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Study of the Effect of the Anti-inflammatory Cytokines IL-10 and TGF-β on the IL-15-induced Activation of Human T Cells

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Resumo

O sistema imunológico humano é composto por múltiplos elementos que trabalham em conjunto para proteger o corpo contra doenças e agentes externos, mantendo a saúde e o equilíbrio. Neste estudo, destacamos a importância das células T e o seu papel fulcral no correto funcionamento do sistema imunológico. Um outro elemento de grande importância são as moléculas do Complexo *Major* de Histocompatibilidade Classe I (MHC-I), frequentemente encontradas em todos os tipos celulares, incluindo células T. Estas moléculas são amplamente conhecidas pela sua conformação fechada, porém, após certas alterações estruturais, podem adotar uma forma aberta, ficando conhecidas como conformadores abertos de MHC-I. As funções destes conformadores abertos vão muito além das habituais funções imunológicas, estando associados a diversas funções não imunológicas, e impactando vários ambientes clínicos e biomédicos. Estudos prévios mostram que as células T podem ser moduladas pela IL-15, afetando a ativação, proliferação e diferenciação, contudo, o efeito desta modulação na expressão de conformadores abertos de MHC-I não é conhecido. A influência que outras citocinas extensamente encontradas no sistema imunológico, nomeadamente a IL-10 e TGF-β, possam ter na modulação induzida por IL-15 e na expressão de conformadores abertos de MHC-I, é também indeterminada.

Neste estudo, linfócitos do sangue periférico (PBL) foram isolados a partir de *buffy coats* de dadores saudáveis, marcados com CFSE e cultivados por 12 dias na presença de IL-15, com a adição de IL-10 ou TGF-β a diferentes dias e em diferentes concentrações. As células foram depois marcadas com vários anticorpos por forma a diferenciar entre células T CD4+ e CD8+, e para possibilitar a deteção de conformadores abertos/fechados de MHC-I. A análise por citometria de fluxo foi posteriormente realizada.

Os resultados mostram de forma clara que a presença de IL-10 e de TGF-β intensifica significativamente a modulação proliferativa já conhecida da IL-15 nas células T, principalmente quando concentrações mais elevadas de citocina secundária (IL-10/TGF-β) estão presentes. Adicionalmente, este estudo demonstrou um impacto direto não apenas da IL-15, como também da IL-10 e TGF-β na expressão de conformadores abertos de MHC-I nas células T, constituindo novas descobertas. Foi observado um aumento destes conformadores aquando da presença de IL-15, enquanto que a presença de IL-10 e TGF-β contrabalançou este efeito, causando uma diminuição nos níveis de conformadores abertos de MHC-I.

Palavras-chave

Células T CD4+ e CD8+; Ativação e Proliferação de Células T; Conformadores de MHC-I Abertos e Fechados; IL-15; IL-10; TGF-β.

Resumo Alargado

O sistema imunológico humano é composto por uma imensidade de componentes que trabalham em sintonia para atingir um objetivo comum, a proteção do corpo contra doenças e agentes exógenos, mantendo a saúde e equilíbrio do organismo. De entre estes componentes, destacamos neste estudo as células T, responsáveis por uma multitude de funções cruciais para o correto funcionamento do sistema imunológico. Na superfície das células T é possível encontramos moléculas de imensurável importância, as moléculas do Complexo Major de Histocompatibilidade (MHC) Classe I, compostas por uma cadeia α pesada, uma cadeia leve denominada beta-2-microglobulina e um péptido de 8 a 12 aminoácidos. Este composto trímero é designado de conformador fechado de MHC-I, contudo, mediante dissociação da cadeia beta-2-microglobulina e na ausência do péptido, as moléculas de MHC-I apresentam uma conformação aberta, sendo designadas de conformador aberto de MHC-I. Estas moléculas de conformação aberta têm funções que vão muito além das habituais funções imunológicas, associando-se a uma variedade de recetores expressos em superfícies celulares, podendo modular a proliferação e diferenciação celular, e ainda ter impacto em ambientes clínicos de doenças autoimunes, transplantação e escape tumoral. Através de estudos prévios, sabe-se que as células T podem ser moduladas por outros componentes, nomeadamente pela IL-15, uma citocina conhecida por ser um potente regulador da ativação, proliferação e diferenciação destas mesmas células. Contudo, esta modulação pode estar sujeita à influência de componentes adicionais, como as citocinas IL-10 e TGF-β, extensamente presentes no ambiente imunológico e também elas com amplo efeito sobre as células T. Adicionalmente, o efeito da influência não só da IL-15, como também da IL-10 e TGF-β na expressão dos conformadores abertos de MHC-I pelas células T, não é conhecido.

Neste estudo, e por forma a estudar o efeito da IL-10 e TGF-β na ativação e proliferação das células T mediadas por IL-15, foram isolados linfócitos do sangue periférico humano (PBL) a partir de *buffy coats* de dadores saudáveis. Estes foram marcados com CFSE e cultivados por 12 dias na presença de IL-15, e na presença de combinações de IL-15+IL-10 ou IL-15+TGF-β, em que a segunda citocina está presente sob diferentes condições de adição, havendo diferenças na concentração adicionada e no dia de adição. Finda a cultura, os PBL foram marcados com os anticorpos primários HC-10 e W6/32, de forma a detetar conformadores abertos e fechados de MHC-I, respetivamente, conjugados com o anticorpo secundário GAM-PE. Seguidamente, houve também a marcação com anticorpos anti-CD3 (CD3 APC) e anti-CD8 (CD8β PE-Cy.7), por forma a detetar células T CD4+ e T CD8+.

Procedeu-se, em seguida, à análise das amostras preparadas por citometria de fluxo e submeteram-se os resultados obtidos a uma análise estatística.

Os resultados demonstram de forma clara que o efeito proliferativo previamente conhecido da IL-15 nas células T foi amplificado de forma notável com a presença tanto de IL-10 como de TGF-β, e mais significativamente na presença de uma maior concentração de citocina secundária no meio de cultura. Adicionalmente, este estudo demonstrou pela primeira vez que, para além de modular a proliferação de células T, a IL-15 é também capaz de influenciar os conformadores abertos de MHC-I nestas células, aumentando a expressão dos mesmos. Ficou também demonstrada a capacidade, até agora desconhecida, da IL-10 e TGF-β para influenciarem a expressão dos conformadores abertos de MHC-I ainda nas células T. Neste estudo, estas citocinas diminuíram a expressão destes conformadores, contrariando o efeito da presença de IL-15, além de simultaneamente aumentarem a expressão de conformadores fechados de MHC-I. Considerando as inúmeras implicações em vários contextos clínicos e em certas doenças dos conformadores abertos de MHC-I, seria de particular interesse a realização de estudos complementares para investigar os possíveis efeitos adicionais da presença de IL-15, IL-10 e TGF-β, bem como os mecanismos específicos pelos quais a expressão de conformadores abertos de MHC-I é impactada.

Abstract

The human immunological system is composed of multiple elements working together to protect the body against deaseases and external agents, maintaining a good health and balance. Here, we highlight the importance of T cells and their pivotal role for the correct functioning of the immunological system. Other elements of great importance are Major Histocompatibility Complex Class I (MHC-I) molecules, commonly found throughout all cells, including T cells. These molecules are best known when in their closed conformation, however, upon certain structural changes, they can adopt an open form, becoming known as open MHC-I conformers. The functions of these open conformers go far beyond common immune roles, participating in non-immunological functions and exerting an impact in several clinical and biomedical settings. Previous studies have shown that T cells can be modulated by IL-15, impacting their activation, proliferation and differentiation. However the effect of this modulation on the expression of open MHC-I conformers is thus far unknown. The influence of other cytokines widely found in the immunological system, namely IL-10 and TGF-β, on this IL-15-induced modulation and on the expression of the same open conformers of MHC-I, is also yet to be determined.

In this study, peripheral blood lymphocytes (PBL) were isolated from buffy coats of healthy donors, labelled with CFSE, and cultured for 12 days in the presence of IL-15, with the addition of IL-10 or TGF-β in different concentrations and at different days. Cells were then labelled with several antibodies in order to differentiate between CD4+ and CD8+ and to detect open/closed MHC-I conformers. Analysis by flow cytometry was later conducted.

Results clearly show that the presence of either IL-10 or TGF-β greatly enhances the already known proliferative modulation of IL-15 on T cells, most noticeably when in combination with higher concentrations of the secondary cytokine (IL-10/TGF-β). Moreover, this study has demonstrated a direct impact not only of IL-15, but also IL-10 and TGF-β, on the expression of open MHC-I conformers on T cells, constituting novel discoveries. The expression of these conformers was seen to increase in the presence of IL-15, while the simultaneous presence of IL-10 and TGF-β counterbalanced this effect, decreasing the levels of open MHC-I conformers.

Keywords

CD4+ and CD8+ T Cells; Activation and Proliferation of T Cells; Open and Closed MHC-I Conformers; IL-15; IL-10; TGF-β.

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List of Acronyms

β2m	Beta-2 microglobulin
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
APS	Penicillin-Streptomycin
BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein Succinimidyl Ester
CO_2	Carbon Dioxide
CSIF	Cytokine Synthesis Inhibitory Factor
DC	Dendritic Cell
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FBSi	Inactivated Fetal Bovine Serum
FSC	Forward Scatter
GAM-PE	PE-Conjugated Goat Anti-Mouse
GDF	Growth and Differentiation Factor
HCl	Hydrochloric Acid
HLA-I	Human Leukocyte Antigen 1
HSA	Human Serum Albumin
HSi	Solution of Human Serum Albumin
IFN-γ	Interferon Gamma
IL-1α	Interleukin 1-alpha
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-18	Interleukin 18
KIR	Killer-cell Immunoglobulin-like Receptor
LIR	Leukocyte Immunoglobulin-like Receptor
MDC	Most Dividing Cells
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II

NDC	Non-Dividing Cells
NH_4Cl	Ammonium Chloride
NK	Natural Killer
NKR	Natural Killer Cell Receptor
NaN_3	Sodium Azide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PBL	Peripheral Blood Lymphocytes
RCF	Relative Centrifugal Force
SB	Staining Buffer
SEM	Standard Error of the Mean
SSC	Side Scatter
TAP	Transporters Associated with Antigen Processing
T_{CM}	T Central Memory
TCR	T-cell Receptor
TDC	Total Dividing Cells
T_{EM}	T Effector Memory
T_{EMRA}	T Effector Memory CD45RA+
TGF-β	Transforming Growth Factor Beta
T_N	T Naïve
TNF-α	Tumor Necrosis Factor Alpha
T_{REG}	Regulatory T Cells
T_{RM}	T Resident Memory
T_{SCM}	T Stem-Cell Memory

Chapter I – Introduction

Chapter I – Introduction

1.1 T Cells

T cells are important lymphocytes in the immunological system and play a vital role in the adaptive immune response. They can be distinguished from other lymphocytes due to the presence of T-cell receptors (TCR) in the cell surface and are originated in the bone marrow from hematopoietic stem cells. Subsequently, they migrate to the thymus to mature, hence their name. Mature T cells are tightly regulated through cell survival, proliferation and apoptosis, in a process known as T cell homeostasis [1].

