impaired antiviral and anti-tumoral $CD8^+$ T cell function (Figure 4).

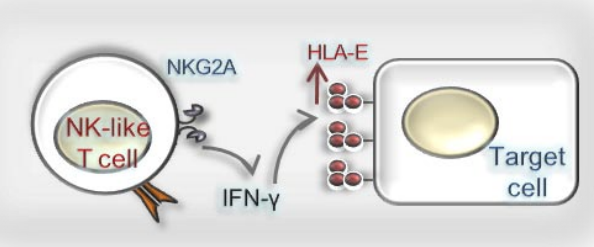

Figure 4. NKG2A crosslinking lead to IFN-γ production that will in turn lead to the increase of the ligand HLA-E in target cells

Schematic representation of a possible regulatory mechanism by which the triggering of inhibitory receptors could increase inhibition by IFN-γ release that will in turn increase the cognate ligand in target cells.

Accordingly to the cytokine secretion pattern, it could be thus suggested that IL-15-induced **$CD8^+NKR^+$ T cells could display an immunoregulatory role**. Interestingly, the term "suppressor $CD8^+$ T cells (Ts)" has been resuscitated. This is a broad term that includes several phenotypes (103-104) namely $CD8^+CD28^-CD56^+$ T cells. Importantly, IL-10, IFN-γ, MIP-1β have been described within the spectrum of cytokines secreted by suppressor CD8+ T cells (103, 105). Also, $CD8^+$CD28-$CD56^+$ T cells have been shown to display strong anti-inflammatory activity in adoptively transfer experiments (106). Thus, in view of our results and taking into consideration all these data, it is plausible to think that $CD8^+NKR^+$ could be endowed with regulatory activity capable of being displayed after inhibitory receptor triggering. Recent reports describing that the IL-10/IFN-γ pathways are essential to the potent immunosuppressive activity of cultured $CD8^+$ NK-like T cells towards naïve responder T cells (107) strongly supports this assumption. Integrating our data it is thus possible to speculate that IL-15 can induce $CD8^+$ T cell differentiation into NK-like T cells by the acquisition of functional NK receptors capable of modulating both their cytotoxic and cytokine release functions and thus conceivably capable of regulating surrounding cells.

IL-15-differentiated $CD8^+$ T cells: could they account for the liver NK-like T cells?

As mentioned above, the human liver contains the highest percentage of NKT cells of the entire body which, contrarily to mice, are mainly $CD8^+CD56^+$ CD1d-unrestricted NKT cells (see General Introduction). However, the majority of the studies on liver NKT cells have been made mainly in mice, due to the difficulty to obtain human hepatic NKT cells from healthy donors, and the few using human sources are often made based in the selection of classical CD1d-restricted NKT cells. Thus, studies regarding human NKT cells, namely at the level of function, disease involvement and also concerning their origin or development, are often confusing and not accurate, based on data extrapolated from mice

to human, making the specific studies about those NK-like T cells found in human livers scarce. Interestingly, Golden Mason and co-workers (2004) have found that the vast majority of human hepatic haematopoietic stem ($CD34^+CD45^+$) cells express IL-2/IL-15Rβ, leading them to hypothesize that IL-15 could act on lymphoid precursors, providing them with the appropriate signals to mature locally into NK/NKT cells (108). Also, as indicated by the expression of IL-15 receptors on mature lymphocyte populations, and accordingly to IL-15 function, it was further suggested that IL-15 could display additional functions in the normal liver, namely to be involved in the recruitment, maintenance and functional regulation of hepatic $CD8^+$, NK and $CD56^+$ T cell populations (108). **In the work present in this thesis, we propose that, in addition to the suggested role in homeostasis, migration or regulation of $CD8^+$, NK and NKT cells, hepatic endogenous IL-15 could also account for the large proportion of NK-like T cells found in human livers by theoretically driving a local $CD8^+$ T cell differentiation program towards the acquisition of NK receptors.** Noteworthy, the few phenotypic and functional studies describing human liver NKT cells further sustain our hypothesis, indicating them as reminiscent of our *in vitro* IL-15-generated NK-like T cells (see box 1).

