Mariana Isabel da Cunha Monteiro Terra

Interleukin-15 and Phytohaemagglutinin: a combination to study human CD8^{+} T cell differentiation *in vitro*

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Orientadora – Doutora Elsa Maria Cardoso
Afiliação – Instituto Superior de Ciências da Sáude do Norte, CESPU

Co-orientador – Doutor Fernando Aguilar Arosa
Afiliação – Instituto Superior de Ciências da Sáude do Norte, CESPU

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Table of contents

Abstract .... **IV**

Resumo .... **V**

Abbreviations .... **VI**

I. **Introduction** **1**

1.1. Long-term cell cultures 1

1.1.1. Antigen-specific *versus* antigen independent cell culturing 1

1.1.2. Long-term antigen-independent $CD8^+$ T cell culture 2

1.2. Interleukin-15 4

1.2.1. IL-15 and its structural cousin IL-2 5

1.2.2. IL-15 expression and regulation of expression 6

1.2.3. IL-15 receptor complex 7

1.2.4. IL-15 and signaling 8

1.3. $CD8^+$ NKT cells and NK receptors 10

1.3.1. NK receptors: CD56, KIR and NKG 11

II. **Aims of the thesis** **14**

III. **Material and Methods** **15**

3.1. Isolation of peripheral blood lymphocytes 15

3.2. Magnetic T $CD8^+$ cell purification 15

3.2.1. Isolation of untouched human $CD8^+$ T cells 15

3.2.2. Positive selection of human $CD8^+$ T cells after CD56 depletion 16

3.3. Cell culture conditions 18

3.4. Flow cytometry analysis 18

3.5. Statistical Analysis 19

IV. **Results** **21**

4.1. IL-15 and PHA are necessary to keep $CD8^+$ T cells growing 21

4.2. IL-15 is necessary to maintain CD8$^+$ T cell viability and cell growth 23

4.3. PHA signals downregulate CD56 expression levels in IL-15 differentiated CD8$^+$ T cells 24

4.4. CD56$^+$ expression takes place preferentially on blast CD8$^+$ T cells 27

4.5. CD56 expression in long-term cultures of CD8$^+$ T cells 29

4.6. Long-term cultures of CD8$^+$ T cells with IL-15 and PHA increases the overall size and complexity of the cells 30

V. **Discussion 33**

5.1. IL-15 and PHA: a good combination to keep CD8$^+$ T cells alive and growing . 33

5.2. IL-15/PHA stimulated CD8$^+$ T cells: a good model to study differentiation 36

5.3. Future prospects 40

VI. **Conclusions 43**

References 45

Abstract / Resumo

Abstract

Previous studies performed in our lab examining the influence of IL-15 on antigen-independent expansion and differentiation of human $CD8^+$ T cells led to the finding that IL-15 was able to induce d*e novo* expression of natural killer receptors (NKR) in purified $CD8^+CD56^-$ T cells after 12 days in culture. NK-like $CD8^+$ T cells induced by IL-15 displayed a regulatory (effector/memory) phenotype, and NKR upregulation was shown to be intrinsically related with CD56 acquisition.

The major objective of this work was to establish optimal culture conditions in order to keep long-term cultures of $CD8^+$ T lymphocytes, and study phenotypical features of the differentiated $CD8^+$ T cells.

To that end, $CD8^+$ T cells were isolated from PBMC of healthy blood donors by magnetic purification and cultured in the continuous presence of IL-15 in combination with TCR-mediated stimuli given by the T-cell specific mitogen phytohaemagglutinin (PHA). Expression of the prototypic NK marker, CD56, was assessed by FACS every seven days, in combination with parameters of cell growth and survival.

By combining IL-15 and PHA we were able to maintain $CD8^+$ T lymphocytes in culture for long periods of time (up to 90 days). During this time, $CD8^+$ T cells acquired a large granular morphology and a fraction of these cells, mostly blast cells, expressed the prototypic marker of NKT cells, CD56. Importantly, the establishment of long-term cultures of differentiating $CD8^+$ T cells was only possible when IL-15 was combined every two weeks with PHA stimuli.

These results suggest that the generation of highly differentiated $CD8^+$ T cells is the result of the combination of TCR-dependent and TCR-independent signals.

Resumo

Estudos prévios realizados no nosso laboratório avaliando a influência da IL-15 na expansão e diferenciação antigénio-independente de células humanas T $CD8^+$, demonstraram que esta citocina é capaz de induzir a expressão *de novo* de recetores natural killer (NKR) em linfócitos T $CD8^+CD56^-$ purificados, após 12 dias de cultura. Células T $CD8^+$ NK-*like* induzidas pela IL-15 apresentavam um fenótipo regulatório (efetor/memória) e a expressão aumentada de NKR estava intrinsecamente relacionada com aquisição de CD56.

O principal objetivo deste trabalho foi estabelecer condições de cultura ideais de modo a manter culturas de longo prazo de linfócitos T $CD8^+$ e estudar caraterísticas fenotípicas destas células T $CD8^+$ diferenciadas.

Para esse fim, linfócitos T $CD8^+$ foram isolados a partir de PBMC de dadores de sangue saudáveis por purificação magnética, e cultivados na presença contínua de IL-15 em combinação com estímulos via TCR através do mitogénio específico para células T, fitohemaglutinina (PHA). A expressão do marcador prototípico de NK, CD56, foi avaliada por citometria de fluxo semanalmente, em combinação com parâmetros de crescimento celular e sobrevivência.

Através da combinação de IL-15 e PHA conseguimos manter em cultura linfócitos T $CD8^+$ por longos períodos de tempo (até 90 dias). Durante este período, as células T $CD8^+$ adquiriram morfologia grande e granular, e uma fração destas células, sobretudo blastos, expressaram o marcador prototípico de células NKT, CD56. Particularmente, o estabelecimento de culturas a longo prazo de células T $CD8^+$ diferenciadas só foi possível quando a IL-15 foi combinada cada duas semanas com o estímulo da PHA.

Estes resultados sugerem o estabelecimento de linfócitos T $CD8^+$ altamente diferenciados em resultado da combinação de sinais TCR-dependentes e TCR-independentes.

Abbreviations

Ab Antibody

Ag Antigen

AICD Activation-induced cell death

Akt Serine/threonine-specific protein kinase

ALL Acute lymphoblastic leukemia

AP-1 Activator protein 1

APC Antigen presenting cell

Bcl-2 B-cell lymphoma 2 protein

Bcl-xL B-cell lymphoma-extra long protein

BSA Bovine serum albumin

CD Cluster of differentiation

CIK Cytokine induced killer

c-myc Avian myelocytomatosis virus oncogene cellular homolog

CTL Cytotoxic T lymphocytes

EBV Epstein Barr virus

EBV-LCL Epstein-Barr virus-transformed lymphoblastoid cell line

EDTA Ethylenediaminetetraacetic acid

FACS Fluorescence activated cell sorter

FBSi Inactivated fetal bovine serum

FITC Fluorescein isothiocyanate

FS Forward scatter

G-CSF Granulocyte colony-stimulating factor

GM-CSF Granulocyte-macrophage colony-stimulating factor

HIV Human immunodeficiency virus

HLA Human leukocyte antigen

HSi Inactivated human serum

IEL Intraepithelial lymphocyte

Ig Immunoglobulin

IL Interleukin

IL-15Rα Interleukin 15 receptor α

IL-2Rγc Interleukin 2 receptor γc

IL-2Rα Interleukin 2 receptor α

IL-2Rβ Interleukin 2 receptor β

ITAM Immunoreceptor tyrosine-based activation motif

ITIM Immunoreceptor tyrosine-based inhibitory motif

JAK Janus kinase

KIR Killer immunoglobulin-like receptor

LAg Lineage antigen

LAK Lymphokine activated killer

Lck Lymphocyte-specific protein-tyrosine kinase

mAb Monoclonal antibody

MACS Magnetic-activated cell sorter

MFI Mean fluorescence intensity

MHC Major histocompatibility complex

mRNA Messenger ribonucleic acid

NCAM Neural cell adhesion molecule

NCR Natural cytotoxicity receptor

NF-kB Nuclear factor-kappa B

NK cell Natural Killer cell

NKG Natural Killer cell lectin-like receptor

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

PE Phycoerythrin

PHA Phytohaemagglutinin

PI3K Phosphatidylinositol 3-kinase

PLC Phospholipase C

PSA Penicillin/Streptomycin/Amphotericin B

MAPK Mitogen-activated protein kinases

SEM Standard error of the mean

SS Side scatter

STAT Signal Transducers and Activators of Transcription

Syk A tyrosine-protein kinase

TCR T cell receptor

Tg Transgenic

T-LGL T large granular lymphocyte

Zap-70 Z-chain-associated protein kinase

Introduction

I. Introduction

1.1. Long-term cell cultures

Human T cell lines and clones are useful tools to obtain information about the role of T cells in the immune response. Therefore, the development of methods to grow T cells *in vitro* in the mid 1970s was a major breakthrough for the study of these cells, providing important insight into the characterization of T cell subpopulations and the understanding of their functional roles [1].

By demonstrating the mitosis-inducing properties of the lectin phytohaemagglutinin (PHA) in 1960 [2], Peter Nowel started the line of research that culminated with the discovery of interleukin-2 (IL-2), so-called T-cell growth factor [3, 4]. For the first time, immunologists were able to grow *ex vivo* T-lymphocytes and obtain clones of cytotoxic T-cells, using IL-2 [3, 4].

Although some studies describing the effects of IL-2 on human T cells appeared between 1976 and 1980, [5], it was not before the beginning of the eighties that the first stable human T cell clones were reported [6-8].

1.1.1. Antigen-specific *versus* antigen independent cell culturing

A large variety of protocols have been described for the *in vitro* expansion of T cells, and today it is possible to easily propagate T cells using conventional multi-well tissue culture plates or flasks [9].

In view of the presumed requirements for antigenic stimulation, a culture system was devised for the generation and expansion of stable allo-antigen-specific cloned human T-cell lines, with a feeder cell mixture, consisting of irradiated peripheral blood mononuclear cells (PBMC), an Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCL), expressing the specific allo-antigen, and phytohemaglutinin, resulting in the amplification and enrichment of T cells directed against the stimulating antigen/peptide [1, 9]. Apparently, PHA can replace the requirement of antigen-mediated triggering of the TCR for T-cell activation by inducing the expression of the IL-2 receptor α (IL-2Rα) chain (CD25) on the T-cells. [1, 10, 11].

Recent findings show that *naïve* CD8+ T cells of human and mouse origin can be stimulated by cytokines without requirement for a nominal antigen [12-15]. Cytokines clearly play an important role in allowing survival and proliferation of activated T cells, and it has been shown that cytokine-mediated signals can control lymphocyte proliferation by regulating the expression of cell cycle proteins that control entry into the S phase of the cell cycle [16].

1.1.2. Long-term antigen-independent CD8+ T cell culture

Due to the aforementioned discovery of mitosis-induced properties of the lectin PHA and the discovery of the T-cell growth factor IL-2, several studies, reported the long-term antigen-independent successful culturing of CD8+ T cells using this combination, and it is worth mentioning that this methodology has been widely used to grow or expand T cells *in vitro*.

In order to test the effects of replicative senescence in the ability of human T cells to undergo apoptosis, Spaulding et al. compared the same population of T cells at different stages of their *in vitro* replicative life span. An *in vitro* culture model of cellular aging was achieved by adding recombinant IL-2 and allogeneic irradiated EBV-transformed B cells (that serve as APC), reaching up to 25 population doublings [17].

A different study with the goal to analyse the mechanisms responsible for regulating CD28 expression in primary human CD8+ T lymphocytes subjected to chronic activation, produced cultures reaching 20 population doublings, using rIL-2 and restimulation with APCs (PBMC combined with EBV-transformed B lymphoblastoid cells) every 21-28 days [18].

Valenzuela and Effros followed a series of long-term CD8+ enriched cultures for 79 to 238 days, using a protocol involving the use of IL-2 and restimulation with APC every 14-25 days, to elucidate the long-term telomerase dynamics in human T-cells [19].

In another study, an experimental approach was undertaken to compare the phenotypic changes of human CD8+ T lymphocytes occurring during *in vivo* ageing and during replicative senescence *in vitro*. Lymphocytes were cultured *in vitro* for 30 to 35 days in the presence of IL-2, and with PHA adding at the time of seeding [20].

To assess the mechanisms that limit the *in vitro* proliferative potential of human $CD8^+$ T lymphocytes, Migliaccio et al. cultured T cells for up to 95 days using IL-2, PHA and irradiated allogeneic PBMC as feeder cells. The restimulation procedure was repeated every 10 or 15 days [21].

Finally, on a different study with the goal to investigate the effect of introducing an exogenous CD28 gene into cultures of chronically activated human $CD8^+$ T cells, these cells were exposed to T cell activation microbeads (anti-CD2/3/28) and stimulated repeatedly every 14 days. Cultures were supplemented with IL-2 and survived after seventh restimulation [22].

More recently, cytokines such as IL-7 and IL-15 have raised considerable interest for their potent effect on activation and expansion of T cells. They therefore present a strong potential for the optimization of *in vitro* expansion, although their effects seem to vary according to the type of cells affected: IL-7 is crucial for *naïve* $CD8^+$ T cells survival, whereas IL-15 exerts its effects on memory $CD8^+$ T cells. [23, 24].

A previous study by our group examined the influence of IL-15 on antigen-independent expansion and differentiation of human $CD8^+$ T cells. All human T-cell subsets, both *naïve* and primed human T cells, divided in response to IL-15, which suggests a generic influence of this cytokine on $CD8^+$ T-cell survival and proliferation, opening the possibility for T-cell antigen-independent culturing with IL-15 [12].