Amongst T cells we can find CD4+ and CD8+ T cells, characterized by the expression of the CD4 and CD8 receptors on the cell surface, respectively. Upon maturation and after leaving the thymus to enter circulation, they can be activated by major histocompatibility complex (MHC) molecules (CD8+ T cells by MHC Class I (MHC-I) and CD4+ T cells by MHC Class II (MHC-II)) expressed by antigen presenting cells (APC) through their TCR clonotypic receptor [2]. The antigen presentation and activation follow a model comprised of three signals. The first signal is the TCR recognition of the antigen, the second signal involves the binding of costimulatory molecules, such as CD28, and the third signal is the modulation of T cell differentiation by cytokines [3,4]. After activation, naïve CD8+ T cells enter a stage of differentiation that results in the generation of effector CD8+ T cells displaying different bioactivities and effector functions, reflected by the expression of different molecules [5–7]. Similarly, naïve CD4+ T cells differentiate into regulatory T cells and various subsets of helper T cells, such as Th1, Th2 and Th17, depending on the cytokine environment and the nature of the antigen presented. These subsets are characterized by distinct cytokine profiles, transcription factors and effector molecules that confer them specialized functions [8]. Upon the resolution of the antigenic stimulus, the effector function of both CD4+ and CD8+ T cells is eventually diminished through homeostatic mechanisms, and a small pool of circulating memory T cells remains [1,9].

Overall, the activation and function of CD4+ T cells is regulated by a complex network of interactions between receptors and ligands [10]. CD8+ T cells are also genetically programmed to express an array of receptors during differentiation, enabling them to receive signals of activation and survival from a variety of receptors and ligands, including those involved in the MHC-I pathway [11,12].

CD4+ T cells can be divided into different subsets based on their differentiation pathways and the expression of certain surface markers. These subsets are: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and regulatory T cells (T_{REG}). CD4+ T_N cells express CD45RA, CD45RO and CCR7, but lack expression of other activation markers such as CD25 or CD27. They respond to new antigenic stimulation and differentiate into other subsets upon activation. CD4+ T_{CM} cells, located in secondary lymphoid organs, express CCR7, CD27 and CD28, but are lacking in the expression of CD45RA [13]. CD4+ T_{EM} cells express CD27, CD28 and CD45RO, but not CCR7. These cells are located in non-lymphoid tissues and have a rapid effector function upon antigen stimulation [14]. Lastly, CD4+ T_{REG} cells, playing a pivotal role in immune tolerance and autoimmunity prevention, express CD25 and FOXP3 [15].

Similarly to CD4+ T cells, based on the different pathways of T cell differentiation and on the expression of mainly CCR7, CD27, CD28 and CD45RA, we are also able to distribute CD8+ T cells into 5 distinct major subsets: T_N, stem-cell memory (T_{SCM}), T_{CM}, T_{EM} and effector memory CD45RA+ (T_{EMRA}). The CD8+ T_N pool is comprised of polyclonal T cells expressing CD28, CD27, CCR7 and CD45RA, and has a similar role to the CD4+ T_N pool [5,7]. CD8+ T_{SCM} cells, much like CD8+ T_N cells, express CD28, CD27, CCR7 and CD45RA, however they also express large amounts of CD95, IL-2Rβ, CXCR3 and LFA-1, and possess numerous functional attributes distinctive of memory cells [16]. The CD8+ T_{CM} cell subset expresses CD28, CD27 and CCR7, but has no expression of CD45RA [5,7]. CD8+ T_{EM} cells, much like CD4+ T_{EM} cells, can express CD27, CD28 and CD45RO, while lacking in the expression of CCR7. At last, CD8+ T_{EMRA} cells can be characterized by the expression of CD45RA and loss of CCR7, and play a role in rapid effector functions [5].

Human CD4+ T_{EM} and CD8+ T_{EM} cells have the ability to migrate to non-lymphoid organs and tissues, including, under certain circumstances, the brain [5,17]. A fraction of these cells may stay in such locations as a pool of non-recirculating tissue-resident memory (T_{RM}) cells, expressing CD69 and CD103 that contribute to their retention and residency in the tissue [18]. In addition to conferring local immune protection against infections, recirculating and non-recirculating T_{EM} cells present in non-lymphoid organs may also, in case of injury, assist in the resolution of the inflammation, tissue regeneration and repair. This is possible because of a complex cross-talking network with the environmental cells of the tissue [5].

1.2 Anti-inflammatory Cytokines: IL-10 and TGF-β

The optimal functioning of the human immunological system relies on continuous regulation to ensure protection against exogenous agents, and tolerance towards self-antigens. To achieve this critical immune balance, various types of regulatory components are therefore necessary. Among them, we can find two immunosuppressive cytokines of invaluable importance: Interleukin 10 (IL-10) and Transforming growth factor beta (TGF-β).

1.2.1 IL-10: Biological Effects on Human T Cells

IL-10 is an anti-inflammatory cytokine that is encoded, in humans, by the *IL10* gene located in a discrete area of chromosome 1 [20]. This cytokine, originally referred to as cytokine synthesis inhibitory factor (CSIF), was first described in 1989 by Fiorentino et al. in a study involving mouse T helper cells [21]. Since this description, the list of cells reported to produce IL-10 has grown swiftly, and has shared the growth with the number of cells that are reported to be capable of responding to it. As a matter of fact, it can be produced by virtually any immune cell, and is a part of the homeostatic response to infection and inflammation [22,23]. As such, it is an important regulatory cytokine involved in diverse areas of the human immunological system, with a wide spectrum of anti-inflammatory activity [20,24].

Unresolved inflammatory responses often turn detrimental to the host, and can even be the basis for various pathologies, ranging from neurodegenerative diseases to cancer. IL-10 is critically important in the protection against damage driven by these acute phases of immune responses, acting by limiting inflammation and promoting a balanced immune response [22,25]. Based on the previously mentioned effects by IL-10, this cytokine can be described as a potent limiter of over-magnified immune responses and can have a significant impact in the prevention of autoimmune diseases [24].

IL-10 has also been reported as a powerful regulator of dendritic cells (DC) effector function and maturation, being able to supress the ability of myeloid-derived DCs to stimulate T cells through their antigen-presenting functions [26,27]. However, it is important to mention that not all IL-10 bioactivities result in the supression of immune responses. In fact, this cytokine is capable of co-stimulating B cells and prolonging their survival, co-stimulating natural killer (NK) cell proliferation and co-stimulating cytokine production [27,28].

IL-10 appears to have contrasting effects on human CD4+ and CD8+ T cells. It can induce anergy and suppress the proliferative response in human CD8+ T cells that are activated in

the presence of an APC. This effect is mediated by the inhibition of the costimulatory functions of the APC, with the down-regulation of costimulatory molecules [27]. Similarly, IL-10 can also induce anergy and inhibit the proliferative response in CD4+ T cells activated in the presence of an APC, again affecting the costimulatory functions of the APC and down-regulating the expression of costimulatory molecules [29]. Ultimately, a TCR stimulation without co-stimulatory signals induces an immunological unresponsive, anergic state in T cells. IL-10 has been demonstrated to induce this state of anergy in T cells by inhibiting CD28 signaling during exposure to antigens, in immunotherapies and in certain disease contexts [27,30–34]. However, in CD4+ and CD8+ T cells that are preactivated and in the absence of an APC, their proliferation may not be directly inhibited by IL-10. In fact, and in combination with small doses of Interleukin 2 (IL-2), IL-10 can display a growth-promoting activity on these cells, suggesting it may act as a cofactor in enhancing the IL-2-driven proliferation of both CD4+ and CD8+ T cells, in yet to be understood mechanisms [27,35]. Certain reports suggest IL-10 may also potentially function as a growth factor to stimulate the proliferation of certain CD8+ T cell subsets, and possibly have an implication in the development of memory CD8+ T cells [22,36,37]. Thus, it can be concluded that IL-10 may stimulate or inhibit CD4+ and CD8+ T cell proliferation, depending on the cell's status at the time of exposure, and whether an APC is present or not.

1.2.2 TGF-β: Unravelling the Impact on Human T Cells

TGF-β is an anti-inflammatory pleiotropic cytokine. The TGF-β subfamily encompasses three different TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), two activins (A and B), a secretory protein named Nodal, and growth and differentiation factors (GDF) 1, 3, 8, 9 and 11, all of which are secreted and function as homodimers or heterodimers. From an immune standpoint, out of all the family members and from the three isoforms, TGF-β1, a 25 kDa disulfide-linked homodimer, is the most relevant and has been the most extensively studied [38–41]. TGF-β is active in the mediation of a wide variety of effects on cell differentiation, activation and proliferation. As such, it acts on the regulation of the effector functions and on the generation of most, if not all, immune cell types [23,39,41,42]. Its regulatory range can reach both adaptive and innate immune systems, regulating T cell development, homeostasis, tolerance and immunity. It can control adaptive immunity through the regulation of T cells, including the modulation of T_{REG} cells, the inhibition of the function of effector T cells, and the regulation of DCs based on the inhibition of their antigen-presenting capabilities through the suppression of MHC genes [38,43]. Similarly to IL-10, this cytokine acts as an enforcer of immune homeostasis and tolerance by inhibiting the function and proliferation of several immunological system components, including the inhibition of pro-

inflammatory cytokines, as is the case with Tumor Necrosis Factor alpha (TNF-α), Interleukin 1-alpha (IL-1α) and Interleukin 18 (IL-18), among others [23], resulting in the control of inflammatory responses. Failure in this action can consequently lead to immune defects and dysregulation, severe inflammatory disorders and disease [38,40,44].

TGF-β exerts a wide influence on CD4+ T cells. This cytokine plays a crucial role in driving the differentiation of CD4+ T cells towards specific subsets. It can, in combination with other cytokines, such as Interleukin 6 (IL-6), promote the differentiation of CD4+ T_N cells into Th17 cells, which are involved in the defence against extracellular pathogens and autoimmune diseases [43]. Moreover, TGF-β, in combination with Interleukin 4 (IL-4), induces the differentiation of Th9 cells, contributing to allergic responses [45,46]. This cytokine also has a profound impact on T_{REG} cell differentiation and function. It acts as a key factor in the induction of T_{REG} cells by promoting the expression of Foxp3, a transcription factor essential for T_{REG} cell identity [43], and, along with IL-2, can also facilitate the development of T_{REG} cells from CD4+ T_N cells, thereby expanding the pool of regulatory cells capable of suppressing immune responses [43,47].