Box1| Phenotypic and functional similarities of human liver NKT cells and our *in vitro* IL-15-generated $CD8^+NKR^+$	
• $CD8^+$ T cells expressing different NK receptors as CD56 and CD94/NKG2, KIR, NKG2D (7, 10, 109-112)	✓
• predominantly express a mature/activated phenotype (10)	✓
• display both TCR-mediated and NK-like cytotoxicity (4, 10)	✓
• secrete a Tc1/Tc2 pattern of cytokines (4)	✓

However, we consider that IL-15 ought to act in concert with the surrounding microenvironment in order to allow the $CD8^+$ reprogramming into NK-like T cells, only likely to take place in favorable and specific conditions. In this context, questions could be raised regarding the liver idiosyncrasies that might place it as a special candidate for an IL-15-driven $CD8^+$ T cell differentiation to occur. Indeed, as already discussed (see Chapter II, 1.), the **liver fulfills requirements that further support it as a favorable place for such a differentiation to happen**: (i) constitutively express endogenous IL-15 (17, 108), available at physiological amounts (14); (ii) contains a resident T cell population, allowing the chronic or continuous exposure to IL-15; (iii) seems to preferentially recruit and retain memory $CD8^+$ T cells (that express higher levels of IL-15R) (12); and (iv) provides survival signals sustained by surrounding cells, namely

hepatocytes as we have shown here. Thus, besides providing preferential conditions for a $CD8^+$ T cell differentiation process into NK-like T cells, the liver characteristics further supports their retention, survival, maintenance thus justifying their increased enrichment (Figure 5). Indeed, although such an IL-15-driven differentiation of $CD8^+$ T cells towards the acquisition of NKR could occur in other conditions, namely accounting for an the increase of those cells in some infections or within tumor infiltrating lymphocytes, the existence of such a higher percentage in the liver must be related with its particular characteristics.

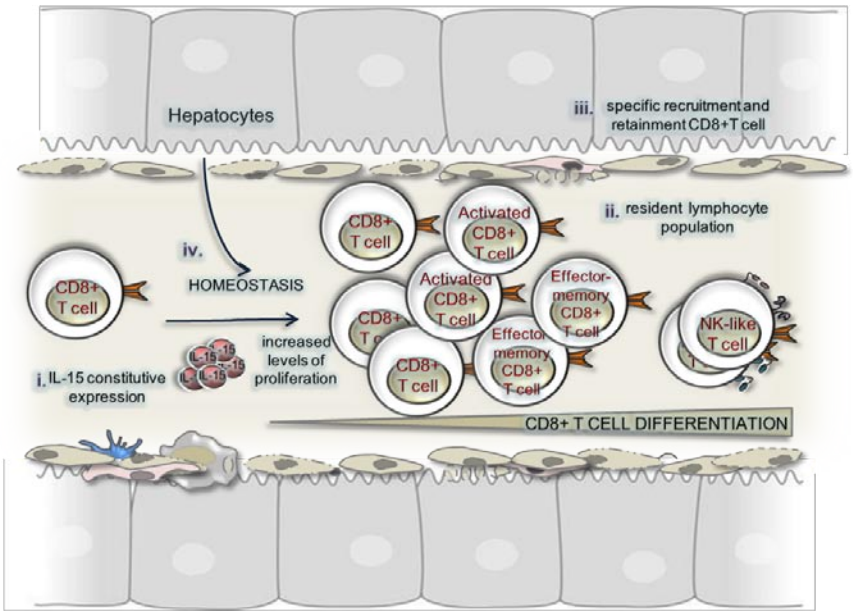

Figure 5. Liver NK-like T cells: local IL-15-driven reprogramming of CD8+ T cell differentiation?