In this context, it is important to note that most studies using IL-15 have focused on short-term T-cell cultures [25-27]. In order to examine functional differences between IL-2 and IL-15 in the regulation of T cell activation, Bulfone-Paus et al. stimulated human T lymphoblasts with IL-15 for 24 to 48 hours [25]. A similar experimental study was developed by Cornish et al., to assess the actions of IL-2 and IL-15 on protein synthesis and cell growth on antigen-activated T $CD8^+$ T cells isolated from spleens and lymph nodes of transgenic mice. Cells were cultured for 10 days with IL-15 [26].

In a more recent study, the effect of exogenous IL-15 added every 4 days on the expansion of $CD3^+CD56^+$ NKT-like cells *in vitro* present on cord blood mononuclear cells was examined for up to 14 days [27].

To this point, only a few studies have focused on long-term cultures of $CD8^+$ T cells using IL-15 as a growth factor. Thus, in order to evaluate the role of IL-15 in the maintenance of antigen-specific CTL in culture and to determine whether these cells would retain their effector function, Lu et al. were able to culture $CD8^+$ T lymphocytes lasting up to 80 days in culture with IL-15 adding every 2 or 3 days, and stimulation with anti-CD3 in the presence of irradiated feeder cells [28]. Subsequent studies by Li et al. used IL-15 stimulation every 10 days, with anti-CD3/CD28 stimulation at seeding, and were able to grow T $CD8^+$ cells for 45 to 80 days, allowing the study of the role of IL-15 in regulating the replicative capacity of memory $CD8^+$ T cells [29].

Studies involving long-term cultures with IL-15 addition are more common for NK cells. With the purpose of demonstrating the potential for IL-15 to support large-scale expansion of clinical-grade LAK-NK effectors, Suck et al. expanded $CD56^+CD3^-$ NK cells with IL-15 for up to 4 weeks [30]. More impressive was the study of Choi et al., in which the effects of IL-15 on neonatal NK cells was examined for up to 12 weeks in culture [31].

1.2. Interleukin-15

Interleukin-15 is a pleiotropic cytokine that belongs to the 4-α-helix bundle cytokine family, which binds to receptors of the hematopoietin family [32, 33], along with 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 [34]. Not surprisingly, being a member of this very diverse family, IL-15 exerts its functions on the proliferation, survival and differentiation of many distinct cell types, hence its designation as pleiotropic [32, 35].

IL-15 was simultaneously discovered in 1994 by two independent research groups [36, 37], having its sudden interest aroused due to its structural similarities with IL-2.

1.2.1. IL-15 and its structural cousin IL-2

A special feature of IL-15 is that it shares with IL-2 the IL-2 receptor beta (IL-2Rβ) and IL-2 receptor gamma common (IL-2Rγc) chains [38-41], which are responsible for downstream cytoplasmic signals [33, 35]. IL-2 and IL-15 also associate with a unique third chain, IL-2Rα and IL-15Rα, respectively [38] (Figure 1.1).

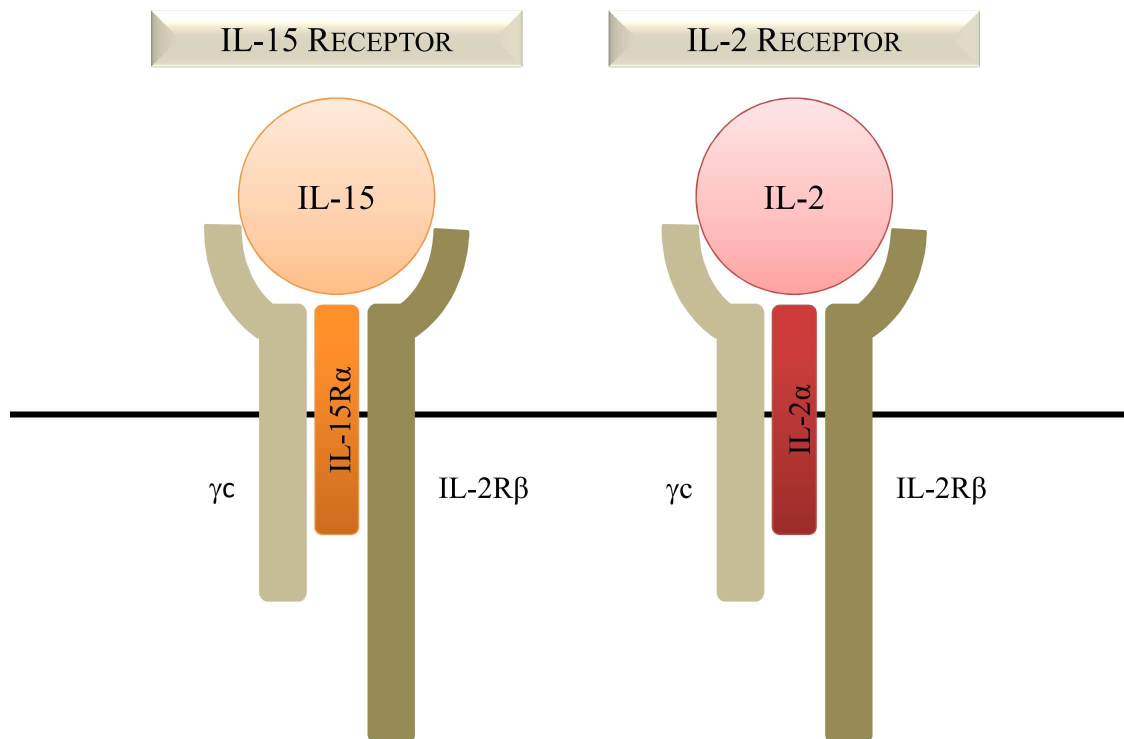


Figure 1.1. Representation of the IL-15 and IL-2 receptors. A special feature of IL-15 is that it shares with IL-2 the IL-2Rβ and IL-2Rγc chains which are responsible for downstream cytoplasmic signals. IL-2 and IL-15 also associate with a unique third chain, IL-2Rα and IL-15Rα, respectively. Adapted from [35, 42-44].

However, since these α-chains have short cytoplasmic domains, they do not appear to contribute directly to signal transduction. The primary role of these α chains appears to be to assist the formation of high-affinity receptor complexes conferring ligand specificity since each α-chain recognizes only it's cognate cytokine [35], and to regulate the size and content of the peripheral lymphoid compartment [45]. In fact, in the absence of an α-chain, the IL-2Rβ-γc dimer can respond to either IL-2 or IL-15, although with lower affinity [45].

Due to the sharing of receptor subunits by IL-2 and IL-15 some authors have proposed that these cytokines may have redundant functions [25]. In fact, they perform a similar spectrum of biological activities, mainly in three major lymphoid cell types: Ig-producing B cells, antigen-specific cytotoxic and helper T cells, and natural killer cells. However, a series of latter studies have shown that, despite their many overlapping functional properties, IL-2 and IL-15 are in fact markedly distinct players in the immune system [25]. While IL-2 is critical in the maintenance of peripheral homeostasis of thymus-derived T cells, IL-15 promotes extrathymic T and NK cell development hence serving a much broader spectrum of bioregulatory purposes [35, 46, 47].

In vivo studies allowed the assessment of the suggested distinct functions for IL-2 and IL-15. The generation of knockout mice in which specific genes have been inactivated has helped to answer the central question of the redundancy IL-2/IL-15. Comparative studies of IL-2Rα – deficient and IL-2/15Rβ – deficient mice suggested that IL-15 might also be important for the differentiation of NK cells and certain subsets of intraepithelial lymphocytes (IEL) [48, 49]. Exogenous IL-15, but not IL-2, selectively induces the proliferation of memory phenotype $CD8^+$ T-cells in normal mice [50]. Mice deficient in the IL-2 pathway ($IL\text{-}2^{-/-}$, $IL\text{-}2R\alpha^{-/-}$ or $IL\text{-}2R\beta^{-/-}$) have normal T-cell development, however exhibit defects in peripheral lymphoid homeostasis and show evidence of incorrect T-cell activation, lymphoid infiltration and autoimmunity [45, 51-53]. The function specificity for IL-15 *in vivo* became clearer with the initial reports of IL-15 deficient ($IL\text{-}15^{-/-}$) mice by Kennedy et al. [54], while $IL\text{-}2^{-/-}$ and $IL\text{-}2R\alpha^{-/-}$ mice spontaneously activate T and B cells, dying from autoimmune disease, $IL\text{-}15^{-/-}$ and $IL\text{-}15R\alpha^{-/-}$ mice are generally healthy, lymphopenic and lack NK cells, NKT cells, IEL subsets and activated $CD8^+$ T-cells [46, 53, 54].

1.2.2. IL-15 expression and regulation of expression

It is worth mentioning that the specificity of IL-15 arises from its pleiotropic characteristics, being expressed by a large variety of cells. While IL-2 is produced by activated T-cells, IL-15 is secreted by a wide variety of cells, including bone marrow stromal cells, gut and skin epithelia, macrophages and other cell types, but not by activated T-cells [35]. IL-15 mRNA is constitutively expressed by monocytes [55], macrophages [56], dendritic cells [57], keratinocytes [58], fibroblasts [59], kidney

epithelial cells [60], IEL [61], neural cells [62] and hepatocyte cells [63]. Additionally, IL-15 was also observed outside the lymphoid compartment, in particular brain [62], kidney [60], liver [64], and intestine [61].

Interestingly, despite this wide cellular distribution, it is extremely difficult to detect IL-15 in cell culture supernatants [32]. Indeed, most primary cells and cell lines which express IL-15 mRNA do not release detectable amounts of this cytokine into the culture medium [65-67]. This "striking" discrepancy between IL-15 mRNA and protein secretion is explained by the several lines of evidence indicating the existence of an intricated control of expression regulation at the levels of transcription, translation and intracellular trafficking and translocation [66-71].

1.2.3. IL-15 receptor complex

As previously mentioned, the IL-15 cell-surface receptor is a complex constituted by a combination of three subunits – the IL-15Rγc, the IL-2/IL-15Rβ (both shared with IL-2) [72], and a private high-affinity subunit IL-15Rα. The ectodomain of IL-2Rβ and γc each consist of two fibronectin-type III domains, which participate in IL-15 binding. Their cytoplasmic domains can sequester signaling molecules such as JAK1 and JAK3 [73] hence being able to participate in both the ligand binding and signal transduction.

As already mentioned, IL-2Rβ and γc are shared subunits of the IL-2 receptor, activating similar signaling pathways upon ligand binding, which may partially account for the overlapping functions of IL-15 and IL-2. A possible mechanism by which IL-15 and IL-2 attain their distinct *in vivo* functions is through differences in the properties of their α-chain receptors.

Recently, is has been demonstrated that IL-15Rα not only exists in a membrane-bound form, but also in a soluble form (sIL-15Rα) [74, 75]. The presence of sIL-15Rα in circulation may negatively affect the availability of free IL-15, by the cytokine competition with the cognate membrane-bound receptors [74, 75], which may be the cause of the difficulty to detect free IL-15 [32].

1.2.4. IL-15 and signaling

Originally, IL-15Rα was thought to be a component of a heterotrimeric receptor complex containing IL-2/IL-15Rβ and γc chains that were required for signaling mediation [76]. Although IL-15Rα may, in some cases, act as a component of this receptor complex, more recent evidence indicates that IL-15Rα predominantly functions by presenting IL-15 to opposing cells expressing the IL-15Rβγ signaling components [76], and may expand IL-15 actions from autocrine or juxtacrine signaling to paracrine and endocrine communication mechanisms, including systemic cytokine effects [32, 77].

The current theory of trans-presentation proposes the intracellular IL-15 binds to high affinity IL-15 binding protein (IL-15Rα), that is shuttled to the cell surface where it stimulates IL-15 signaling components on neighboring cells through cell-cell interaction [78]. In fact, supporting this theory there are accumulated evidence indicating that under physiological conditions, the biologically active form of IL-15 is actually surface-bound IL-15 [55, 59, 76, 79, 80].

Another important aspect of membrane-associated IL-15 is its ability to mediate reverse signaling events [32].

Each cytokine receptor seems to be coupled to a specific array of JAK and STAT, and given the similarities between IL-2 and IL-15 receptor, Johnston et al. found that IL-15 activates similar signaling pathways throughout JAK1 and JAK3, and then these rapidly induce the phosphorylation and activation of STAT3 and STAT5 [81].

The Janus-family tyrosine kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway is the major mechanism used by cytokines to transducer intracellular signals [82, 83]. Briefly, receptor-associated JAK, following ligand binding, become activated and catalyze phosphorylation of tyrosine residues in the receptor cytoplasmatic domains. These phosphorylated tyrosine residues, in turn, serve as “docking” sites for STAT proteins, which, at this point dissociate and are able to translocate to the nucleus where they act as transcription factors [82, 83].

In fact, it has been found that JAK-1 deficient mice exhibit defective lymphopoiesis, due to incorrect signaling by γc-dependent cytokines [84]. Additionally, mutation on the JAK3 gene was shown to result in abnormal NK-cell development, due to defective IL-15 signalling [85, 86]. Similarly, mice lacking STAT3 were found to

exhibit perinatal lethality [87] and STAT5a/b double-knockout mice show a severe defect in T-cell proliferation [88] and a total absence of NK-cell development [89].