In peripheral T cells, an important function of TGF-β signaling is to hinder T cell expansion and to restrain its activity in response to exogenous stimuli [38]. TGF-β-mediated signaling may also actively suppress the effector functions of CD4+ T cells. It can, for instance, inhibit the production of pro-inflammatory cytokines, such as IL-2, thus regulating inflammatory responses and preventing possible tissue damage [48].

TGF-β also has broad impact on CD8+ T cells, having an influence on CD8+ T cell differentiation during thymic development, however it's biggest impact on CD8+ T cells lies in the modulation of their effector functions [43]. In CD8+ T cells, TGF-β-mediated signaling can directly inhibit their cytotoxicity by regulating the production of cytotoxic molecules such as granzyme B and perforin. This signaling has been shown to inhibit the expression of these cytotoxic molecules, resulting in impaired cytotoxicity. This regulatory role of TGF-β prevents excessive tissue damage, ensuring a balanced immune response. Furthermore, TGF-β-stimulated SMADs, proteins that are the main signal transducer for TGF-β receptors, in combination with the transcription factor ATF1 are implicated in the suppression of promoters for various genes involved in the normal function of CD8+ T cells [38,49]. However, when these cells lack or have a deficiency in the expression of TGF-βRII, a protein playing a crucial role in the TGF-β signaling pathway, they exhibit enhanced effector phenotypes and functions, as seen with the increase of Interferon gamma (IFN-γ) and granzyme B production [50]. TGF-β can also influence the expression of key surface receptors and co-stimulatory molecules on CD8+ T cells, as is evidenced with the

downregulation of the expression of the CD8 co-receptor, having an impact in their interaction with APCs and subsequent activation, and consequently influencing the strength of CD8+ T cells in immune responses [51].

TGF-β can additionally act as a negative regulator for the formation of new CD8+ T_{RM} cells, but it may still play an important role on the maintenance of already established ones [3]. CD8+ T_{RM} cells are maintained associated to tissues due to their expression of CD103, which can bind with E-cadherin present in cell junctions on the epithelium. Studies have elucidated that TGF-β signaling can actually promote CD103 expression, thus facilitating the residence of CD8+ cells in the tissue [3,52,53].

1.3 IL-15: A Potent T Cell Modulator

Interleukin 15 (IL-15) is a 14-15 kDa pro-inflammatory cytokine produced by activated monocytes and macrophages, DCs and epithelial cells, mostly under stress conditions, as in infections [54,55]. This cytokine has been evidenced as a potent regulator for the homeostasis, activation, survival, proliferation and differentiation of CD4+ T cells, as well as naïve and memory CD8+ T cells [3,19,56–63].

IL-15 exerts a significant influence on CD4+ T cells. Although this cytokine is not expressed by CD4+ T cells themselves, it can be trans-presented by other cell types, such as DCs and macrophages [63]. Studies have shown that this cytokine can induce the proliferation and survival of CD4+ T cells through the upregulation of anti-apoptotic proteins encoded by the Bcl-2 gene, conferring them a higher resistance to cell death signals [64,65]. Additionally, IL-15 can shape CD4+ T cells by enhancing the production of pro-inflammatory cytokines, as well as having an influence on their differentiation into certain subsets, such as Th17, albeit with some degree of uncertainty [63,66,67].

On top of being known for stimulating the proliferation and function of certain CD4+ T cell subsets, IL-15 may also have an impact on the activity of T_{REG} cells by modulating the expression of Foxp3 on T_{REG} cells, and therefore modulating their suppressive function [68–71]. Furthermore, IL-15 has been shown to promote the effects of TGF-β on the generation of T_{REG} cells, enhancing the acquisition of regulatory functions [72].

IL-15 also has profound influence on CD8+ T cells. Through studies focused on the differentiation of these cells upon stimulation by IL-15, our group has previously shown that this cytokine induces phenotypic and functional changes in different CD8+ T cell subsets [19,56,73]. When cultured in the presence of this cytokine, CD8+ T cells are strongly

induced to produce a wide array of pro and anti-inflammatory cytokines upon stimulation, as can be seen with IFN-γ, TNF-α or even IL-10, among others [19,56]. While IFN-γ is a pro-inflammatory cytokine associated with immune activation, IL-10 possesses anti-inflammatory and regulatory properties, as discussed earlier. This contrast in actions may imply IL-15-induced CD8+ T cells as immunoregulatory agents. The production of cytotoxic effector molecules, namely perforin and granzyme B, is also induced [19,56,74]. Post-stimulation, IL-15-stimulated CD8+ T cells keep on manifesting strong cytotoxic activity, contrary to unstimulated cells [56].

CD8+ T_N cells undergoing homeostatic proliferation are phenotypically and functionally transformed into memory-type cells [5,19]. Nevertheless, such transformations may be reverted with the ceasing of this proliferation [75–77]. As studies have showcased, IL-15 can induce the expression of natural killer cell receptors (NKR) on CD8+ T cells, including CD56, differentiating them into NK-like T cells displaying a regulatory T_{EM}/T_{EMRA} effector memory phenotype, with increased anti-apoptotic levels (evidenced by the higher expression of Bcl-2 after IL-15 stimulation), high expression of cytotoxic effector molecules, cytokine secretion, and the ability to display both MHC-unrestricted and TCR-mediated cytotoxicity characteristic of NK-like T cells [19]. In this scenario, IL-15 could have the ability to regulate NKR expression, shaping the functional phenotype of CD8+ T cells and inducing the formation of CD8+ T_{EMRA} cells [19,73].

1.4 MHC Class I: Antigen Presentation and Cytokine Influence

MHC class I molecules, also known as Human leukocyte antigen 1 (HLA-I) in humans, play a vital role in innate and adaptive immune responses, and are expressed on the cell surface of all nucleated cells, including both resting and activated CD4+ and CD8+ T cells [78–80]. They are composites of a heavy α chain (about 45 kDa), a light beta-2 microglobulin (β2m) chain (12 kDa), and a peptide of 8-12 amino acids [78,80]. The extracellular region of the heavy α chain folds into three domains (α1, α2 and α3), with the α1 and α2 domains being highly polymorphic and forming the groove that will serve as the peptide-binding site, and the α3 remaining conserved. Adjacent to this conserved domain there is a transmembrane domain and an intracellular cytoplasmic tail containing tyrosine and serine motifs [81]. The α1-α2 groove is located on the upper surface of the MHC-I molecule and binds peptides with a length of 8-12 amino acids [79]. These peptides have their origin in the cytosol after mostly endogenous proteins are processed by the proteasome and are later translocated into the endoplasmic reticulum (ER) lumen by transporters associated with antigen processing

(TAP), where MHC-I molecules are folded and assembled with the help of molecular chaperones, including calnexin, calreticulum and ERP57 [82]. Once the peptide is loaded, the MHC-I molecule carrying the peptide is transported to the plasma membrane [78,79]. When in the plasma membrane, MHC-I molecules indulge in their two immunological functions: to present peptides to CD8+ T cells, and to trans-interact with receptors specific for NK cells [83].

IL-15 has been shown to be able to influence the expression of MHC-I molecules in select cell types, such as in DCs, however, there appears to be no direct evidence that this cytokine influences the expression of MHC-I molecules on both CD4+ and CD8+ T cells [84,85]. In a similar way, and despite being capable of regulating the MHC-I and MHC-II expression in certain cells, IL-10 also lacks evidence to associate it to a direct effect on the expression of MHC-I molecules on T cells [27,86,87]. Apart from recent studies showing that TGF-β also appears to be able to modulate the expression of MHC-I on certain cell types, yet again, there is no concrete evidence to support the regulation of MHC-I molecules induced by this cytokine on CD4+ or CD8+ T cells [88,89].

1.5 Open MHC Class I Conformers

The formerly described MHC-I trimeric composites have been designated as closed MHC-I conformers. However, this trimeric composite can dissociate and generate α heavy chains free of the light β2m chain and lacking the peptide. These new composites have been designated open MHC-I conformers but can also be referred to as misfolded/peptide-empty MHC-I molecules or β2m-free MHC-I heavy chains [81,83]. The emergence of open MHC-I conformers at the plasma membrane can be induced by cell activation and proliferation, cell growth and differentiation, and inflammation [81,90]. According to several studies, open human HLA-I conformers are commonly found at the cell surface of activated T cells, but not resting T cells [83,91,92].

There is growing evidence that MHC-I molecules present in the cell surface can exist in an equilibrium between open and closed conformers, a process that is regulated by the endocytosis and phosphorylation of a conserved tyrosine residue in the cytoplasmic tail of MHC-I heavy chains, allowing α heavy chains to cis-associate and form structures called class I homodimers (**Figure 1**) [81,83]. These structures have the ability to trans-interact with killer-cell immunoglobulin-like receptors (KIR) and leukocyte immunoglobulin-like receptors (LIR), present in NK cells and NK-like T cells, modulating immune responses, and having an implication in autoimmune disorders [83,93]. Disulfide bonds between extracellular and cytoplasmatic unpaired cysteines are indispensable for the formation of

these α heavy chain homodimers, and for the subsequent trans-interaction with NKRs [94,95].

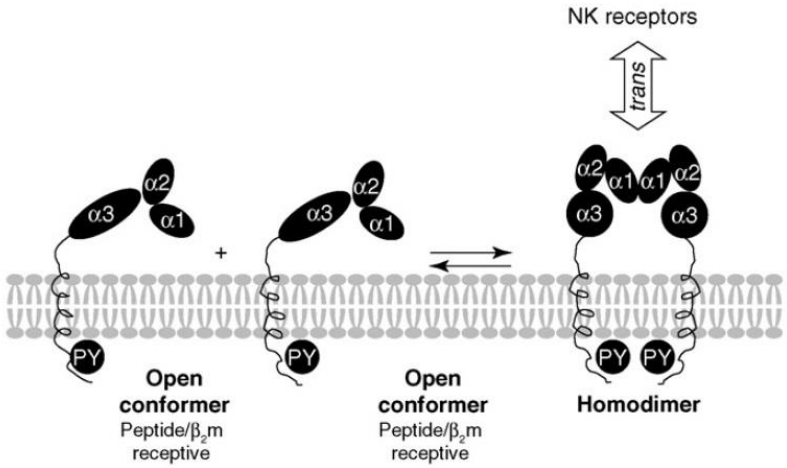

Figure 1. Cis-interaction between MHC-I open conformers resulting in the formation of homodimers capable of trans-interacting with NKR. Adapted from [81].