IL-15 could contribute for the enrichment of CD1d-unrestricted NKT cells in human livers due to its unique characteristics and only by acting in concert with tissue-specific signals that might favor their maintenance during and after $CD8^+$ T cell differentiation, for which hepatocytes could have an important role.

- ***$CD8^+$T cell expressing NKR in human liver: tolerance vs. effective immune response - a way for tolerance?***

Besides the importance of the finding that the observed IL-15-drived induction of NK receptor expression by $CD8^+$ T cells could contribute to explain the increased amounts of NK-like T cells in the liver, it is important to speculate about possible meanings of this expression in the hepatic context. Due to the particular functions of the liver, it presents a high antigenic environment. Accordingly, mechanisms that could lead to an immunological tolerance are required to avoid chronic inflammation. Indeed, NK receptor expression in T cells has been proposed to be important to the maintenance of peripheral tolerance by modifying the Ag activation threshold in mucosa (113) and Jabri and colleagues proposed

that NK receptors expressed by T cells in the intestine could be of major importance in regulating CTL in tissues (15). In a very general way, in normal conditions epithelial cells do not or barely express activating receptor ligands driving the balance in the direction of tolerance. Remarkably, accordingly to our results, it could also be proposed that expression of **inhibitory NKR in $CD8^+$ T cells may favor immunotolerance in normal conditions, not only by the described ability to inhibit T cell activation, but also by inducing a significantly increase in cytokine secretion that could potentially lead to the establishment of a potential immunosuppressive microenvironment.**

However, this **tolerance should be balanced with the requirement of an effective immune response** by the lymphocytes present in the liver. This balance between tolerance and immune responses requires a specific tissue-associated regulation of T cell responses. In this context, it can be proposed that **tissue-specific factors such as IL-15** could function as putative regulators of NKR expression which in turn may confer $CD8^+$ T cells with differing effector functions in a **tissue and site-specific manner**, therefore shaping $CD8^+$ T cell mediated effector responses. Indeed, it has been reported an increased expression of IL-15 in infection, inflammation or distressed cells (14, 18, 114-115). Accordingly to our results, in conditions of increased IL-15 expression it is conceivable that the percentage of $CD8^+$ T cells expressing both activating and inhibitory NKR will increase, and that **the direction of the response would be delineated by the expression of ligands in the epithelial cells**. It has been reported that in stress, tumor or infection conditions, cells are known to upregulate NKG2D ligands, namely MICA/B, that are barely expressed on normal cells (116-118). Indeed, while in normal conditions the expression of NKR by $CD8^+$ T cells might lead to the maintenance of tolerance, the increased expression of ligands would instead drive to an effective immune response. Interestingly, IL-15 has been reported to be involved also in MICA/B expression increase (119). In this context, it could be proposed that IL-15 upregulation could lead to an increased immunosurveilance (120). However, it has been described that tumors could developed escape strategies, namely by blocking of NKG2D by the secretion of a soluble form of MICA from certain tumor cells (121-124). Thus, since IL-15 also increases the expression of inhibitory NK receptors, the chronic exposure to this cytokine could also contribute to tumor escape, being the **balance between increased immunosurveillance and tumor escape could be determined by the expression levels of activating NKR, by other microenvironmental factors, and by the pattern of NKR ligands in the target cells**.

$CD8^+NKR^+$ T cells "beyond the liver context": **Cytokine-induced killer cells**

As referred, IL-15-cultured $CD8^+$ T cells were shown to be capable of NK-like cytotoxicity and TCR-mediated cytotoxicity and secretion of a Tc1/Tc2 pattern of cytokines described to modulate immune responses. The dual innate and adaptive immune functions place $CD8^+NKR^+$ T cells alongside natural killer T (NKT) cells and subsets of γδ T cells as frontline innate immune effectors and potential regulators of adaptive immune responses against microorganisms and tumors (125-126). These properties make $CD8^+NKR^+$ T cells attractive potential targets for therapy for infectious and immune-mediated diseases as well as cancer. In support of this notion, numerical and functional deficiencies and phenotypic alterations of $CD56^+$T cells have been reported in patients with various infectious and autoimmune diseases and cancer (6, 45, 110, 127-128).