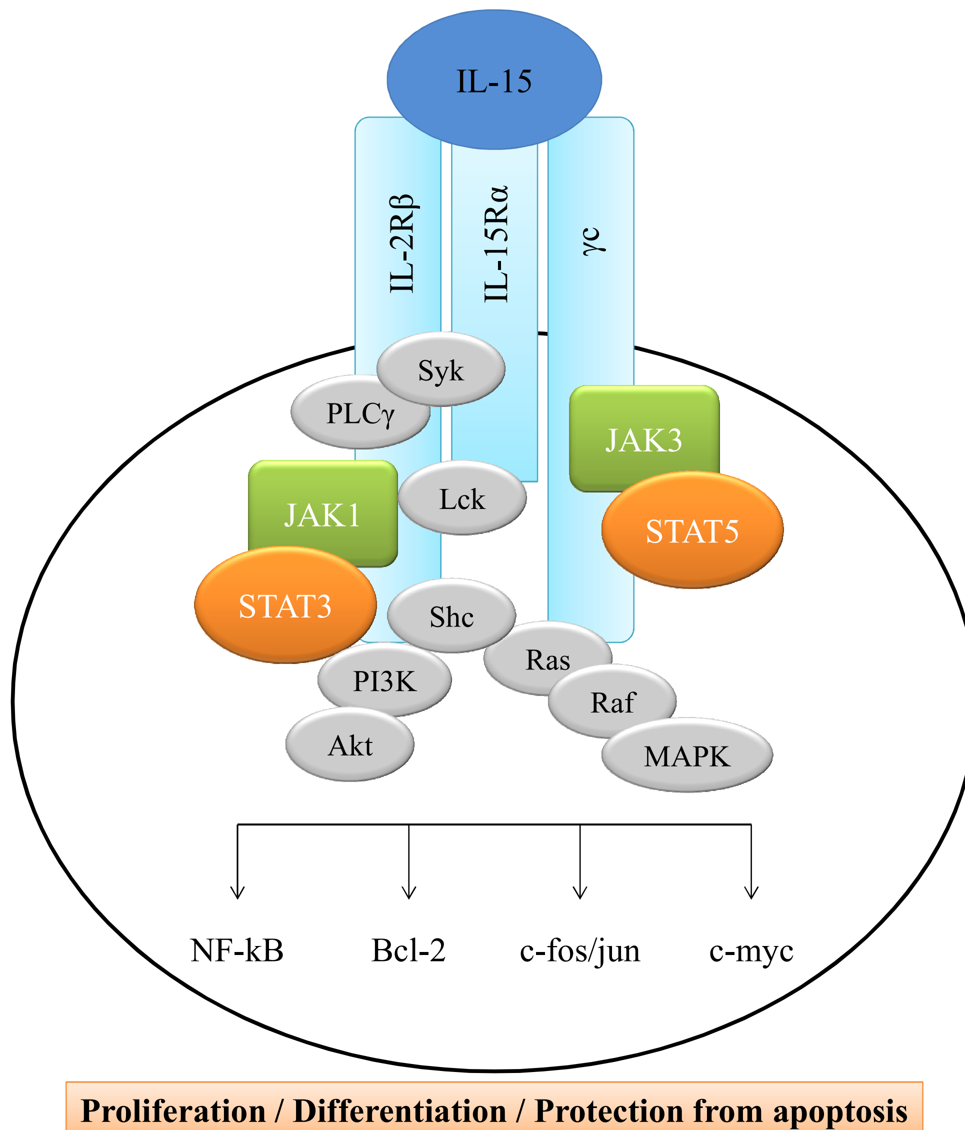


Figure 1.2. IL-15 receptor mediated intracellular signal transduction events in lymphocytes. IL-15 activates JAK1/JAK3 and STAT3/STAT5. Additional pathways involve Syk kinase and PLCγ, Lck kinase, Shc, resulting in the activation of PI3K/Akt and Ras/Raf/MAPK cascades, that could lead to the subsequent expression of bcl-2, c-myc and c-fos/jun genes and NF-kB activation. Adapted from [32].

The specific signaling mediated by the IL-15 receptor takes place after receptor-ligand interaction, when the associated JAK are brought into close apposition. In particular, the IL-2Rβ chain recruits JAK1, whereas IL-2Rγc activates JAK3, which in turn lead to the activation of STAT3 and STAT5, respectively. The cytoplasmatic

domain of the IL-2Rβ and γc subunits contains specific tyrosine residues that become activated upon ligand binding, serving as docking sites for STAT3 and STAT5 via the STAT Src homology 2 (SH2) domains. Subsequently, recruited STAT are phosphorylated by activated JAK. Hence, the phosphorylated STAT transcription factors form either homo-and/or heterodimers and translocate to the nucleus to bind to their target DNA regulatory elements and participate in the activation of gene expression [32] (Figure 1.2).

Additionally, there have been described a number of other signaling pathways activated by IL-2 and IL-15R, namely, the phosphorylation of the Src tyrosine kinases family (Lck, Fyn and Lyn), Syk-kinases and Plc-γ and Shc, resulting in the activation of PI3K/Akt and Ras/Raf/MEK/MAPK cascades [32, 90, 91]. IL-15 has been shown to activate NF-kB and AP-1 transcription factors [32, 92] and to control c-myc [93]. Moreover, it can induce Bcl-2 or alternatively result in fos/jun activation [94] (Figure 1.2).

1.3. CD8^{+} NKT cells and NK receptors

We have previously shown that IL-15 was able to induce antigen-independent expression of NKR in purified CD8^{+}CD56^{-} T cells, after a 12 day culture period [63], including Ig-like receptors (KIR2DL2/3, KIR2DL4 and KIR2DL2), lectin-like receptors (NKG2A), and *de novo* expression of CD56 and NKp46 [63]. To our knowledge, this was the first evidence of an antigen-independent NKR *de novo* expression by IL-15 in resting human peripheral blood CD8^{+}CD56^{-} T cells [63, 95]. Supporting our observations, Tang et al., have shown induction of natural cytotoxic receptors on umbilical cord blood T cells by IL-15, and that these cells were mainly on CD8^{+} and CD56^{+} T cells [96]. A different study reported that IL-15 could induce CD94/NKG2A expression, but not NKR belonging to the Ig-like family, in T cells activated by superantigens or allogenic cells [97].

In fact, in the last years it has been shown that NK receptors could be expressed, not only by NK cells, but also by T cells, however, in contrast to NK cells, for which stimulatory and inhibitory functions of NKR could be conveniently demonstrated, their function on CD8^{+} T cells is less clear.

Initial reports showed that minor subsets of T cells, mostly $CD8^+$ T cells, from normal donors could express KIR [98, 99]. Mingari et al., have found that, along with KIR, CD94/NKG2A was also expressed in $CD8^+$ T cells [97, 99]. In the last years, also activating receptors have been described to be expressed in T cells, namely, the NKG2D receptor has been found to be normally expressed in almost all $CD8^+$ T cells, contrarily to the other NKR [100].

Curiously, contraction of TCR repertoire in old age may be accompanied by induction of a diverse array of NKR [101], and it has been proposed that induction of NKR/KIR expression in aging T cells can be seen as a compensatory adaptation to maintain immune diversification [101, 102], representing the best example for a secondary level of diversification of the immune repertoire, most likely as an adaptation to the extension of lifespan [101, 103],

Importantly, we have preliminary evidence indicating that some of the NKR expressed on IL-15-activated $CD8^+$ T cells are functional and these cells display an *in vitro* cytotoxicity, show increased anti-apoptotic levels of bcl-2 and high granzyme B/perforin expression. Moreover, these cells secrete regulatory cytokines, such as IFN-γ, TNF-α, IL-1β and IL-10, upon cross-linking of NK receptors [95]. These results suggest that IL-15 is capable of differentiating $CD8^+$ T cells into NK-like T cells displaying a regulatory (effector/memory) phenotype.

1.3.1. NK receptors: CD56, KIR and NKG

The NK receptor CD56 is a membrane glycoprotein belonging to the Ig superfamily [104] and an immunophenotypic marker for several unique populations of PBL [105], being present on approximately 10% to 25% of peripheral blood lymphocytes, and on approximately 5% of $CD3^+$ peripheral blood lymphocytes [106]. CD56 appears to be identical to the neural cell adhesion molecule (NCAM), a well-characterized structure mediating homotypic and heterotypic cell-cell interactions [107-110].

The core polypeptide of the CD56 adhesion molecule appears to be the 140 kDa isoform of NCAM, which contains six potential sites for *N*-linked glycosylation

extensively modified with polysialic acid [104, 110, 111]. The molecular weight of the CD56 heavily glycosylated protein (NKH-1 molecule) is 200 – 220 kDa [112, 113].

In humans, CD56 represents a prototypic marker of NK cells and is also found on a subset of $CD4^{+}$ and $CD8^{+}$ T cells [104]. On NK cells it defines two different subpopulations according to the cell-surface density of this marker, $CD56^{dim}$ and $CD56^{bright}$ NK cells, with differential phenotype and function [104, 114-116]. Regarding the $CD3^{+}CD56^{+}$ T lymphocytes it comprises a unique subset of cytotoxic T lymphocytes that mediates non–major histocompatibility complex (MHC)-restricted cytotoxicity [117].

In contrast to NK cells, CD56 expression on T cells is not constitutive. It has been observed that $CD56^{+}$ T cells are almost absent at birth and in adults are found as oligoclonal expansions [104]. $CD8^{+}CD56^{+}$ T cells generally contain high amounts of intracellular perforin and granzyme B, and CD56 expression on $CD8^{+}$ T cells correlates with cytolytic activity [95, 104]. The finding that $CD8^{+}CD28^{-}$ T cells have low telomerase activity and frequently express CD56, further supports the idea that $CD56^{+}$ T cells have an effector/memory phenotype [118]. $CD56^{+}$ T lymphocytes are believed to be major players in immune-surveillance and anti-tumor responses. Thus, the mechanisms by which these cells home into tumors and areas of inflammation are of great relevance to human health and disease [105].

In addition to CD56, human NK cell responses result from the integration of signals from both cytokine receptors and germline-encoded NK cell inhibitory and activation receptors [119]. Major families of these cell surface receptors that inhibit and activate NK cells to lyse target cells have been characterized, including killer cell immunoglobulin-like receptors (KIRs), C-type lectins, and natural cytotoxicity receptors (NCR) [119, 120]. It is now clear that NK cell function is strictly regulated by the balanced triggering between positive and negative signals provided by their activating or inhibitory NKR [121, 122]. Activating receptors (activating KIR, CD94/NKG2C, NKG2D) are characterized by short cytoplasmic domains lacking signal transduction elements, and they associate with common transmembrane adaptor proteins such as CD3ζ, FcεRγ and DAP12, containing cytoplasmic tails with immunoreceptor tyrosine-based activation motifs (ITAM) [120, 121, 123]. Ligation of NK-activating receptors with membrane-bound molecules of target cells results in NK cell blastogenesis, cytokine production, cytotoxicity, and migration [120]. On the other

hand, inhibitory receptors (inhibitory KIR, CD94/NKG2A) recognize and engage their ligands, MHC class I molecules (HLA), on the surface of the target cell, thereby initiating an inhibitory signal [120, 121, 123]. Although the extracellular domains of NK cell inhibitory receptors are diverse, the intracytoplasmic signaling motifs of these transmembrane receptors are remarkably similar, known as immunoreceptor tyrosine-based inhibitory motifs (ITIM) [121, 124].

Even though NK cells are prepared to rapidly kill abnormal cells, it is important to mention that they are normally restrained by these inhibitory receptors, and it has been proposed that NK cells all express at least one inhibitory receptor to provide NK cell tolerance and to prevent inappropriate NK cell responses directed at self [119, 124].

Aims of the thesis

II. Aims of the thesis

The main objective of this thesis was to ascertain the optimal culture conditions for the long-term culture of *ex vivo* $CD8^+$ T cells in order to study their phenotypic features.

The specific aims were:

1. To compare the effect of chronic stimulation with IL-15 alone or in combination with PHA on the growth and survival of *ex vivo* $CD8^+$ T cells.

2. To monitor cellular parameters of long-term cultured $CD8^+$ T cells, namely size and complexity.

3. To correlate growth and survival with expression of CD56.

Material and Methods

III. Material and Methods

3.1. Isolation of peripheral blood lymphocytes

In this study we have used Buffy Coats of healthy blood donors from *Instituto Português do Sangue* and *Hospital de São João, Porto*.

Peripheral mononuclear cells (PBMC) were obtained from these buffy coats after gradient density centrifugation over Lymphoprep (Nycomed, Norway). First, buffy coats were diluted on PBS 1x (13 mM Na_2HPO_4, 7mM $NaHPO_4$, 100 mM NaCl, pH 7.0) 1:1, put over Lymphoprep (2:1) and centrifuged at 2200 rpm for 20 minutes without brake. The PBMC were collected from the ring at the interface between Lymphoprep and plasma, and washed with PBS 1x solution followed by centrifugation at 1500 rpm, 10 minutes to remove traces of Lymphoprep.

Contaminating red blood cells were lysed in pre-heated lysis solution (10 mM Tris, 150 mM NH_4Cl, pH 7.4), for 10 minutes at 37 ºC. Subsequently, PBMC were plated in non-adherent surface Petri dishes in 10 mL RPMI (5% FBSi, 1% Penicilin/Streptomycin/Amphotericin B (PSA)), at a density of $5x10^6$ PBMC/mL. Following overnight culture peripheral blood lymphocytes (PBL) were obtained, and counted. Two brands of RPMI were used (Gibco, Invitrogen, UK; and Lonza, BioWhittaker, Belgium). Fetal bovine serum was from PAA, Germany and PSA was purchased from Gibco, Invitrogen, UK.

3.2. Magnetic T $CD8^+$ cell purification

Starting from the previously isolated PBL, purified $CD8^+CD56^-$ were obtained by magnetic isolation, using one of the following approaches.

3.2.1. Isolation of untouched human $CD8^+$ T cells

Magnetic labelling

Isolation of untouched $CD8^+$ T cells was done using a $CD8^+$ T cell isolation kit (see below) (Miltenyi Biotec, Germany). PBL were centrifuged at 1500 rpm, 10

minutes, and the pellet was ressuspended in 400 μL of wash buffer (PBS 1x, 2mM EDTA, 10% BSA) per $100x10^6$ total cells. Afterwards, the $CD8^+$ T cell Biotin-antibody Cocktail (Cat #130-096-495 or #130-094-156) was added, 50 μL per $100x10^6$ total cells, and cells were incubated for 10 minutes at 4 ºC. Next, 300 μL of wash buffer per $100x10^6$ cells were added, followed by 100 μL of $CD8^+$ Cell Microbead Cocktail (Cat #130-096-495 or #130-094-156) per $100x10^6$ total cells. Then, cells were incubated, for the second time, for 15 minutes at 4 ºC. Finally, cells were washed with 1-2 mL of wash buffer by centrifuging at 1500 rpm, 10 minutes, and ressuspended in 500 μL of wash buffer per $100x10^6$ of total cells.