Even though peptide-bound MHC-I molecules have an important role in the immune response through trans-interactions with TCR and NKR, the role of open MHC-I conformers is more ambiguous, and appears to be related to their capability of cis-association both with themselves and with other receptors [81,83]. In early studies, it was shown that MHC-I molecules were able to interact in cis with insulin, epidermal growth factor (EGF) and IL-2 receptors [96–99]. Today, the catalogue of molecules expressed in the cell surface that are reported to cis-associate with MHC-I molecules is much larger, and includes receptors whose functions are both immune and non-immune [81]. Open conformers can take part in non-immunological functions at the plasma membrane of metabolically active cells by cis-associating with receptors for hormones, cytokines, growth factors, CD8αβ, CD82 and a variety of members of the NKR family, forming heterodimers of α heavy chains, capable of fine-tuning receptor-mediated signaling (**Figure 2**) [83,100,101]. This points to MHC-I molecules as having a multifunctional role in recognition processes and being capable of modulating cell growth, differentiation and communication [81,83,102]. Furthermore, open MHC-I conformers have also been shown to impact certain clinical and biomedical settings such as autoimmune responses, transplantation, neuronal development and even tumor escape [83].

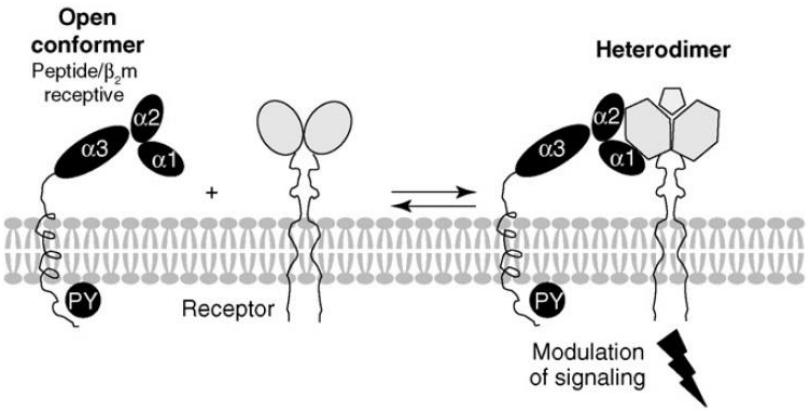

Figure 2. Cis-interaction between an open MHC-I conformer and a receptor resulting in the formation of a heterodimer, with the subsequent signal modulation. Adapted from [81].

There is also the possibility that MHC-I conformers, open or closed, end up being removed from the plasma membrane, becoming soluble conformers, capable of trans-interacting with other receptors and inducing the formation of new specific antibodies [83].

Several *in vitro* studies have demonstrated the correlation between T cells and open MHC-I conformers. One study found that LA45, an antibody recognizing an epitope in the α1 domain of the MHC-I heavy chain when the β2m and the peptide are not present, is expressed on activated human T cells and even induced by them [91]. Another study demonstrated that open MHC-I conformers expressed in activated T cells are involved in mechanisms that regulate the function of these activated T cells [92]. Additionally, it has been shown that the expression of open MHC-I conformers is sharply increased upon T cell activation and division, capable of even exceeding the expression of closed MHC-I conformers, and is therefore proportional to the level of proliferation of T cells [90,103]. Other *ex vivo* studies have shown further capabilities of open MHC-I conformers. The binding of such conformers to a KIR receptor was shown to be able to regulate NK cell function and promote lymphocyte survival. This interaction can also play an important role in the regulation of immune homeostasis and in the recognition of certain tumours [104]. However, some studies have shown a correlation between an increased expression of open MHC-I conformers and some diseases, including Polycythemia Vera and Spondyloarthropathy, raising questions about a possible involvement in these pathologies [90,105].

Chapter II – Aims of the Study

Chapter II - Aims of the Study

IL-15, IL-10 and TGF-β are all part of an intricate interplay that continuously takes place in the human immunological system. They can be described as small gears in a much larger mechanism, functioning as a crucial layer of protection in our bodies. These small gears have the capability of impacting and interacting with one another. Therefore, studying these interactions and their potential effects is of great interest. It is known that one of these gears, IL-15, is capable of inducing the activation and proliferation of T cells, a component that is critical for the proper functioning of the immunological system. However, the influence that IL-10 and TGF-β may have on this IL-15-induced mediation remains unknown. Additionally, there is no concrete evidence suggesting that IL-15, IL-10 or TGF-β have an impact on the expression of open or closed MHC-I conformers on T cells.
In light of these uncertainties, several questions arose:

1) Can IL-10 and TGF-β influence the IL-15-driven activation and proliferation of CD4+ and CD8+ T cells?
2) As a side effect of the IL-15-induced activation of T cells, do adjustments occur in the expression of open MHC-I conformers?
3) What effect do IL-10 and TGF-β have on the expression of open/closed MHC-I conformers in IL-15-activated T cells?

The present study was conducted to answer these questions and to seek for a better understanding of the implications of these interactions.

Chapter III – Materials & Methods

Chapter III – Materials & Methods

3.1 Cells and Reagents

Human peripheral blood cells were obtained from buffy coats of healthy donors provided by the Blood and Transplantation Centre of Coimbra (IPST, IP), as indicated below. Ammonium Chloride (NH_4Cl), Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), Tris base, the RPMI-1640 Medium, Penicillin-Streptomycin (APS) and the Antibiotic-Antimycotic solution containing Amphotericin were all obtained from Sigma-Aldrich (Madrid, Spain). Sodium azide (NaN_3) was obtained from Amresco (Solon, United States of America). Lymphoprep was obtained from STEMCELL Technologies (Grenoble, France). The antibodies W6/32 and HC-10, along with the cell proliferation kit *CellTrace™ Carboxyfluorescein diacetate succinimidyl ester* (CFSE), were obtained from eBioscience (San Diego, United States of America). The PE-conjugated goat anti-mouse antibodies (GAM-PE), CD8β PE-Cy.7, CD3 APC and IgG2a were obtained from Immunotools (Germany). The cytokines rhIL-15, rhIL-10 and rhTGF-β1 were all obtained from R&D Systems/Bio-techne (United States of America).

3.2 Cytokine Reconstitution

Both cytokines were reconstituted according to the R&D Systems/Bio-techne guidelines. IL-10 was reconstituted in 100 μg/mL of 1X Phosphate-buffered saline (1X PBS) with 0,1% HSA, and TGF-β was reconstituted in 100 μg/mL of 4mM Hydrochloric acid (HCl) with 0,1% HSA. Afterwards, and separately for each cytokine, aliquots containing 1 μL of cytokine and 1 mL of RPMI Medium filtered and supplemented with 1% APS and a solution of 5% Human Serum Albumin (HSi) were prepared. The final cytokine concentration in each aliquot was 100 ng/mL.

3.3 PBL Isolation and Count

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats after centrifugation with Lymphoprep at 800g (Relative Centrifugal Force (RCF)) for 30 minutes. Cells were later washed twice with 1X PBS and with a red blood cell lysis solution (10mM Tris, 150mM NH_4Cl, pH=7,4) for 10 minutes at 37ºC. Cells were then washed again with 1X PBS and filtered. The cellular suspension of PBMC was subsequently placed in two petri

dishes for 1 hour at 37ºC and 5% carbon dioxide ($CO_{2)}$. The plates contained RPMI medium supplemented with 5% inactivated fetal bovine serum (FBSi), and approximately 50 million cells each, amounting to a total of 100 million cells. From this incubation, isolated peripheral blood lymphocytes (PBL) were obtained.

In order to calculate the number of viable PBL, cells were counted. A solution was prepared containing 10 µL of cell suspension and 90 µL of Trypan blue. A fraction of this solution was transferred to a Neubauer chamber, the number of viable cells in each quadrant were counted and a mean was calculated from the obtained values. With this, it was possible to calculate both the number of viable cells per mL of cell suspension, using a formula (1), and the total number of cells per cell suspension, using another formula (2).

$$(1)\ \frac{Cells}{mL} = mean\ of\ cells\ in\ quadrants\ \times\ dilution\ factor\ \times 10^4$$

$$(2)\ Total\ Number\ of\ Cells = \frac{Cells}{mL}\ \times\ Volume\ of\ cell\ suspension$$

3.4 CFSE Labelling

After counting, 20 million PBL were labelled with CFSE for 5 minutes at a final concentration of 5mM, protected from light and with frequent mixing. Subsequently, cells were washed twice with RPMI medium supplemented with 10% FBSi and resuspended in 2mL of the culture medium, consisting of RPMI medium supplemented with 5% HSi and 1% APS.

3.5 Cell Culture Conditions

PBL labelled with CFSE were cultured in a 24-well plate. About 1 million cells were added to each of 10 wells, and the volume was adjusted up to 1mL per well with RPMI medium supplemented with 5% HSi and 1% APS. 10 µL of IL-15 were added to all the wells, and subsequently, on the same day (day 0), IL-10 and TGF-β were added to 2 wells each. 1 µL of IL-10 was added to one well, 10 µL of IL-10 were added to another well, 1 µL of TGF-β was added to another well, and at last 10 µL of TGF-β were added to another well. The cell culture was then incubated at 37ºC, 5% CO_2. On Day 6 of cell culture, the incubation was

briefly stopped in order to replenish all 10 wells with 200 μL of RPMI medium supplemented with 5% HSi and 1% APS, along with an additional 10 μL of IL-15, in order to ensure a continuous IL-15-mediated proliferation. Additionally, on Day 6, IL-10 and TGF-β were once again added to 2 wells each, in the same concentrations of 1 μL and 10 μL. The incubation at 37ºC with 5% CO_2 was then continued up to a total of 12 days. In the plate, 4 wells contained cells that underwent proliferation in the presence of IL-15 + IL-10/TGF-β added on Day 0, 4 wells contained cells that underwent proliferation in the presence of IL-15 + IL-10/TGF-β added on Day 6, and in the remaining 2 wells, cells underwent proliferation merely in the presence of IL-15, serving as negative controls.

3.6 Flow Cytometry

After cell culture, cells were centrifuged and prepared for analysis by flow cytometry. Five hundred thousand cell aliquots were transferred each into different wells, in a 96-round-bottom well plate and centrifuged at 150×g for 2 minutes at 4ºC. After resuspension of the pelleted cells, they were first incubated with unlabelled mouse W6/32 and HC-10 antibodies in Staining Buffer (SB) (1×PBS, 0,2% BSA, 0,1% NaN_3). After three washes, cells were incubated with GAM-PE. After three more washes, cells were then incubated with fluorochrome-conjugated mouse anti-human CD3 (CD3-APC) and CD8 (CD8β PE-Cy.7) antibodies. All incubations were performed at 4ºC during 30 minutes in SB. Cells incubated only with GAM-PE were used as negative control to define background staining.

W6/32 recognizes a monomorphic epitope on all classical HLA-I heavy chains dependent on the presence of both the α heavy chain and the light β2m chain, allowing for the detection of closed MHC-I conformers [106]. HC-10, on the other hand, can recognize particular amino acid sequences present on the α1 domain of the heavy chain, particularly between residues 57-84, that become exposed whenever a peptide and β2m dissociation occurs (**Figure 3**), allowing for the detection of HLA-I chains not associated with β2m and, as such, the presence of open MHC-I conformers [81,90].