In this line, another important point raised as result of our work is the establishment of a possible **parallelism between our generated differentiated $CD8^+CD3^+CD56^+$ population with the so-called cytokine-induced killer (CIK) cells**, largely used in clinical trials. CIK cells are highly efficient cytotoxic effector cells capable of lysing tumor cell targets (81) obtained by expansion of peripheral blood lymphocytes (PBL) namely with anti-CD3 mAb and IL-2 pre-incubated or not with IFN-γ, that correspond to a population enriched in $CD3^+CD56^+$ T cells (68, 129). Indeed, it has been described that the major effector cell in CIK cultures expresses both the T cell marker CD3 and the NK cell marker CD56 (68). However, the exact molecular structures that account for this functional activity are still largely unknown. The cytotoxicity of those cells seems to correlate with the levels of CD56 expression (69), which lead to attempts to relate the CD56 molecule function with the cytotoxic effects, however the results mainly point that CD56 itself does not account to cytotoxicity (69). More recently, some studies have shown that CIK cells also co-express NKG2D, suggesting that this NK receptor could have a role in the recognition and signaling in cytotoxic function (130). However, the expression of other NK receptors by CIK cells is an unexplored field. Interestingly, in our *in vitro* study, we have obtained about 20% $CD8^+CD56^+$ T cells, a percentage similar to the percentage normally obtained in the classical culture conditions with IL-2 and α-CD3. Importantly, in this context, we have demonstrated that $CD8^+CD56^+$ T cells not only express CD56 marker but also co-express other NK receptors, both activating and inhibitory. Indeed, the fact that almost all IL-15-cultured $CD8^+$ T cells express NKG2D at high levels, while only small percentages of cells express the other NK receptors, lead to the fact that those *in vitro* generated cells are indeed tendentiously cytotoxic. In fact, our results pointed

NKG2D as the receptor with the major outcome in modulating cytotoxicity of the bulk population, which is in agreement with the previous studies suggesting NKG2D as the receptor involved in CIK cells cytotoxicity. However, malignant tumors have been described to develop several strategies in order to control the expression of activating versus inhibitory receptors on immune cells and their ligands in favor of tolerance (124). Indeed, a decrease in the levels of NKG2D or blocking of this activating receptor, namely by the secretion of a soluble form of MICA from certain tumor cells, are within strategies described (121-124). Therefore, the fact that those *in vitro* generated cells also express inhibitory receptors will be of major importance, since that their triggering might contribute to tumor escape, not only by a possible decrease in cytotoxicity but also, and importantly, as we have demonstrated, by inducing a significantly increase in cytokine secretion that could potentially lead to the establishment of a potential immunosuppressive microenvironment. In our view, this could be of major importance in further studies in which CIK cells will be intended to be used to kill tumors. These findings could be this way extended and open a new window in understanding CIK cells function, and in the way that their cytotoxicity could be regulated.

IL-15: the missing link between $CD8^+$ and NK-likeT cells?
Linking innate and adaptive immunity?

Immune system and immune cells are unquestionably dynamic. Indeed, it is increasingly known that lymphocytes could continuously change and adapt, shaping and being shaped by the surrounding microenvironment. Interestingly, by proposing that a fraction of human NKT cells could be instead $CD8^+$ T cells that entered in an IL-15-driven differentiation program and do not necessarily belong to a different lineage, we are opening a new door in NKT cell origin. Indeed, we have found that in the presence of IL-15, $CD8^+$ T cells acquire similarities with NKT cells not only at the phenotype level, by sharing the same repertoire of receptors, but also at functional level by displaying NK-like cytotoxicity and secreting a varied pattern of cytokines. Thus, our findings unambiguously **blur the distinctions between $CD8^+$ T cells and NKT cells, suggesting IL-15 as the missing link between both cell types** (Figure 6).