Magnetic separation using LS columns

A LS column (Cat #130-042-401, Miltenyi Biotec, Germany) was placed in the magnetic field of a suitable magnetic-activated cell sorter (MACS) separator (MidiMACS separator, Miltenyi Biotec, Germany) and prepared by rinsing with 3 mL of wash buffer. The cell suspension previously magnetically labeled was applied onto the column (maximum number of labeled cells/column - 10^8), and the total effluent, representing the enriched $CD8^+$ T cells was collected. Further column washing steps (3x1 mL) were performed with wash buffer to increase $CD8^+$ T cell recovery.

3.2.2. Positive selection of human $CD8^+$ T cells after CD56 depletion

Magnetic labelling – CD56 depletion

PBL were centrifuged at 1500 rpm, 10 minutes, and the pellet was ressuspended in 800 μL of wash buffer per $100x10^6$ total cells. The CD56 Microbeads (Cat #130-050-401, Miltenyi Biotec, Germany) were added, 200 μL per $100x10^6$ total cells, and cells were incubated for 15 minutes at 4 ºC. Next, cells were washed with 1-2 mL of wash buffer and centrifuged at 1500 rpm, 10 minutes, to remove non-binded beads, and ressuspended in 500 μL of wash buffer per $100x10^6$ of total cells.

Magnetic separation using LD columns

The LD column (Cat #130-042-901, Miltenyi Biotec, Germany) was placed in the magnetic field of a suitable MACS separator (MidiMACS separator) and prepared by rinsing with 2 mL of wash buffer. The cell suspension previously magnetically labeled was applied onto the column (maximum number of labeled cells/column - 10^8), and the total effluent, representing the CD56 depleted cells was collected. Further column washing steps (2x1 mL) were performed with wash buffer to increase cell recovery.

Magnetic labelling – CD8 positive selection

CD56 depleted cells were centrifuged at 1500 rpm, 10 minutes, and the pellet was ressuspended in 800 µL of wash buffer per $100x10^6$ total cells. The CD8 microbeads (Cat #130-045-201, Miltenyi Biotec, Germany) were added, 60 µL per $100x10^6$ total cells, and cells were incubated for 15 minutes at 4 ºC. Next, cells were washed with 1-2 mL of wash buffer by centrifuging at 1500 rpm, 10 minutes, to remove un-binded beads, and ressuspended in 500 µL of wash buffer per $100x10^6$ of total cells.

Magnetic separation using LS columns

The LS column was placed in the magnetic field of a suitable MACS separator (MidiMACS separator) and prepared by rinsing with 3 mL of wash buffer. The cell suspension previously magnetically labeled was applied onto the column (maximum number of total cells/column - 10^8), and the total effluent, representing the unlabelled cell fraction was collected. Further column washing steps (3x1 mL) were performed with wash buffer to optimize separation. Subsequently, the column was removed from the separator and placed in a suitable collection tube, and 5 mL of wash buffer were pipetted onto the column. Using the plunger supplied with the column, the enriched $CD8^+$ T cells were flushed out.

After collection of $CD8^+$ T lymphocytes, cells they were centrifuged at 1500 rpm for 5 minutes and counted using trypan blue exclusion test.

3.3. Cell culture conditions

After purification, $CD8^+CD56^-$ T cells were cultured in RPMI (10% HSi, 1% PSA), at 37 ºC, 5% CO_2, 95% humidity. Inactivated human serum was from PAA, Germany. Cultures were set-up at $1.0x10^6$ cells/mL on 24-well plates, and IL-15 (R&D Systems, USA) was added to a final concentration of 10 ng/mL. Cells were monitored daily through visual and microscopic observation to ensure optimal cell growth and contamination control and prevention.

Every 7 days cells were washed (1500 rpm, 5 minutes), counted and ressuspended on fresh RPMI (10% HSi, 1% PSA) to a final concentration of $1.0x10^6$ cells/mL. Fresh IL-15 was added to a final concentration of 10 ng/mL. Every 14 days besides renewal of IL-15, cyclic cell restimulation was done by TCR-dependent stimuli using PHA (Sigma Aldrich, USA) at a final concentration of 1 μg/mL.

Besides this cell culture condition, the human enriched $CD8^+$ T-cells isolated from PBMC were cultured *in vitro* under two other different culture conditions: on day 7, some cultures were splitted in two conditions: those stimulated with IL-15 and those washed and replated without IL-15, hence with medium alone (Figure 4.1). The cell cultures that were restimulated on day 7 with IL-15, when reached 14 days of culture were splitted again and some were cultured with only IL-15. These will be the cultures further designated and treated as controls. The remaining cultures were restimulated with PHA, alongside with IL-15, as described above. Those cultures where early IL-15 withdrawn was performed, from day 14 cell cultures were restimulated on a TCR-dependent manner with PHA and IL-15.

In some experiments, 100 U/mL of IL-2 (Tecin, USA) was added on day 14 instead of PHA (Figure 4.4)

3.4. Flow cytometry analysis

Cells were harvested, counted and between $0.25x10^6$ to $1x10^6$ cells were used for extracellular FACS staining. Then, cells were centrifuged at 1200 rpm, 2 minutes in 96 well round-bottom plates, and incubated with 50 μL of diluted appropriate antibody (ies) (Table 3.1) on staining buffer (PBS 1x, 0.2% BSA, 0.1% NaN_3) at 4 ºC for 30 minutes, in the dark and on ice. Negative controls (without anti-CD56 antibodies) were

also performed in each staining. Afterwards, cells were then washed again with staining buffer at 1200 rpm, 2 minutes. Cells were ressuspended in 200 μL of PBS 1x, and transferred to a FACS tube in a final volume of 300 μL.

Table 3.1. List of antibodies used on FACS extracellular staining.

Antibody	Clone	Isotype	Conjugate	Company	Dilution
CD8	MEM-31	IgG2a	PE	Immunotools	1:10
CD8	DK25	IgG1	FITC	Dako	1:10
CD56	B159	IgG1 k	Alexa 488	BD Biosciences	1:20
CD56	B159	IgG1	PE	Immunotools	1:10

Extracellular stained samples were acquired in a Coulter Epics XL (Beckman-Coulter) flow cytometer. For each sample 10.000 events were normally acquired in the lymphocyte gate defined using FS/SS parameters. Data were subsequently analyzed using the System II Coulter Epics XL software.

The individual analysis was done using the following approaches: 1) Setting of gates to delimit four different regions (A - resting lymphocytes, B - blast cells, C -dead cells (as determined by propidium iodide positivity), D – Live cells), 2) Determination of cell size and complexity according to FS/SS parameters and 3) CD56 expression – the relative percentage of $CD56^{+}$ T cells from total $CD8^{+}$ T cells was calculated.

3.5. Statistical Analysis

Statistical analysis was performed using Excel 2007 and IBM SPSS Statistics 2.0 (SPSS Inc. USA) softwares. Student T-test (for paired and independent samples) was used to determine the significance of the differences between group means. Pearson's correlation was used to assess the correlation between continuous variables (FS/SS and weeks in culture). Statistical significance was defined as $p<0.05$.

Results

IV. Results

4.1. IL-15 and PHA are necessary to keep $CD8^+$ T cells growing

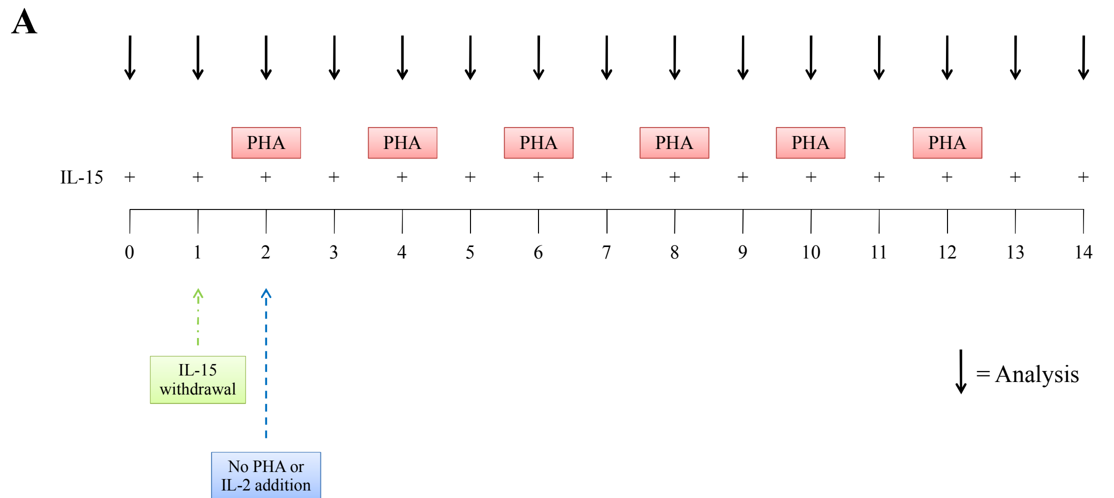
In order to establish the best approach to perform long-term cultures of isolated $CD8^+$ T lymphocytes, the number of live cells at the end of each 7 day period was registered, as described in material and methods. The concentration of cells in all cultures from days 0 to 21 was calculated, and the results obtained are depicted on Figure 4.1 (mean$\pm$SEM).

Since the initial lymphocyte concentration each week was $1x10^6$ cells per mL, this was the reference value, and the cultures quality was examined verifying fluctuations above or below this cut-off.

Thus, starting from an average of $1x10^6$ cells per mL (number of experiments, n=10), it was observed a slight but significant decrease (p=0.002) on cell number after the first week to $0.72x10^6 \pm 0.02x10^6$ cells per well (mean$\pm$SEM). On day 14, the splitted cultures refreshed with IL-15 on day 7 showed a statistically significant (p=0.010) doubling on lymphocyte numbers to $1.43x10^6 \pm 0.07x10^6$ cells per well (n=10). In contrast, the same cultures subjected to IL-15 removal, drastically decreased ($p<0.001$) their average cell concentration, by more than 50% ($0.33x10^6 \pm 0.01x10^6$ cells per well, n=3). These cells, were not able to recover optimal cell growth by day 21 ($0.40x10^6 \pm 0.14x10^6$ cells per well; p=0.786, n=2), even after IL-15 and PHA addition on day 14, eventually disappearing thereafter. The effect of restimulation with PHA on day 14 was quite striking. Indeed, while where PHA was omitted, the cell recovery decreased from $1.43x10^6 \pm 0.07x10^6$ cells per well to $0.30x10^6 \pm 0.02x10^6$ cells per well (n=2, p=0.001), and these lymphocytes eventually disappeared, thereafter cell cultures restimulated via TCR with PHA maintained on day 21 a cellular concentration well above the one million cut-off ($1.67x10^6 \pm 0.08x10^6$ cells per well, n=9), revealing a slight increase when compared to day 14, although not statistically significant (p=0.449).

Based on these results, subsequent cultures were carried out with the continuous presence of IL-15 (added in fresh medium every 7 days) and addition of PHA every 14 days.

A



B

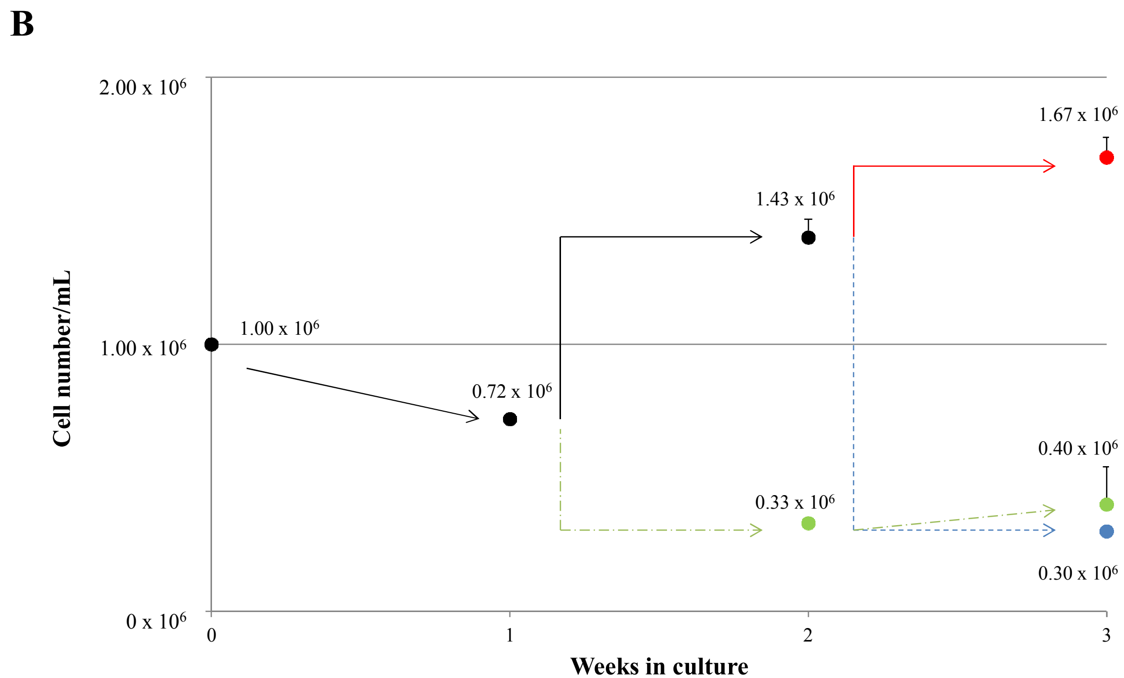


Figure 4.1. Study of different culture conditions. (A) Representative scheme of the different culture conditions performed. (B) Plot showing the cell concentration (mean±SEM) over time of culture. Green dash dot lines represent cell cultures subjected to IL-15 withdrawal on day 7; blue dash lines correspond to cell cultures restimulated on day 14 only with IL-15; red solid line stands for cell cultures restimulated with PHA, every 14 days.