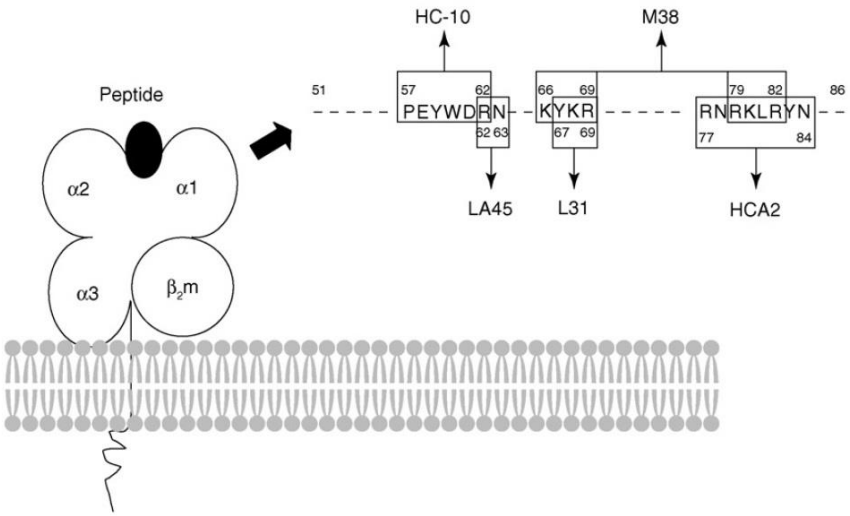

Figure 3. Representation of a closed MHC-I conformer composed by an α heavy chain, a lighter β2m chain and a binding peptide. Upon peptide dissociation, certain amino acid sequences present in the α1 domain will become exposed, and open for recognition by several antibodies, including HC-10. Adapted from [81].

After the labelling process, cells were harvested and 20 000 events were acquired in a BD Biosciences Accuri C6 flow cytometer. Results were analysed with the use of the BD Accuri C6 software. In the analysis process, gating strategies were employed in order to correctly select different cell subpopulations based on their light scattering and fluorescence emission characteristics. Given that the four fluorescence detectors of the Accuri were occupied (FL1: CFSE; FL2: W6/32-GAM-PE and HC-10-GAM-PE; FL3: CD8β PE-Cy.7; and FL4: CD3-APC), it was not possible to directly identify CD3+CD4+ T cells. However, by analysing CD3 versus CD8 we could identify a clear population of CD3+CD8− T cells that by inference can be designated as CD3+CD4+ T cells. Therefore, for the sake of this study, when CD4+ T cells are mentioned, it is in reference to CD3+CD8− T cells.

3.7 Statistical Analysis

Data obtained from flow cytometry was processed, and a normalization was performed comparing all values from dividing T cells with non-dividing T cells, with the use of a formula (3), and most dividing T cells with non-dividing T cells, using a similar formula (4). The values corresponding to all the different conditions of addition of IL-15 + IL-10/TGF-β, for most dividing, dividing and non-dividing cells, were also normalized with the values for the addition of only IL-15 (5).

$$(3)\ Normalised\ MFI\ value\ of\ dividing\ T\ cells = \frac{MFI\ value\ of\ dividing\ T\ cells}{MFI\ value\ of\ non-dividing\ T\ cells} \times 100$$

$$(4)\ Normalised\ MFI\ value\ of\ most\ dividing\ T\ cells = \frac{MFI\ value\ of\ most\ dividing\ T\ cells}{MFI\ value\ of\ non-dividing\ T\ cells} \times 100$$

$$(5)\ Normalised\ MFI\ value = \frac{MFI\ value\ corresponsing\ to\ cells\ in\ the\ presence\ of\ IL-15 + IL-10/TGF-\beta}{MFI\ value\ corresponsing\ to\ cells\ in\ the\ presence\ of\ IL-15} \times 100$$

An example on how these populations were determined is illustrated in the dot-plot below, in **Figure 4**, where:

- Q1-Upper Right Quadrant: Non-dividing T cells
- Q2-Upper left Quadrant: Dividing T cells
- Gate A: Most dividing T cells

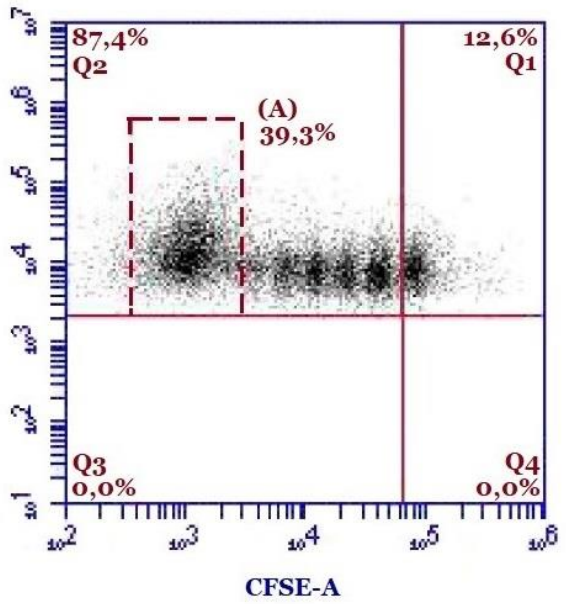

Figure 4. Dot-plot demonstrating the different cell divisions that occurred during proliferation. After culture, cells were analysed by flow cytometry, and dot-plots were obtained. 3 regions of interest can be observed on the basis of CFSE characteristics, one corresponding to the cells that did not divide, represented in the first quadrant, located on the upper right, one for the cells that divided, represented by the second quadrant located on the upper left, and one for the cells that divided the most, represented by the area in Gate A.

The software Graph Pad Prism 8 was employed in order to conduct the statistical analysis. Differences between the means of the groups were ascertained with analysis of variance (ANOVA) tests with correlation for multiple comparisons, and statistical significance was established at a threshold of $P < 0,05$.

Chapter IV – Results

Chapter IV – Results

4.1 Effect of IL-10 and TGF-β on the Percentage of Blast Cells

In order to assess the impact of IL-10 and TGF-β on the activation of T cells mediated by IL-15, we first started by analysing the percentage of blast cells at the end of the cell culture. Upon stimulation with IL-15, peripheral blood lymphocytes, namely T cells and NK cells, start to increase 2-3 times in size becoming blastoid cells, which are larger and more intracellularly complex, followed by cell division and a return to their original smaller size. These cell changes can be monitored by flow cytometry when the forward scatter (FSC detector, measuring size) and the side scatter (SSC detector, measuring complexity) are determined, as is exemplified in **Figure 5**, shown below.

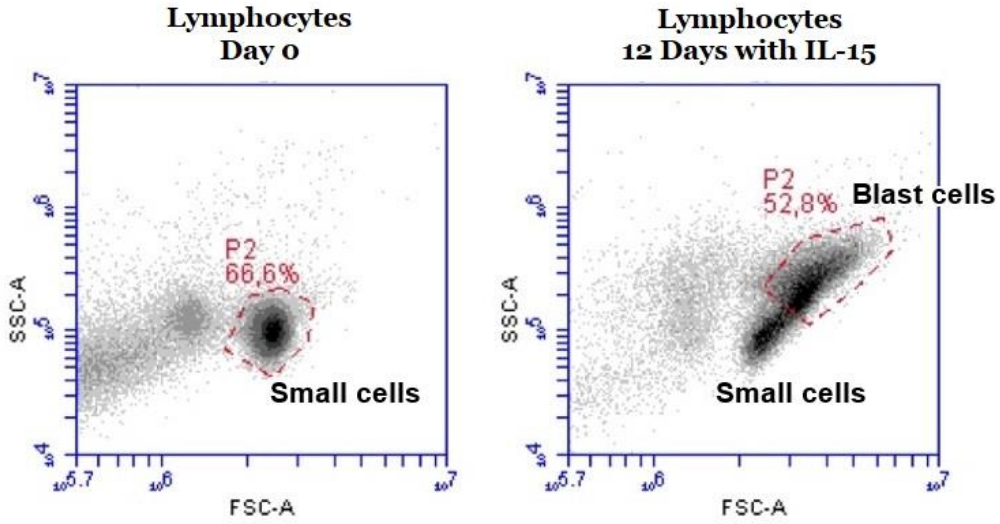

Figure 5. Dot-plot illustrating the blast transformation of PBL in response to IL-15. Left dot-plot shows FSC versus SSC parameters of lymphocytes at day 0. Small "resting" cells can be observed. Right dot-plot shows FSC versus SSC parameters of the same cells after culture with IL-15. Small and larger (blasts) cells can be observed.

A mean for the values of all the different experiments involving IL-10 and TGF-β was calculated, and the data was put into graphs to simplify the analysis. The results shown in **Figure 6** correspond to the total % of blasts in the presence of IL-15 alone, and in the presence of IL-15 + IL-10 added at different times and in different concentrations. In this graph, a small and non-statistically significant reduction in the total % of blast cells can be observed when IL-10 is present together with IL-15, in comparison to when only IL-15 is present.

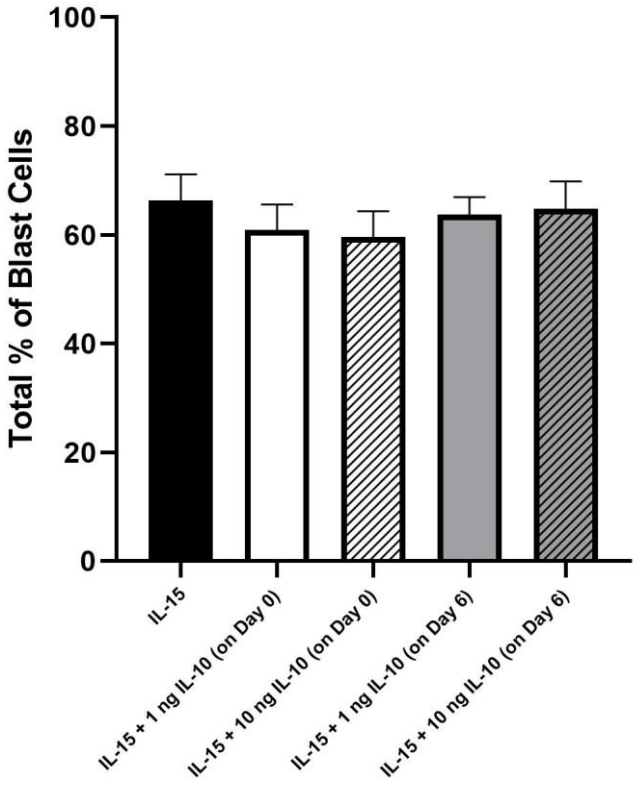

Figure 6. Total % of Blast Cells in the presence of IL-15 alone, and IL-15 + IL-10. After the culture in the presence of both IL-15 and IL-10, PBL were analysed by flow cytometry, and dot-plots were obtained. The total % of blast cells was selected in the obtained dot-plots, according to FSC/SCC characteristics and using BD Accuri C6. The values were obtained under 5 different conditions: Cells cultured in the presence of IL-15, cells cultured in the presence of IL-15 and 1ng of IL-10 added on day 0 of culture, cells cultured in the presence of IL-15 and 10ng of IL-10 added on day 0 of culture, cells cultured in the presence of IL-15 and 1ng of IL-10 added on day 6 of culture and cells cultured in the presence of IL-15 and 10ng of IL-10 added on day 6 of culture. A mean was calculated from the values of all the different experiments (n=4).