The dichotomy “innate” and “adaptive” immunity has been brought into discussion in the last years. Indeed, it has been argued that there is not a strict boundary between innate and adaptive immunity, but instead a closely interplay between cells from both classifications. Beyond an interrelation between “innate” and “adaptive” cells, it is known

that cells as NKT cells and γδ T cells can exhibit characteristics of both, straddling the interface of innate and adaptive immunity (Figure 6). Moreover, recently, Sun *et al*, 2009 have shown that NK cells, thought to be strictly "innate", appear to be capable to display "memory", a hallmark of "adaptive" immunity (131). Thus, being claimed that such a binary fashion of categorization oversimplifies the intricacies of the immune system.

In this context, our work broadens the field of discussion by showing, not only that cells can display characteristics of both "innate" and "adaptive", but importantly that "adaptive" cells as $CD8^+$ T cells can be induced to acquire "innate" phenotype and functions. Thus, IL-15 besides bridging innate and adaptive immunity, smudges the division between the concepts by bringing the two worlds together - reprogramming CD8+ T cells into NK-like T cells through the acquisition of "innate" phenotype and functions.

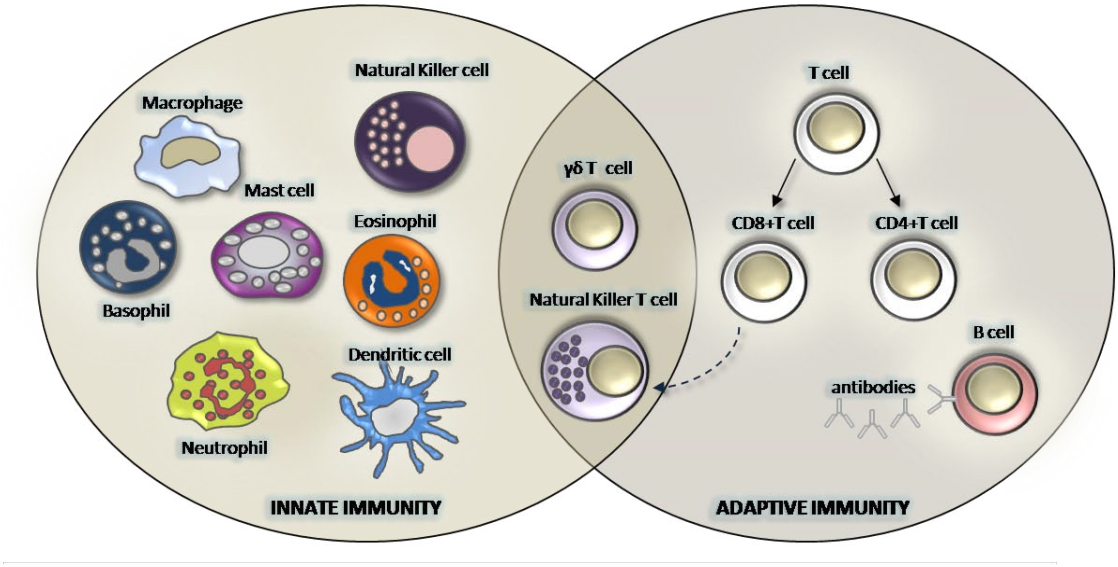

Figure 6. Innate and adaptive immunity: is there a strict boundary?

Schematic representation of immune cells belonging to "innate" or "adaptive" immunity, with NKT cells and γδ T cells as cells lying in the interface. CD8+ T cells can be reprogrammed into NK-like T cells, linking "innate" and "adaptive" immunity (dashed arrow). Adapted from (131).

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