4.2. IL-15 is necessary to maintain CD8$^+$ T cell viability and cell growth

In order to further analyze the effect of IL-15 withdrawal from cell cultures on day 7, we analysed cell viability and cell growth. While the percentage of surviving cells was calculated after subtraction of dead cells (as determined by a decrease in FS), the percentage of cells that increased their size was calculated by gating on cells with an overall increase in FS and SS. We have previously shown that dead cells have a reduced FS and are positive for propidium iodide, a marker of non-viable cells [125] positivity (see also Fig. 4.6A).

As can be seen in Figure 4.2, IL-15 withdrawal affected negatively cell viability and the percentage of blast cells. Hence, from a cell viability of 75.3±3.9 % on day 7 (n=5), cultures subjected to IL-15 removal decreased to 32.3±12.0 % on day 14 (p=0.008). This was in marked contrast to cultures in the presence of IL-15, where the number of live cells was about 63.2±6.9 %. The difference observed on day 14 between these two culture conditions was statistically significant, p=0.007.

Regarding the percentage of blast cells (Figure 4.2B), cultures subjected to IL-15 withdrawal showed a marked decrease from 20.1±6.7 % on day 7 (p=0.050) to 1.9±0.9 % (n=5) on day 14. In marked contrast, cultures with IL-15 showed a 21.7±3.9 % of blasts on day 14 (n=5). The difference on the percentage of blast cells between these two culture conditions on day 14 was statistically significant (p=0.004).

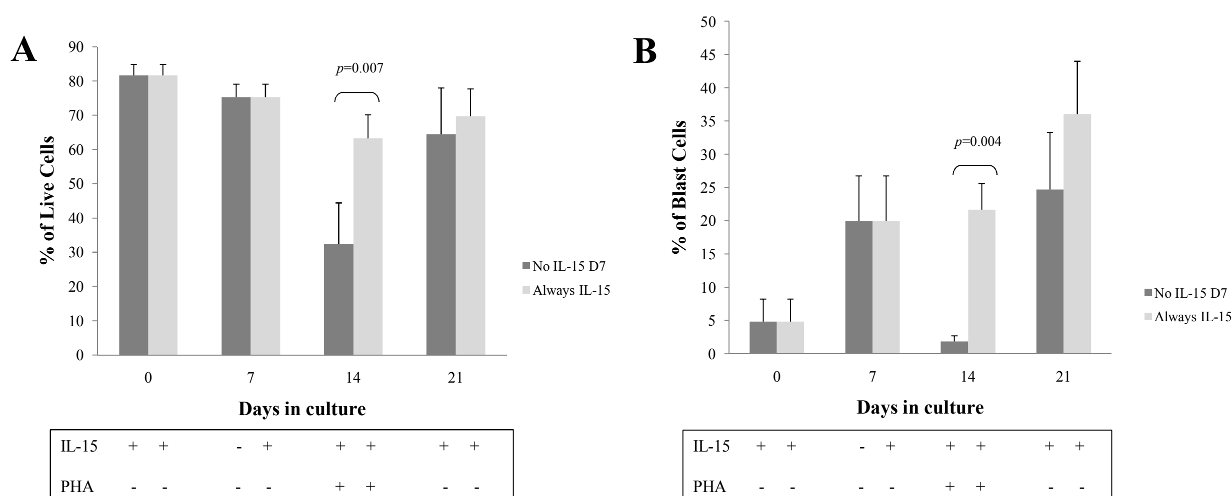


Figure 4.2. Early IL-15 withdrawal markedly affects cell viability and blast cells count. (A) Percentage of live cells over time of culture. (B) Percentage of blast cells over time of culture. (A) and (B) represent mean±SEM values from five experiments. Paired student T-test. The level of significance was set at p<0.05.

4.3. PHA signals downregulate CD56 expression levels in IL-15 differentiated $CD8^+$ T cells

A detailed study regarding the percentage of $CD8^+$ $CD56^+$ cells was done along a 28 days culture period. Cell cultures where PHA was absent on day 14 were used as controls (Figures 4.3 and 4.4).

The results suggest a progressive increase on the percentage of $CD56^+$ cells until day 14. Accordingly, in the cell cultures (condition D14 + PHA), after one week it was observed a statistically significant increase ($p=0.001$) on the relative percentage of $CD56^+$ cells from 4.6 ± 1.8 to 14.5 ± 3.7 (mean$\pm$SEM, $n=11$). The same behavior was verified on day 14, when the percentage of $CD56^+$ cells accounted for 25.4 ± 4.6 of total $CD8^+$ cells ($n=11$, $p<0.001$) (Figure 4.4).

Interestingly, IL-15 withdrawal on day 7 affected negatively the percentage of $CD56^+$ cells (Figure 4.3). Thus, while cells grown in the presence of IL-15 for 14 days showed a percentage of $CD56^+$ cells of 14.3 ± 2.8%, cell cultures where IL-15 was removed showed only 3.8 ± 1.3% of $CD56^+$ cells ($n=5$). Again, the difference between these two conditions was statistically significant ($p=0.004$).

A striking pattern modification took place on day 21 in cultures where PHA was added on day 14, where a clear decrease on the percentage of $CD56^+$ cells was observed (25.4 ± 4.6 *versus* 7.0 ± 1.0 %, $n=8$, $p=0.004$). Contrarily, in control cultures without PHA, such decrease was not verified (25.4 ± 4.6 *versus* 20.2 ± 8.3; $n=3$; $p=0.132$).

This pattern was not seen when IL-2 was added on day 14 instead of PHA (Figure 4.5).

Even though the addition of PHA affected the percentage of $CD56^+$ cells, it was a temporary and reversible effect. In fact, from day 21 to day 28 it was found that cell cultures recovered to a percentage of $CD56^+$ cells of 15.1 ± 3.4 % ($n=4$, $p=0.047$, Figure 4.3A).

No addition of PHA on day 14 led to a decrease of $CD56^+$ cells on day 28. However, this refers to the only one experiment that achieved this day in this culture condition.

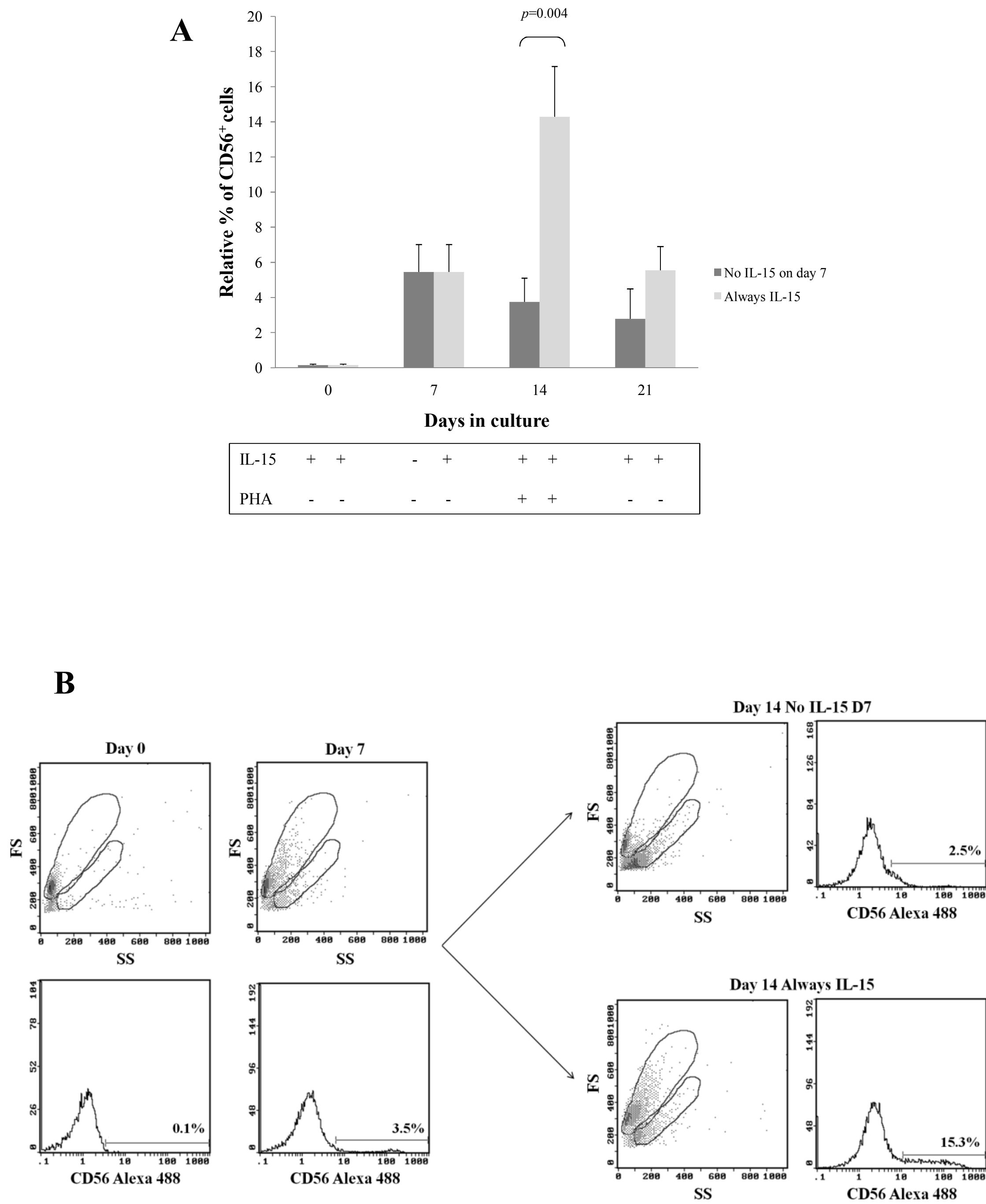


Figure 4.3. Early IL-15 withdrawal markedly affects CD56^{+} cells. (A) Percentage of live cells over time of culture. (A) Represents mean±SEM values from five experiments. Paired student's *t*-test. The level of significance was set at $p<0.05$. (B) Representative flow cytometry data showing the differences on the percentage of CD8^{+}CD56^{+} T cells after cytokine removal at day 14. Histograms show CD56 expression on live CD8^{+} T cells (region D on dot-plots).

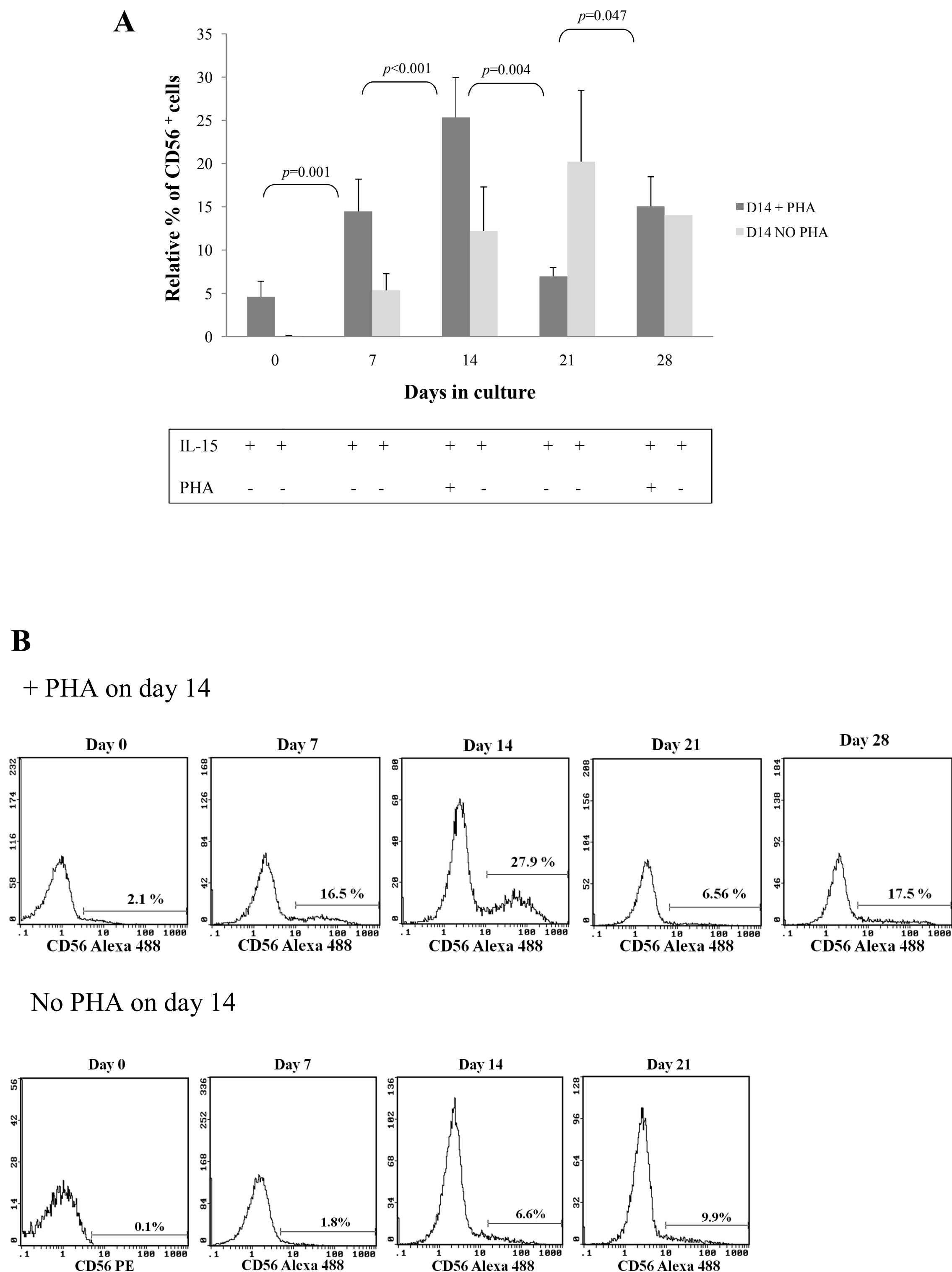


Figure 4.4 PHA addition on day 14 negatively affected the percentage of CD56$^+$ cells. (A) Relative percentage of CD56$^+$ cells over days in culture. Data depicted are the mean±SEM of relative percentage of CD56$^+$ cells, gated on live CD8$^+$ T cells. The level of significance was set at $p<0.05$ using unpaired student T-test.. (B) Representative histograms, gated on live CD8$^+$ T cells from two different experiments where PHA was added (upper graphs) or omitted (lower graphs) on day 14.