Figure 7 corresponds to the total % of blasts in the presence of IL-15 alone, and in the presence of IL-15 + TGF-β, added again at different times and in different concentrations. This graph demonstrates a different outcome, in which the total % of blast cells is very slightly increased, without statistical significance, when 1ng of TGF-β, along with IL-15, is added to the cell culture. However, when IL-15 and a higher concentration of TGF-β are added to the culture, a small decrease in the total % of blast cells is noted.

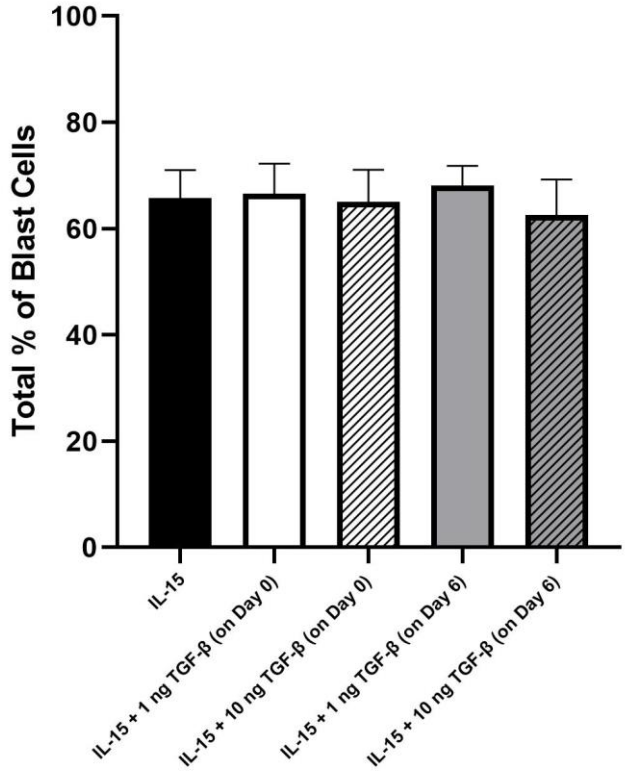

Figure 7. Total % of Blast Cells in the presence of IL-15 alone, and IL-15 + TGF-β. After the culture in the presence of both IL-15 and TGF-β, PBL were analysed by flow cytometry, and dot-plots were obtained. The total % of blast cells was selected in the obtained dot-plots, according to FSC/SCC characteristics using BD Accuri C6. The values were obtained under 5 different conditions: Cells cultured in the presence of IL-15, cells cultured in the presence of IL-15 and 1ng of TGF-β added on day 0 of culture, cells cultured in the presence of IL-15 and 10ng of TGF-β added on day 0 of culture, cells cultured in the presence of IL-15 and 1ng of TGF-β added on day 6 of culture and cells cultured in the presence of IL-15 and 10ng of TGF-β added on day 6 of culture. A mean was calculated from the values of all the different experiments (n=4).

4.2 Impact of IL-10 and TGF-β on the Percentage of CD4+ and CD8+ T Cell Blasts After Culture With IL-15

One of the objectives of this study was to determine whether IL-10 and TGF-β were capable of influencing the IL-15-driven activation and proliferation of T cells. To that end, we determined the percentages of CD4+ and CD8+ T cell blasts at the end of the cell culture. As referred in the Materials & Methods chapter, we could not include an antibody against the CD4 receptor due to the limitations of the fluorescence detectors of the Accuri cytometer (only four detectors). However, we assumed that the CD3+CD8− T cells corresponded to the CD4+ T cells. This is demonstrated in **Figure 8**, where regions corresponding to CD4+ T cells (i.e., CD3+CD8− T cells) and CD3+CD8+ T cells can be observed. Since this

assumption was applied to all the culture conditions, any possible error in the determination of the percentage of CD4+ T cells was equally distributed.

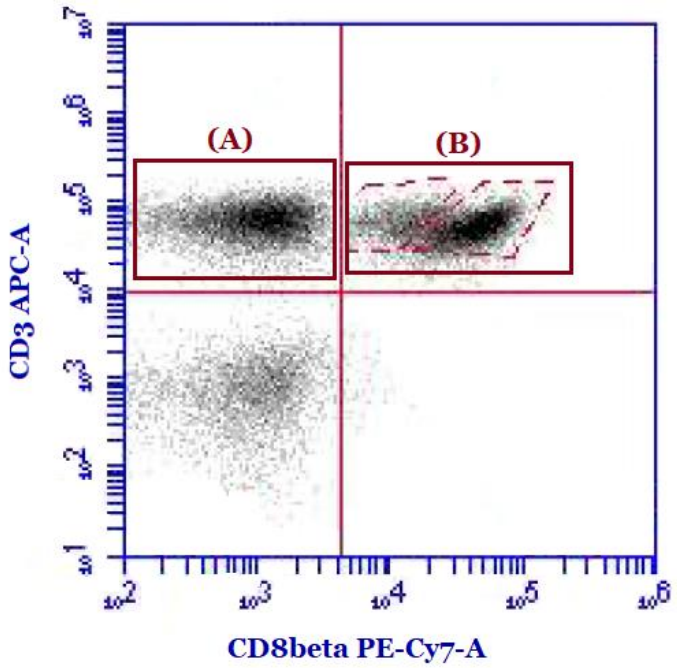

Figure 8. Determination of the percentage of CD4+ and CD8+ T cell blasts. After the 12-day culture in the presence of cytokines, cells were acquired and analysed as indicated in the Materials & Methods chapter. Dot-plot shows CD8 versus CD3 expression on gated blast cells, allowing for the identification of CD3+CD8+ T cell blasts (Upper Right Quadrant and Gate B) and CD3+CD8− T cell blasts (i.e., CD4+ T cells) (Upper Left Quadrant and Gate A).

Upon analysing the results of the different experiments performed, the percentage of CD4+ T cell blasts was noted to increase when IL-15-activated PBL were cultured in the presence of IL-10, even more noticeably when 10ng of IL-10 were used. Similar results were observed when IL-15-activated PBL were cultured in the presence of TGF-β. However, no statistical significance was found due to an increased standard error of the mean (SEM) (P≥0,05).

The analysis of the percentage of CD8+ T cell blasts revealed interesting results. As illustrated in **Figure 9**, the percentage of CD8+ T cell blasts obtained after the 12-day culture with IL-15 was statistically significantly increased when 1ng and 10ng of IL-10 were added to the cultures on day 0 (p=0.0073 and p=0.0216, respectively). Interestingly, this statistical significance was observed when cells were labelled with W6/32 antibodies (**Figure 9**), but not when cells were labelled with HC-10 antibodies (**Figure 10**).

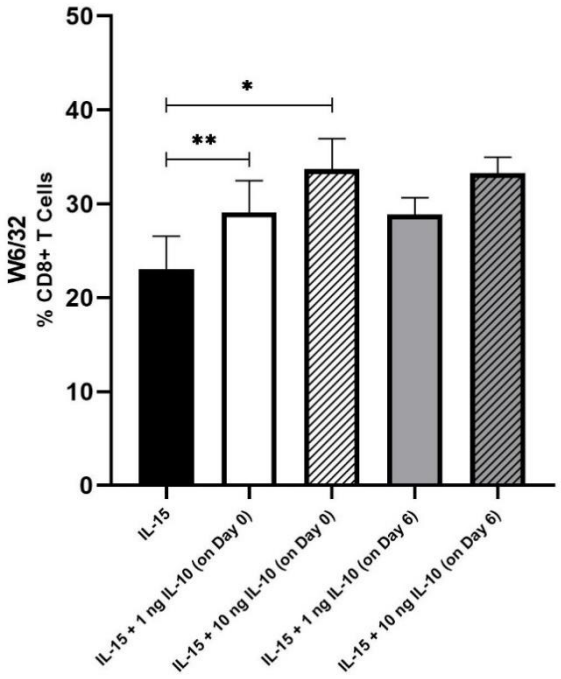

Figure 9. Effect of IL-10 on the percentage of IL-15-activated CD8+ T cell blasts. After 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the percentage of CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions. *P=0,0216; **P=0,0073.

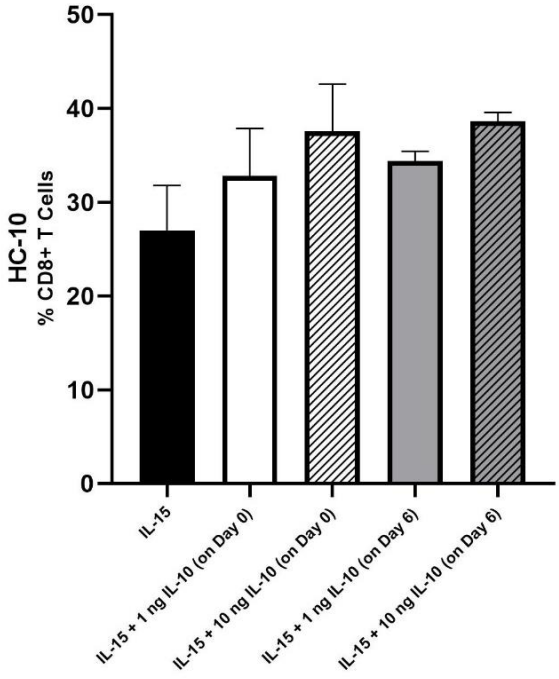

Figure 10. Effect of IL-10 on the percentage of IL-15-activated CD8+ T cell blasts. After 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the percentage of CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions.

Furthermore, the percentage of CD8+ T cell blasts cultured in the presence of IL-15 and TGF-β was also increased when compared to the percentage obtained after culture with IL-15 alone. As shown in **Figure 11**, this increase was statistically significant (p=0.0097) when 10 ng of TGF-β were added on day 0. However, when HC-10 antibodies were used to label the cells, the statistical significance was lost (**Figure 12**).

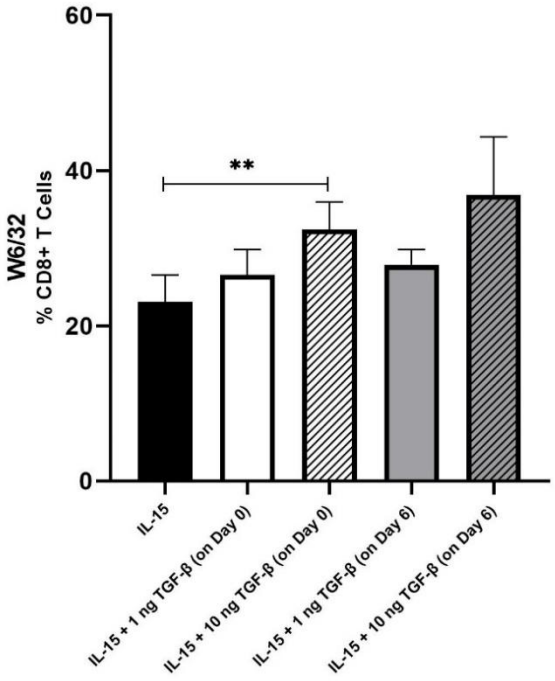

Figure 11. Effect of TGF-β on the percentage of IL-15-activated CD8+ T cell blasts. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the percentage of CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions. **P=0,0097.

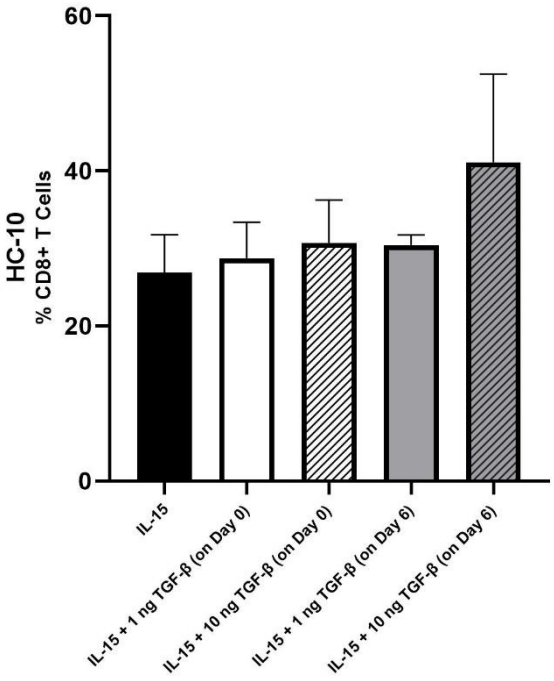

Figure 12. Effect of TGF-β on the percentage of IL-15-activated CD8+ T cell blasts. After the 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the percentage of CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions.

4.3 T Cell Proliferation and Cell Division Induced by IL-15: Effect of the Addition of IL-10 and TGF-β

To further study the impact of IL-10 and TGF-β on the activation of T cells present in the PBL preparations by IL-15, we analysed CFSE loss of fluorescence on CD3+ T cell blasts, which allows to quantify the extent of T cell proliferation and determine the cycles of cell division. Generally, in all the experiments performed, the cycles of cell division ranged between 4 and 7. In addition, this analysis allowed us to determine the percentage of cells that did not enter cell division, the percentage of cells that entered cell division and, finally, the percentage of cells that divided more than 5 times (see **Figure 13**).

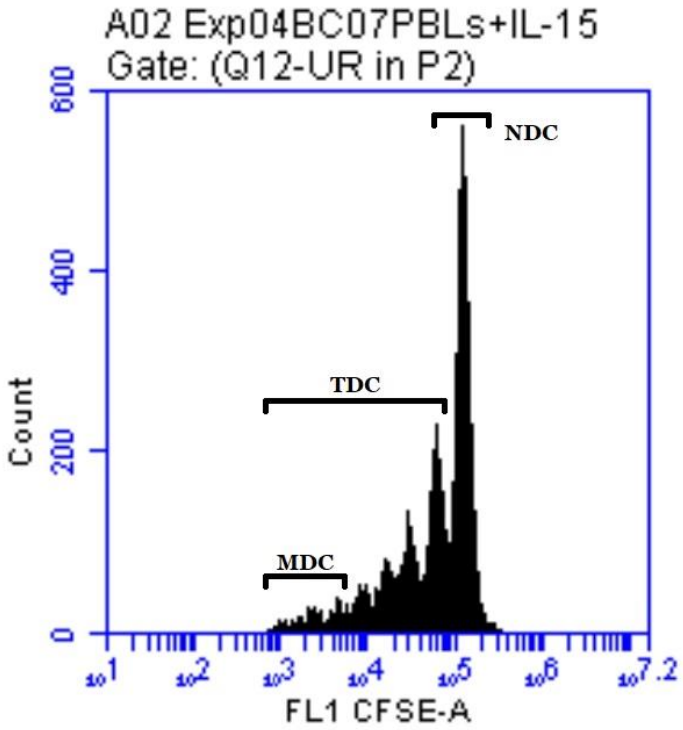

Figure 13. Proliferation of T cells after culture with IL-15. Histogram shows CFSE halving in gated CD3+ T cells after 12-day culture of PBL with 10ng of IL-15. Based on the CFSE profile of non-stimulated PBL, the histogram allows for the determination of the percentage of non-dividing cells (NDC), the total percentage of dividing cells (TDC) and the percentage of the most dividing cells (MDC, >5-6 cell divisions), as indicated.

Analysis of the effect of IL-10 and TGF-β on the total percentage of dividing CD4+ T cells in cultures of IL-15-activated PBL showed small increases without statistical significance (data not shown). Simultaneously, both IL-10 and TGF-β had a significant impact on the total percentage of dividing CD8+ T cells when added to cultures of IL-15-activated PBL. As shown in **Figure 14**, IL-10 significantly increased the total percentage of dividing CD8+ T cells when added at day 0 (10ng, p=0.0175) and at day 6 (1ng, p=0.0114). As previously observed for other parameters, this difference was only observed when cells were labeled with W6/32, but not HC-10 antibodies (**Figure 15**).

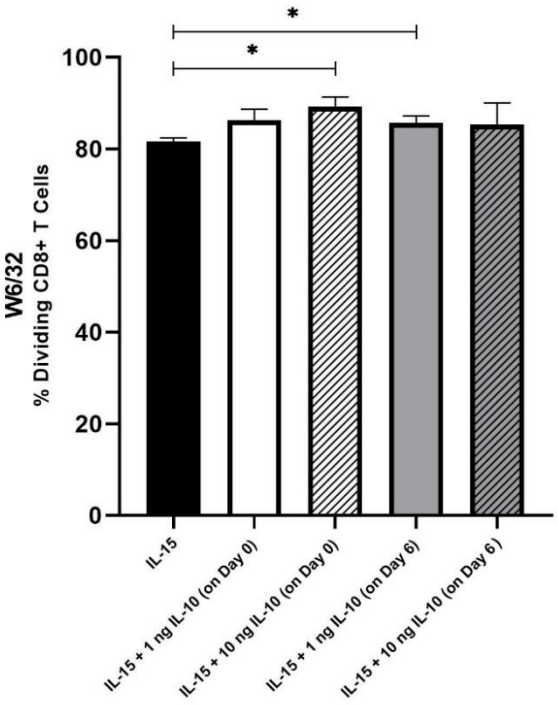

Figure 14. Effect of IL-10 on the total percentage of dividing CD8+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the total percentage of dividing CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions. *P≤0,0175.

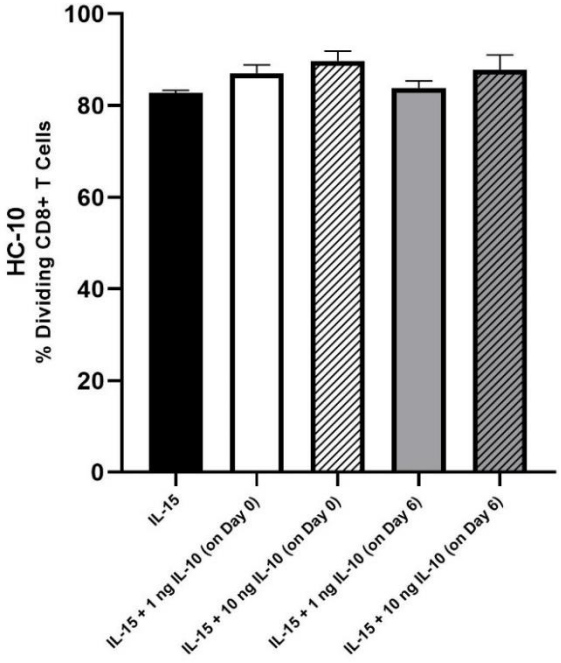

Figure 15. Effect of IL-10 on the total percentage of dividing CD8+ T cells. After the 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the total percentage of dividing CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions.

Similar results were obtained when TGF-β was added to the cultures of IL-15-activated PBL. The statistically significant increase in the percentage of total dividing CD8+ T cells was observed when 1ng and 10ng of TGF-β were added to the cultures, and the use of the HC-10 antibody did not interfere with the results, as shown in **Figure 16** and **Figure 17**.

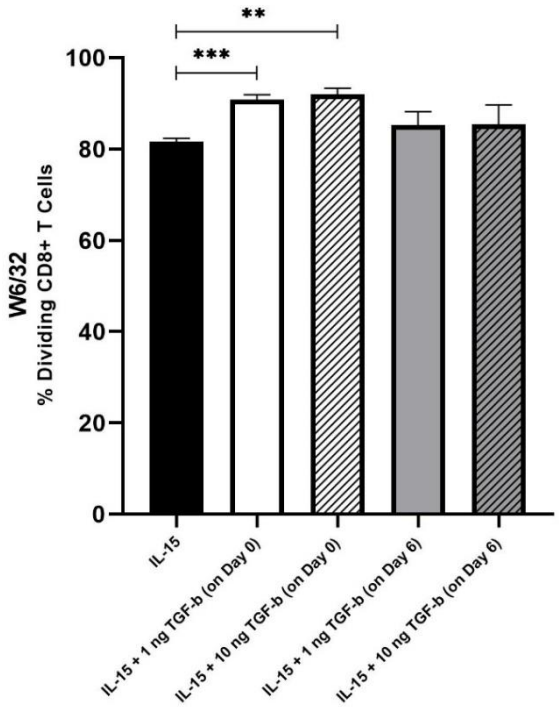

Figure 16. Effect of TGF-β on the total percentage of dividing CD8+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the total percentage of dividing CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions. **P=0,0015; ***P=0,0004.