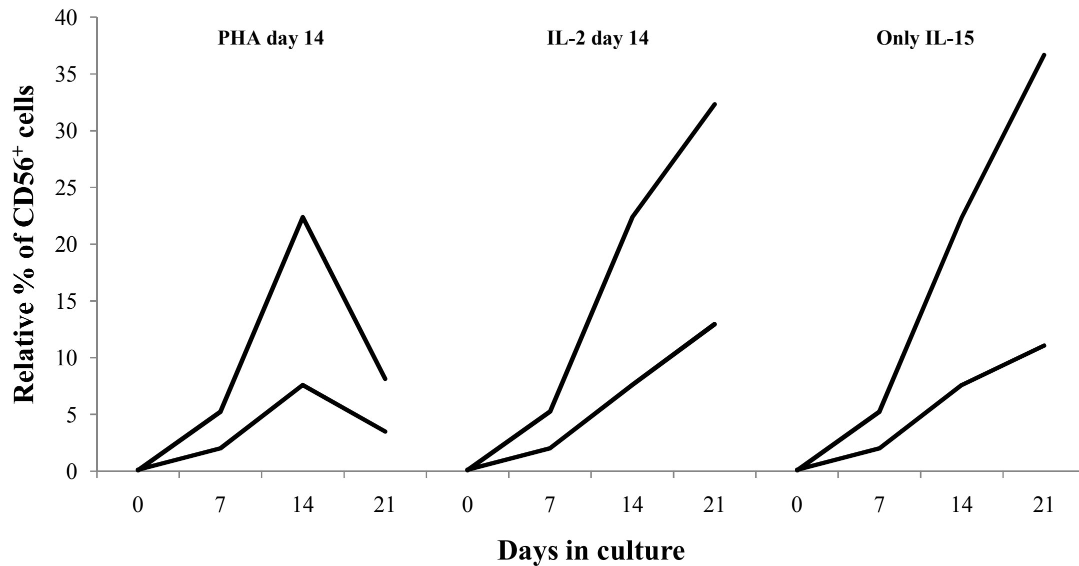
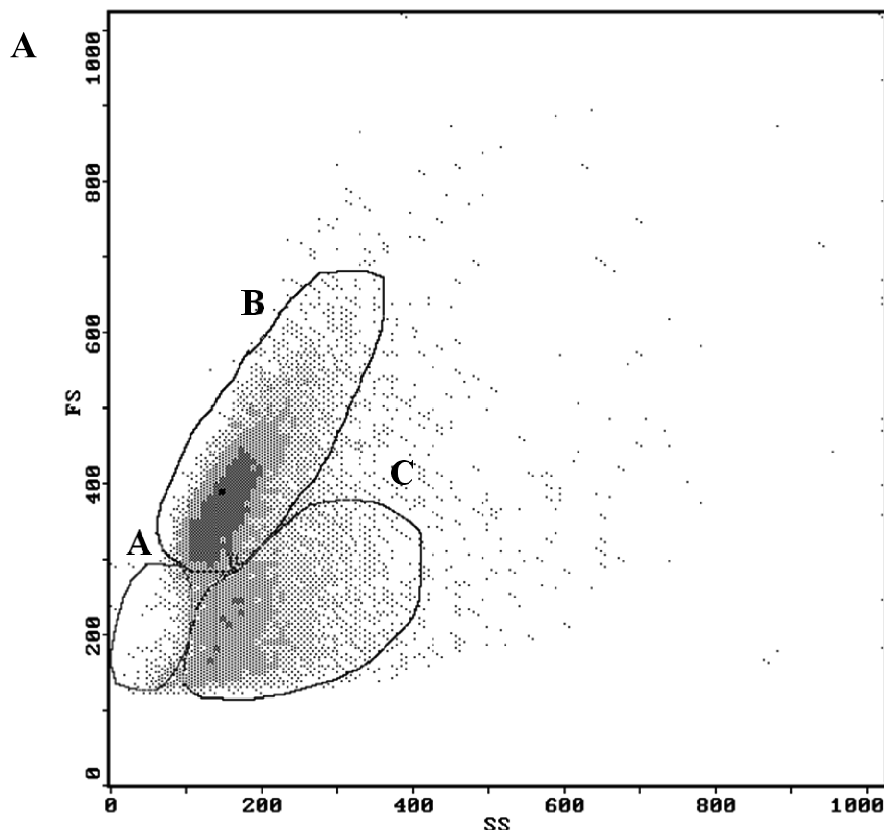


Figure 4.5. PHA, but not IL-2, adding negatively affects the relative percentage of CD56$^+$ cells. Data from two different experiments.

4.4. CD56 expression takes place preferentially on blast CD8$^+$ T cells

Next, we wanted to ascertain whether cellular transformation influenced the expression of CD56. It was found that CD56 was preferentially expressed by blast cells at any point ($p<0.05$, Figure 4.6).



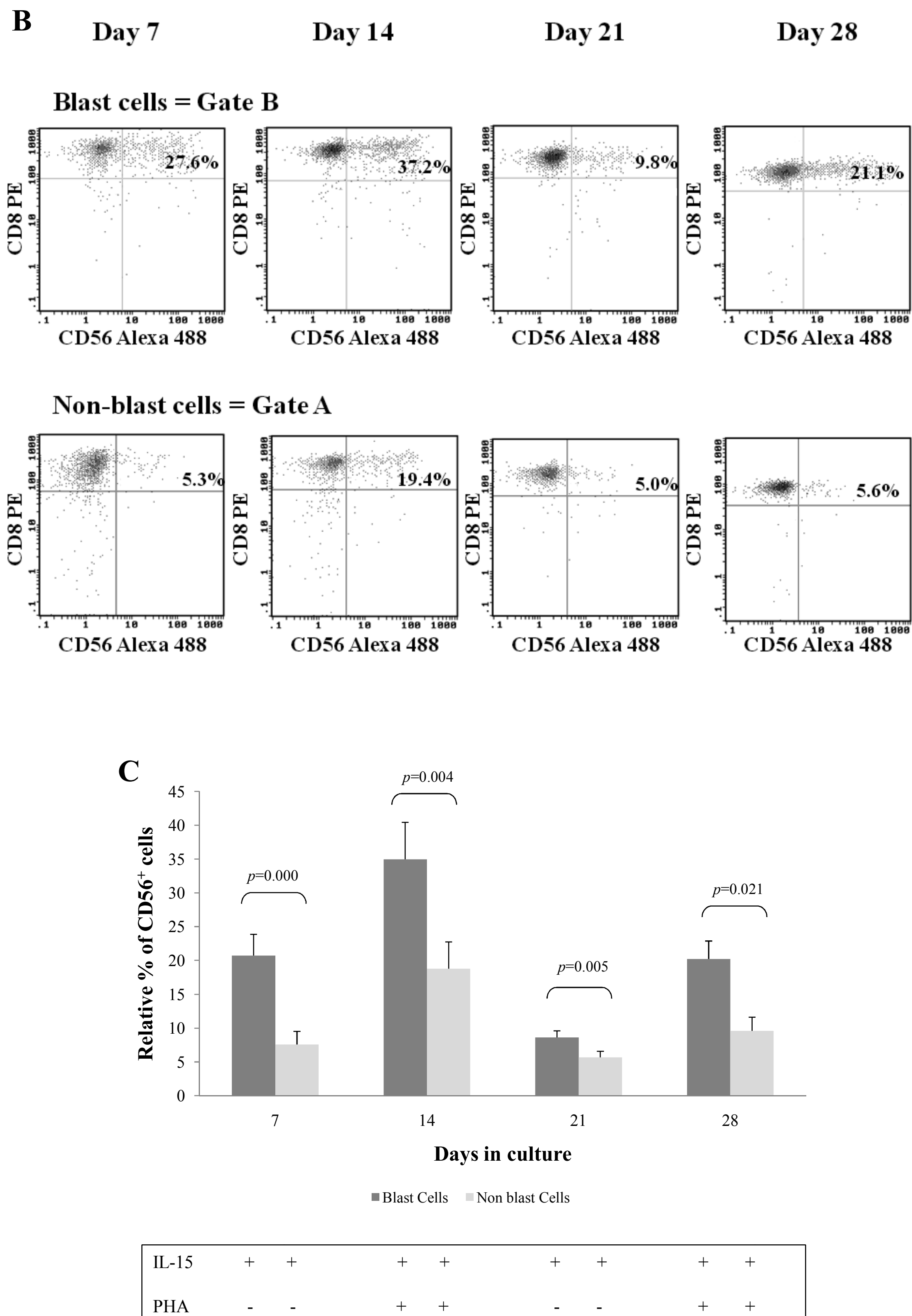


Figure 4.6. CD56 is preferentially expressed on blast cells. (A) Representative dot-plot indicating the aforementioned analysis gates (A stands for non-blast cells, B denotes blast-cells and C represents dead cells). (B) Representative dot-plots for two experiments over days in culture. (C) Relative percentage of $CD56^+$ cells over days in culture. Data depicted are the mean±SEM of relative percentage of $CD56^+$ cells, gated in blast and non-blast cell region. Paired student T-test. The level of significance was set at $p<0.05$.

4.5. CD56 expression in long-term cultures of CD8$^+$ T cells

To ascertain whether the up and down CD56 modulation seen when PHA was added on day 14 could be observed in long-term cultures, three CD8$^+$ T cells samples were cultured up to nine weeks in the continuous presence of IL-15 and the addition of PHA at weeks 2, 4, 6 and 8. As illustrated in Figure 4.7, all three cultures behaved similarly during the first 4 weeks, with CD56$^+$ cells decreasing one week after addition of PHA and increasing two weeks later. This spiky pattern (up and down) was maintained in sample 1 until the end of the culture, while in sample 2 only lasted until week six, at which time a steady increase in the percentage of CD8$^+$CD56$^+$ T cells was observed until week nine. This increase was most striking in sample number 3, where the percentage of CD8$^+$CD56$^+$ T cells increased by more than two fold between week four and nine.

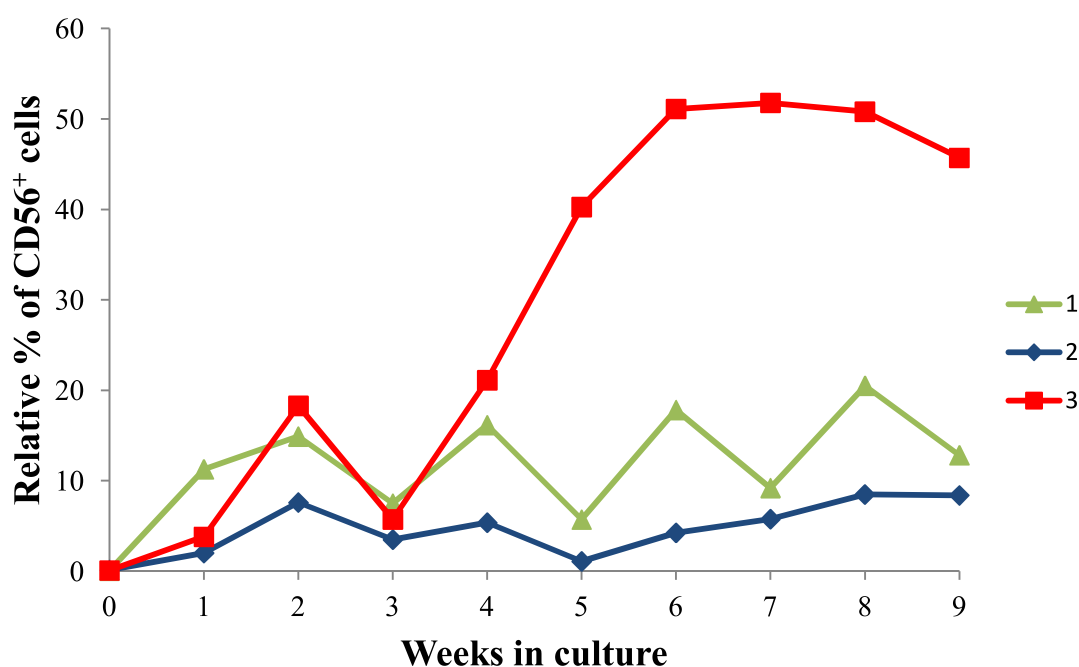


Figure 4.7. Percentage of CD56$^+$ cells in long-term cultures. Data of three different experiments. The percentage of CD56 was calculated on gate of alive cells and CD8$^+$ T cells.

4.6. Long-term cultures of $CD8^+$ T cells with IL-15 and PHA increases the overall size and complexity of the cells

In general, the percentage of alive cells exceeded the percentage of dead cells, throughout the culture period (Figure 4.8). However, after eight weeks, most cultures showed a decline in the percentage of live cells.

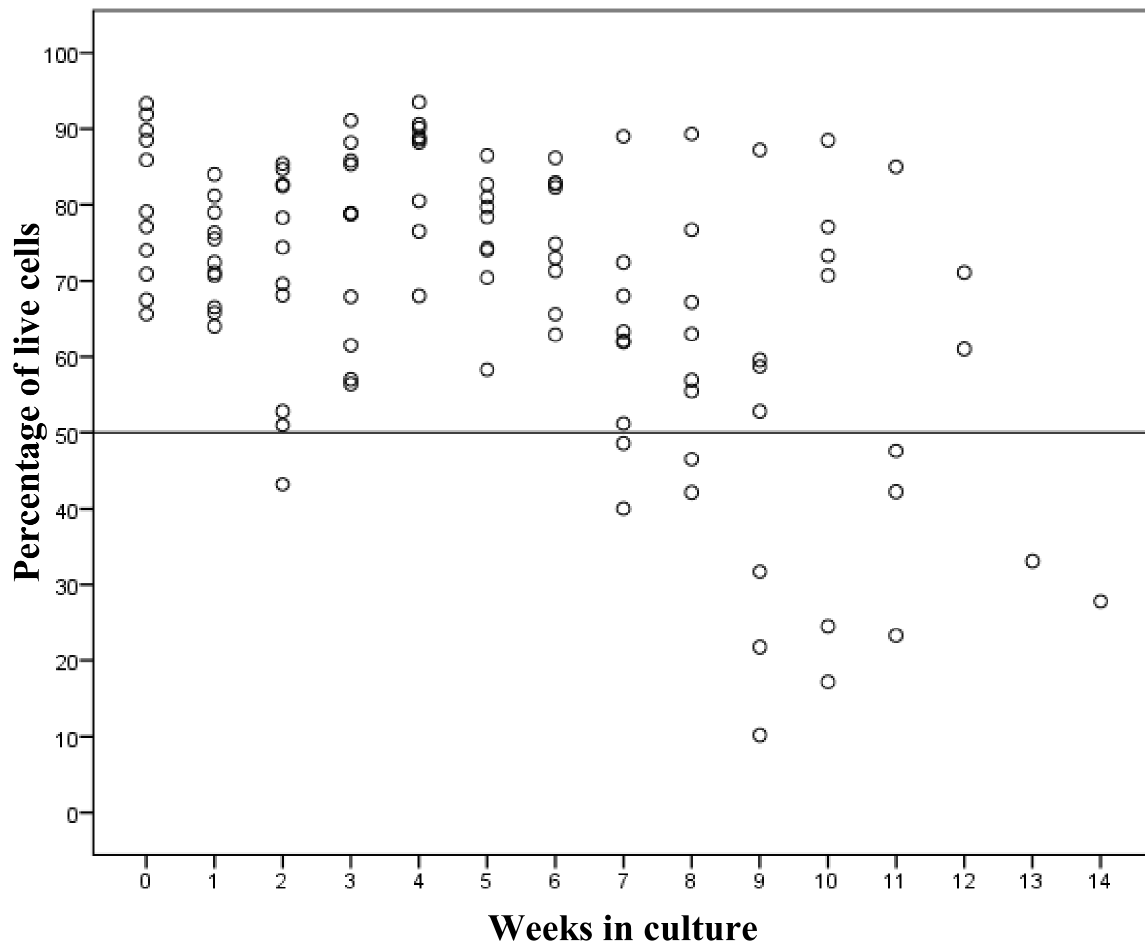


Figure 4.8. Percentage of live cells in long-term cultures. Scatter plot showing the percentage of live cells along the culture period. Each circle represents a different experiment (n=11). Only 50% of the cultures survived until week 10. This number was reduced to two by week 12.

The following phenotypic features studied were the forward scatter (FS) and side scatter (SS) parameters, in order to analyze $CD8^+$ T cell size and cellular complexity throughout time (n=11). Figure 4.8 plots the dispersion of mean fluorescence intensity (MFI) values of FS/SS over the days in culture.

There was a positive and statistically significant (p<0.001) correlation between both FS (r=0.494) and SS (r=0.584) parameters and time of cell culture period.

Interestingly, once cells become larger and more complex they did not return to their initial status exhibited on day 0.

In spite of this global increase of FS/SS parameters, the day-specific analysis provides additional data. Thus, one week after adding PHA (week 3, 5, 7…), a notorious enhancement on cell size and on lymphocyte complexity and granularity (although less perceptible) was observed.

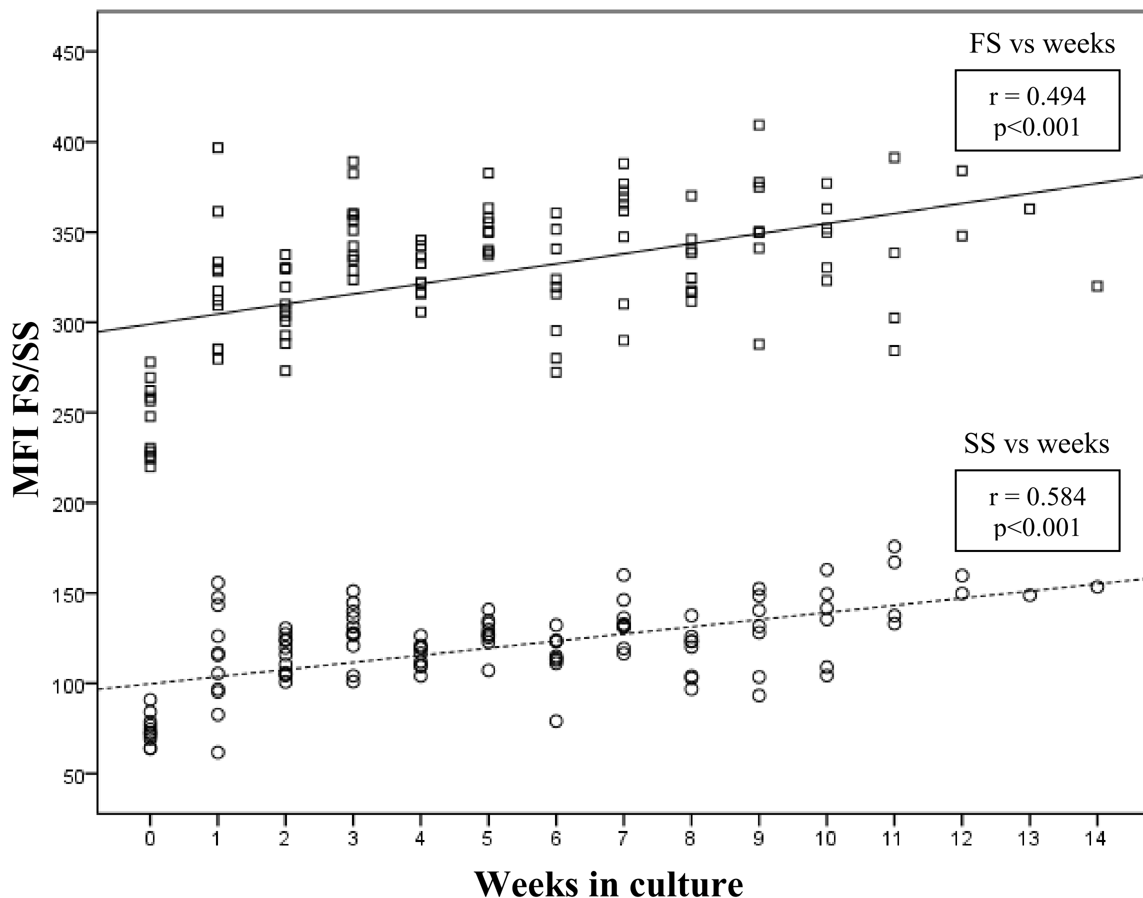


Figure 4.9. Cellular parameter (FS/SS) of cells over time. Data from 11 experiments. Pearson coefficient correlations and p values are depicted. The level of significance was set at p<0.05. Squares and straight line denote FS and circles and dotted line denote SS. Each square/circle represents the live cell gate MFI of an experiment on each week (n=109).

Discussion

V. Discussion

5.1. IL-15 and PHA: a good combination to keep CD8$^+$ T cells alive and growing

The main objective of this work was to establish a good and reliable cell culture method to keep in culture *ex vivo* CD8$^+$ T lymphocytes for long periods of time using IL-15 as the main stimulus. By combining IL-15 with addition of PHA every two weeks we have been capable to maintain *ex vivo* human CD8$^+$ T cells in culture for more than 90 days.

It is well known that IL-15 is a T cell growth factor which main biological functions are the inhibition of apoptosis and the stimulation of cell proliferation. *In vivo* studies have demonstrated that IL-15 functions during all four phases of the T-cell immune response: initiation, expansion, clonal deletion and memory generation [126], and specifically regulates the survival and proliferation of memory CD8$^+$ T cells under normal physiological conditions [127]. Indeed, long-term survival of CD8$^+$ memory T cells is critically dependent on this cytokine [46, 128, 129]. The key role played by IL-15 *in vivo* became clear with the initial reports showing that IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice are lymphopenic and lack NK cells, NKT cells, IEL subsets and activated CD8$^+$ T cells [46, 53, 54]. Interestingly, administration of recombinant IL-15 has been shown to selectively stimulate the proliferation of memory phenotype CD8$^+$ T cells both *in vivo* and *in vitro* [50].

Previous *in vitro* studies have focused on IL-15 effects on short-term T cell cultures [12, 25, 130]. Thus, we have previously shown that IL-15 is capable to activate and drive into proliferation human CD8$^+$ T cells in an antigen-independent, that is without TCR stimulation, during a 7-day culture period, suggesting a generic influence of this cytokine on CD8$^+$ T cell proliferation and survival [12]. In another study, the effect of exogenous IL-15 on the *in vitro* expansion of CD3$^+$CD56$^+$ NKT-like cells present in cord blood mononuclear cells samples was explored in 14-day cultures [27]. Despite these remarkable effects CD8$^+$ T cell homeostasis, IL-15 biological activities *in vitro* are not long lasting and to our knowledge, there are no reports of long-term cultures of CD8$^+$ T cells with only IL-15.

In the present study, we were able to keep optimal numbers of live $CD8^+$ T cells in culture with IL-15 for up to 21 days, but not beyond. Importantly, IL-15 had to be freshly added each week to the cultures in order to keep $CD8^+$ T cell alive, indicating that its continuous presence is necessary to avoid $CD8^+$ T cells apoptosis. In this regard, it is important to refer that one of the main biological properties of IL-15 is related with its capacity to inhibit apoptosis in multiple systems [131-133], including prevention of IL-2 dependent activation induced cell death (AICD) [134], presumably by upregulating anti-apoptotic proteins such as Bcl-2 and Mcl-1 [135-140]. Most likely, the continuous presence of IL-15 in the cultures is improving the survival of our *in vitro* cultured $CD8^+$ T cells by upregulating pro-survival factors. Our recent work showing that $CD8^+CD56^+$ T cells cultured for 12 days with IL-15 express high levels of the anti-apoptotic protein Bcl-2 [95], supports this assumption. The results of this study are also in agreement with the fact that in the absence of proliferative IL-15 signals, memory $CD8^+$ T cells undergo a slow atrophy in number, until they become essentially undetectable [127, 128, 141], which is in line with our observation that IL-15 withdrawal at day 7 drastically decreased $CD8^+$ T cell viability and numbers on day 14.

It is well known that human $CD8^+$ CTL are difficult to maintain in culture for long periods of time (>3 weeks) without the eventual loss of viability, requiring periodic stimulation with either antigen, anti-TCR antibodies or mitogenic agents such as PHA, which ideally should be provided in combination with feeder cells that function as antigen presenting cells (APC) and the appropriate cytokines to proliferate [28]. Having this in mind, and in order to overcome the temporal limitations of the cell growth and survival bioactivities of IL-15, we decided to combine IL-15 with a TCR-mediated stimulus. PHA was the choice because it binds to the TCR/CD3 complex, mimicking all the intracellular activation events triggered by anti-CD3 antibodies [142-144], and does not interfere with functional and phenotypic characteristics of the activated T cells. Previous studies have already shown that it is possible to carry out long-term cultures human $CD8^+$ T cells. Lu et al, by using IL-15 added every 2/3 days in combination with anti-CD3 antibodies and the presence of irradiated feeder cells were able to grow $CD8^+$ T cell for up to 80 days [28]. Also, Li et al. used IL-15 stimulation every 10 days, with anti-CD3/CD28 stimulation at seeding, providing the crucial TCR signal, and were able to grow human T $CD8^+$ cells for 45 to 80 days in culture [29].

Noteworthy, when PHA was included in the stimulus protocol, we found that it helped to keep IL-15-stimulated $CD8^+$ T cell cultures for much longer periods of time when added every two weeks. Under these conditions, some cultures of $CD8^+$ T cells lasted up to 90 days. The molecular basis for this successful combination is presently not known, but could be related with the establishment of a correct balance between proliferative signals (PHA) and pro-survival signals (IL-15). Thus, PHA is a potent T cell mitogen that induces T cell blastogenesis and proliferation when accessory signals are present [145]. Yet, proliferating T cells, especially at S phase of the cell cycle, are susceptible to apoptosis [146]. Thus, in spite of inducing cell proliferation, PHA also promotes cell apoptosis due to Fas-FasL interactions [147], which can be inhibited by IL-15 [131]. Thus, the combination of PHA and IL-15 signals apparently creates a favorable environment for $CD8^+$ T cells to grow and expand. While PHA mainly promotes the entrance into the cell cycle, IL-15 contributes to basal levels of proliferation and protection from apoptosis. In this context, it has been proposed that IL-15 acts as a survival factor that induces a quiescent state in T cells that helps them to survive in the absence of TCR engagement [148].

In support of this assertion are studies showing that IL-15 can augment the anti-CD3 and PHA proliferation and cytokine production of both mouse and human $CD8^+$ T cells induced by anti-CD3 antibodies and PHA [37, 130, 149-151]. Moreover, a recent study comparing the differential effect of IL-15 and IL-2 on survival of PHA-activated cord blood and adult peripheral blood T lymphocytes showed that IL-15 enhanced the survival of cord blood PHA-activated T cells by decreasing the caspase-3^+ population and by increasing the Bcl-2^+ population [152]. Finally, Seder et al. demonstrated that IL-15 enhanced proliferative responses by PBMC obtained from HIV-1– infected individuals in response to antigens or mitogen [153].

Overall, our results suggest that the combination of TCR-independent (IL-15) and TCR-dependent (PHA) signals keeps $CD8^+$ T cells into a continuous proliferation and survival process. Yet, even though our IL-15/PHA cell cultures were viable during most of their life span, viability started to decline after two/three months, indicating that that IL-15 signaling combined with alternated PHA stimulation is not enough for maintaining long-term $CD8^+$ T cell growth. Most likely, and in resemblance with senescence models or natural ageing, this suggests that proliferation/survival signals other than those provided by IL-15 and PHA are lacking in our *in vitro* system.

5.2. IL-15/PHA stimulated $CD8^+$ T cells: a good model to study differentiation

Taking advantage of the positive effect of the combination of IL-15 and PHA on $CD8^+$ T cell growth and survival, we decided to examine the differentiation status of the cultured $CD8^+$ T cells by analyzing cell size and complexity akin to CD56 expression. The results of this analysis showed that IL-15/PHA stimulated $CD8^+$ T cells acquired a large granular morphology and a fraction of these cells, mostly blast cells, expressed CD56, the prototypic marker of NKT cells.

We have recently shown that IL-15 *per se* is capable of driving purified $CD8^+CD56^-$ T cells into a TCR-independent program of differentiation that is associated with the acquisition and upregulation of typical NK receptors, resulting in the generation of $CD8^+CD56^+$ T cells co-expressing other NK receptors, such as several KIR and NKp46 [95]. Although these studies were performed during a short window of time (7-12 days), they laid the grounds to explore further the biological activities of IL-15 on $CD8^+$ T cells during longer period of times.

Taking into account that CD56 is a good marker of *de novo* NKR expression by IL-15-differentiated $CD8^+$ T cells, we assessed the expression profile of this typical NK receptor on *ex vivo* $CD8^+CD56^-$ T cells cultured for periods longer than 12 days with the IL-15/PHA combination.

In view of the influence of IL-15 on CD56 upregulation by $CD8^+$ T cells, the cytokine early withdrawal from cell cultures resulted in a decrease the percentage of $CD8^+CD56^+$ T lymphocytes. Since IL-15 *per se* is capable of inducing *de novo* expression of some typical NK receptors, such as NKp46, it is tempting to speculate that IL-15 withdrawal would likely affect also their expression.

A detailed study of the percentage of $CD8^+CD56^+$ cells along the whole culture period revealed the negative impact that PHA stimulation had on CD56 expression, with a consistent decrease following PHA addition during the first 4-6 weeks of culture. These data suggest the existence of a close relationship between PHA stimulation of chronically activated $CD8^+$ T cells and CD56 expression.

One could hypothesize that, perhaps, PHA was "masking" some CD56 epitopes. This comes from the fact that PHA binds to glycosylated residues on the TCR/CD3 complex [10, 11, 143, 144]. Being CD56 a heavily glycosylated molecule [112, 113]

there is the possibility that PHA was blocking some anti-CD56 antibody binding sites. However, since the binding of PHA to the TCR/CD3 complex is temporary, the same may be argued for its putative binding to CD56, ruling out a possible “masking” effect. Therefore, the PHA-induced CD56 decrease must be due to other mechanisms, for instance, the hypothetic activation of common signaling pathways between TCR and IL-15.

Interestingly, both IL-15 and TCR-engagement (through CD3 stimulation) are capable of inducing $CD8^+$ memory T-cells proliferation, suggesting that these two pathways share some common downstream events, despite apparent differences in the initial ligand-receptor interaction [154]. Moreover, it was found that these two stimuli induce highly similar responses, showing that many of the genes involved in signal transduction, transcription regulation, T cell activation, cell cycle, apoptosis, and effector functions, which are characteristic changes of TCR-mediated activation, are expressed upon IL-15 activation, suggesting that both stimuli effectively turn on the cell cycle machinery in memory phenotype $CD8^+$ T cells [154]. Therefore, we suggest that TCR activation following PHA stimuli could interfere with IL-15 intracellular signaling, affecting the CD56 expression induction by IL-15 (Figure 1.2).

Due to this seemingly overlapping downstream events and induction of similar gene expression and effector function, we hypothesize that PHA may be competing with IL-15 for intracellular cascade ligands, decreasing the available molecules that mediate the IL-15-induced CD56 upregulation (Figure 5.1). Indeed, the addition of IL-2 on day 14, instead of PHA, did not result on a decrease in CD56 expression. Only when restimulation was done with PHA $CD8^+$ T cells downregulated CD56 expression, suggesting that TCR-mediated activation works as a negative feedback for CD56 expression. Moreover, the negative effect of PHA on the percentage of $CD8^+CD56^+$ T cells from day 21 to day 28 was reversed by replacing PHA by fresh IL-15, suggesting a temporary and reversible effect. Alternatively, the decrease on CD56 expression could be the result of an enhanced apoptosis after PHA addition, suggesting that CD56 down-modulation is a consequence of PHA-stimulation.

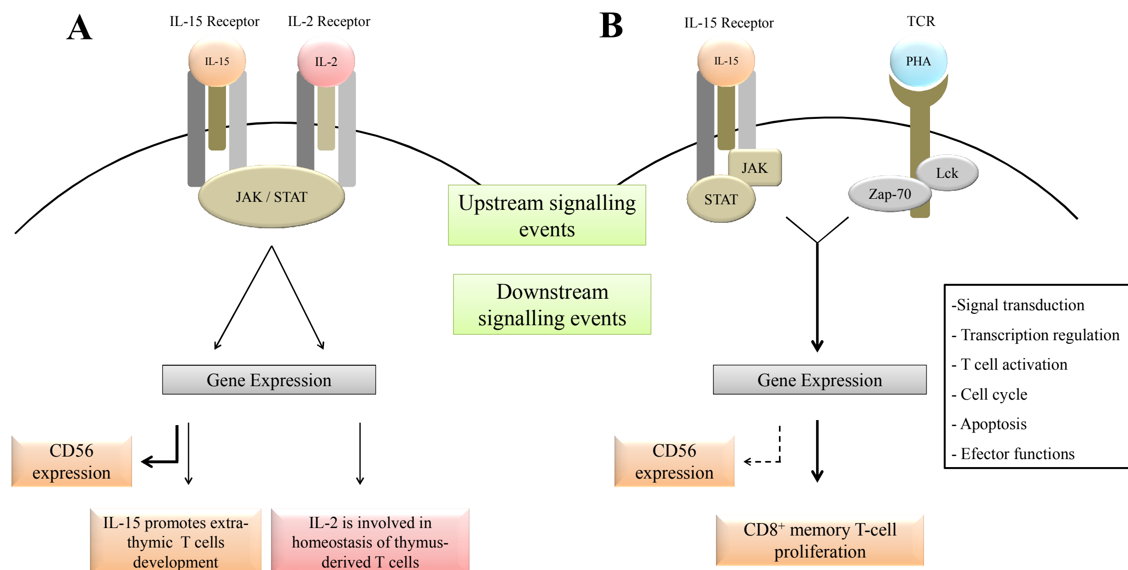


Figure 5.1. Signalling pathways of IL-15, IL-2 and PHA-TCR. (A) IL-15 and IL-2 activate similar signaling pathways throughout activation of JAK/STAT, meaning they should dictate a common biologic outcome. However, the fact they do not means there are differences in the signaling pathways triggered by each cytokine, explaining their unique functions [26, 35, 46, 47] (B) The different initial signaling events of both IL-15 receptor and TCR complex deliver significantly overlapping downstream signals [154] in memory phenotype CD8$^+$ T cells *in vitro*, leading to similar gene expression. PHA may be competing with IL-15 for intracellular cascade ligands, affecting negatively the expression of CD56 (dotted line).

It appears that the signaling pathways through IL-15 receptor and the TCR complex deliver significantly overlapping downstream signals in CD8$^+$ T cells *in vitro*. These preliminary results need to be corroborated and extended in order to unravel the molecular mechanism behind the PHA interference on CD56 expression

In this study, we have also shown that CD56 is preferentially expressed by blast-like CD8$^+$ T cells. Although the reason for this finding is presently unknown, a possible explanation might take into account an epigenetic mechanism, such as modulation of the methylation status of the transcriptional active region, taking place during active cell proliferation [155]. Accordingly, it was found that the promoting region of CD56 gene is located on a GC rich domain, containing high number of CpG dinucleotides, which renders this promoter an excellent candidate to methylation [156]. Hence, while increased methylation in the promoter region of a gene leads to reduced expression, demethylation could render the gene constitutively expressed [155]. In fact, in contrast to NK cells, CD56 expression on T cells is not constitutive. It has been observed that CD56$^+$ T cells are almost absent at birth and in adults are found as oligoclonal

expansions [104], suggesting a link between CD56 expression and number of division cycles.

PHA-induced down-modulation of CD56 expression by $CD8^+$ T cells was not a lasting effect. It took place during the first four weeks in the large majority of samples studied but differed thereafter. In three long-term cultures, and from day 28 onwards, cultures began to diverge with one sample keeping the up-down pattern until week nine, and two other samples displaying a gradual increase in $CD8^+CD56^+$ T cells. This was most striking in one culture, where after day 42 the $CD8^+CD56^+$ T lymphocytes reached a plateau-like phase, showing an impressive percentage of CD56 of around 50%. Thus, these two cell cultures apparently became "refractory" to the purported PHA-signaling interference with the IL-15 pathway. One possible mechanism rendering these $CD8^+$ T cells refractory is the possible "constitutive" expression of CD56 due to a complete demethylation of the gene promoter, as referred above [156]. Whether this is the result of the "replicative past" of the $CD8^+$ T cells under study, remains to be ascertained.

Finally, we were also able to show a positive correlation between size (FS) and complexity/granularity (SS), and the time of cell culture under our culture conditions. This may be a consequence of the combined use of IL-15/PHA on cell proliferation, resulting in a progeny of blastoid/activated cells [12, 50, 153, 157-162]. The observed increase in SS along the culture time imply an increase on $CD8^+$ T cell granularity, indicating that long-term IL-15/PHA cultured $CD8^+$ T cells are functional $CD8^+$ T cells displaying an increased cytotoxic potential, as determined by high granzyme B/perforin content [12, 95]. Due to their features, IL-15/PHA cultured $CD8^+$ T cells can be considered large granular lymphocytes endowed with cytolitic activity and capable to secrete and array of regulatory cytokines, as described by us [95, 104]. Interestingly, $CD8^+$ T cells with these characteristics are being used *in vivo* both in animals and in humans for the treatment of melanoma, renal cell carcinoma, non-Hodgkin's lymphoma, and lung and colorectal cancers [163, 164]. Last, but not least, the morphological characteristics of our *in vitro* $CD8^+CD56^+$ T cells are reminiscent of the large granular lymphocytes described in leukemic patients. Indeed, IL-15 has been proposed to play a role in the development of T cell large granular lymphocyte (T-LGL) leukemia [165], an indolent lymphoproliferative disorder that represents a highly significant clonal expansion of cytotoxic T cells [166-168]. There are several evidences that aggressive variants of human T-cell large granular lymphocyte leukemia are

characterized by an immunophenotype that includes surface expression of CD56, along with a variable surface expression pattern of NK cell and/or T cell markers [166, 169-171].

5.3. Future prospects

In the present investigation we were able to maintain $CD8^+$ T lymphocytes in culture with IL-15/PHA for long periods of time. Moreover, during this period these cells acquired a large granular morphology and increased the expression of a prototypic marker of NKT cells (CD56), suggesting a process of cellular differentiation induced by IL-15. The successful establishment of these long-term cultures is the starting point to set up a solid cell culture method, scientifically accurate, to study the effects of IL-15 on the upregulation of other NKR for long periods of time.

The observed downmodulation of CD56 expression after PHA addition to cell cultures remains to be experimentally substantiated, and more work needs to be done to unravel the reason behind the PHA interference on $CD56^+$ T cells percentage. In fact, it would be interesting to validate or exclude our hypotheses. Both the signaling pathways through IL-15 receptor and the TCR complex deliver significantly overlapping downstream signals in memory phenotype $CD8^+$ T cells *in vitro*, which could actually be an interesting pathway to uncover, since it remains to be determined at what level these two signals converge, and what molecules are involved in the overlapping processes. Furthermore, it would be interesting to perform similar experiments but replacing TCR triggering with anti-CD3 stimulation instead of PHA, which would provide more information to assess if the downmodulation of CD56 expression is indeed related with intracellular cascade ligands competition. Alternatively, the decrease on CD56 expression could be explained by enhanced apoptosis after PHA addition, which in the future could be experimentally assessed.

As already discussed, a possible reason explaining the expression of CD56 on dividing blasts, could be an epigenetic mechanism, such as modulation of the methylation status of the transcriptional active region, taking place during development

and cell proliferation [155]. Hence, it would be interesting to experimentally uncover if the apparent evasion on the effect of PHA downmodulating CD56 expression is justified by demethylation, rendering the CD56 gene to be expressed constitutively.

Finally, inadequate expression of cytokines and/or abnormal activation of cytokine receptors have been suggested to complement genetic aberrations in leukemogenesis, thus, supporting survival and proliferation of leukemic cells [172, 173]. In this context, IL-15 may have a role in the development of T cell large granular leukemias (T-LGL) [165]. Therefore, our *in vitro* model might allow to determine whether IL-15 is a leukemogenic factor capable of T-cell leukemic transformation, and to unveil the molecular mechanisms underlining this process.

Conclusions

VI. Conclusions

In the present investigation we were able to maintain $CD8^+$ T lymphocytes in culture for long periods of time (over two months), when maintained in the continuous alternate presence of IL-15 and PHA, respectively. During this period, $CD8^+$ T cells acquired a large granular morphology and expressed CD56, the prototypic marker of NKT cells. Overall, the data suggest that the combination of TCR-independent (IL-15) and TCR-dependent (PHA) signals keeps $CD8^+$ T cells into a continuous differentiation process.

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