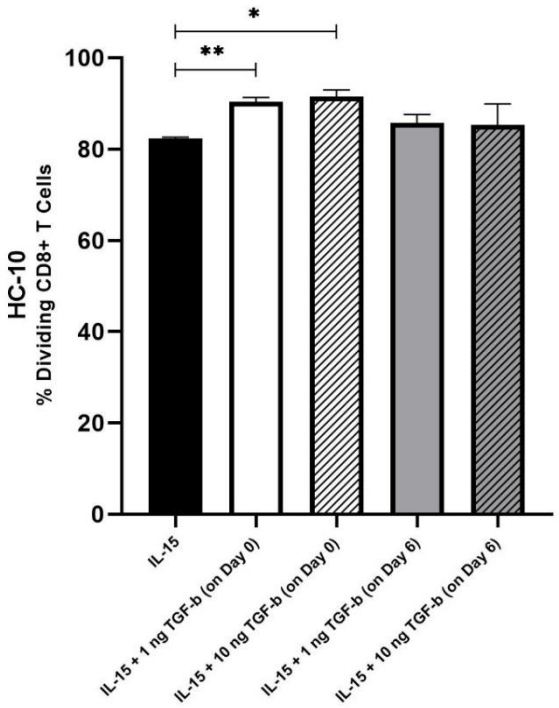

Figure 17. Effect of TGF-β on the total percentage of dividing CD8+ T cells. After the 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the total percentage of dividing CD8+ T cells (Mean±SEM, n=4), on gated blast cells, in the different culture conditions. *P=0,0111; **P=0,0079.

4.4 IL-15 Increases the Expression of Open Conformers (HC-10 Epitope) at the Cell Surface of IL-15- activated T Cells

In this study, we were also interested in documenting possible changes in the expression of the two main conformational states of MHC-I molecules at the cell surface of activated T cell cultures with IL-15 alone or in combination with IL-10 and TGF-β. These two major conformational states are the following: a) closed conformers, formed by the transmembrane MHC-I heavy chain, the β2m light chain and a small peptide, recognized by the W6/32 antibody; and b) open conformers, formed only by the transmembrane MHC-I heavy chain, recognized by the HC-10 antibody, as previously mentioned.

In the initial experiments, we noted that in cultures of PBL with IL-15 alone, the expression of the W6/32 epitope (closed conformers) on dividing CD4+ and CD8+ T cells remained steady or even had a tendency to slightly decrease with the cell division cycles (**Figure 18**

and **Figure 20**). In contrast, we consistently observed that the expression of the HC-10 epitope (open conformers) on dividing CD4+ and CD8+ T cells had a tendency to increase with the cell division cycles, being even more evident in the most dividing T cells (**Figure 19** and **Figure 21**). As expected, the level of expression of closed conformers was always much higher than the level of expression of open conformers.

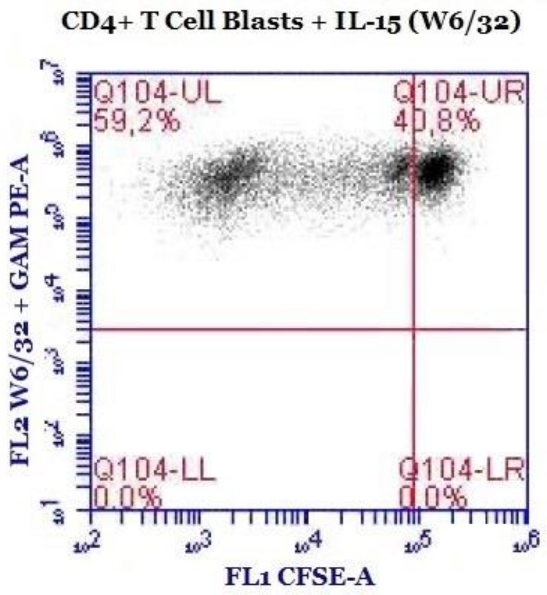

Figure 18. Expression of W6/32 epitopes on CD4+ T cell blasts as per cell division cycles. After the 12-day culture in the presence of IL-15, cells were acquired and analysed as indicated in the Materials & Methods chapter. Dot-plot shows CFSE versus W6/32 + GAM-PE expression on CD4+ T cell blasts, obtained from a single experiment.

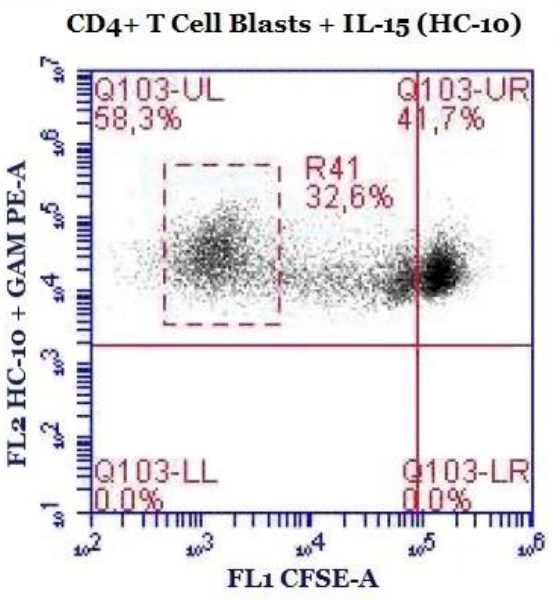

Figure 19. Expression of HC-10 epitopes on CD4+ T cell blasts as per cell division cycles. After the 12-day culture in the presence of IL-15, cells were acquired and analysed as indicated in the Materials & Methods chapter. Dot-plot shows CFSE versus HC-10 + GAM-PE expression on CD4+ T cell blasts, obtained from a single experiment.

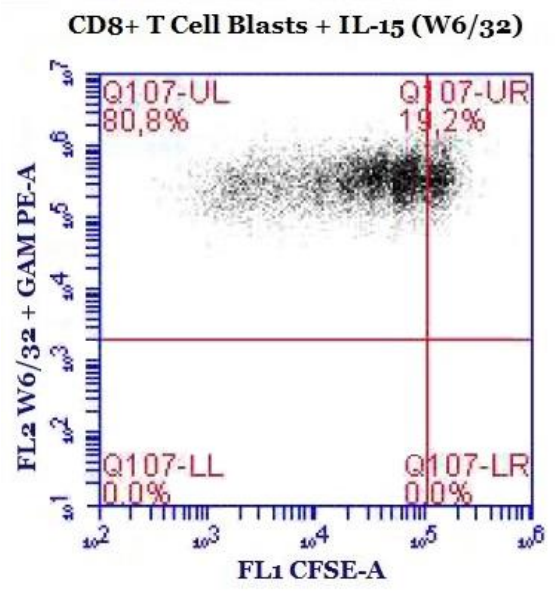

Figure 20. Expression of W6/32 epitopes on CD8+ T cell blasts as per cell division cycles. After the 12-day culture in the presence of IL-15, cells were acquired and analysed as indicated in the Materials & Methods chapter. Dot-plot shows CFSE versus W6/32 + GAM-PE expression on CD8+ T cell blasts, obtained from a single experiment.

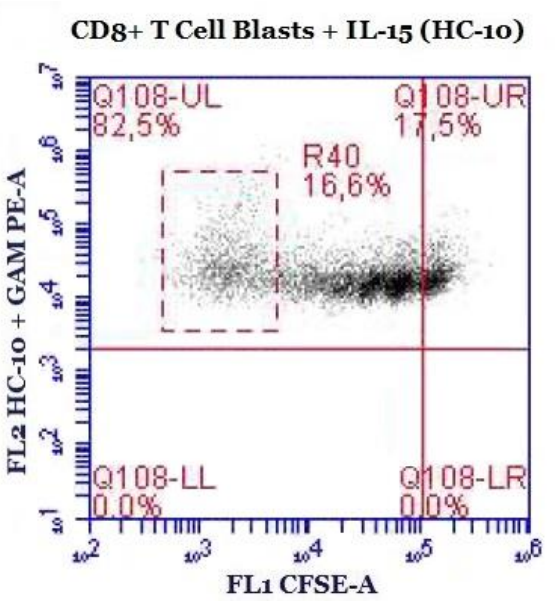

Figure 21. Expression of HC-10 epitopes on CD8+ T cell blasts as per cell division cycles. After the 12-day culture in the presence of IL-15, cells were acquired and analysed as indicated in the Materials & Methods chapter. Dot-plot shows CFSE versus HC-10 + GAM-PE expression on CD8+ T cell blasts, obtained from a single experiment.

Figure 22 and **Figure 23** summarize the mean fluorescence intensity (MFI) values for the open conformers present in non-dividing cells, total dividing cells and most dividing cells among CD4+ and CD8+ T cells after the 12-day culture with IL-15. It can be seen that the expression of the HC-10 epitope (i.e., open conformers) increased among CD4+ by 1.5-fold between non-dividing cells and total dividing cells, and by approximately 2-fold between non-dividing cells and most dividing cells (p=0.0144) (**Figure 22**). Among CD8+ T cells cultured in the presence of IL-15 alone, the expression of the HC-10 epitope remained unchanged between non-dividing cells and total dividing cells, and increased by 1.5-fold between non-dividing cells and most dividing cells, though without statistical significance (**Figure 23**). The expression of the W6/32 epitope (i.e., closed conformers) remained largely unchanged between non-dividing cells, total dividing cells and most dividing cells, among CD4+ and CD8+ T cells.

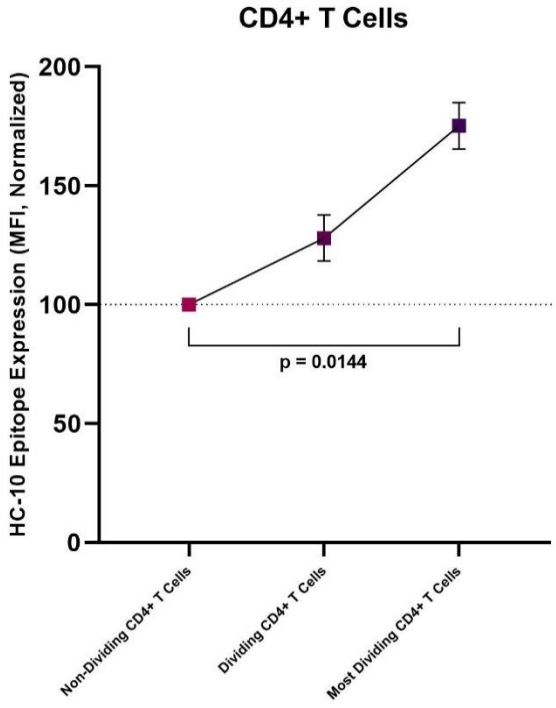

Figure 22. HC-10 epitope expression by non-dividing cells, total dividing cells and most dividing cells among CD4+ T cells after 12-day culture of PBL with IL-15. After culture in the presence of IL-15, PBL were labelled with HC-10+GAM-PE, CD3 and CD8, acquired by flow cytometry, and MFI values calculated on gated CD4+ T cells. The graph shows HC-10 MFI values in non-dividing cells (Mean±SEM, n=4), total dividing cells (Mean±SEM, n=4) and most dividing cells CD4+ T cells (Mean±SEM, n=3). Data was normalized in accordance to formulas (3) and (4), indicated in the Materials & Methods chapter. P=0,0144.

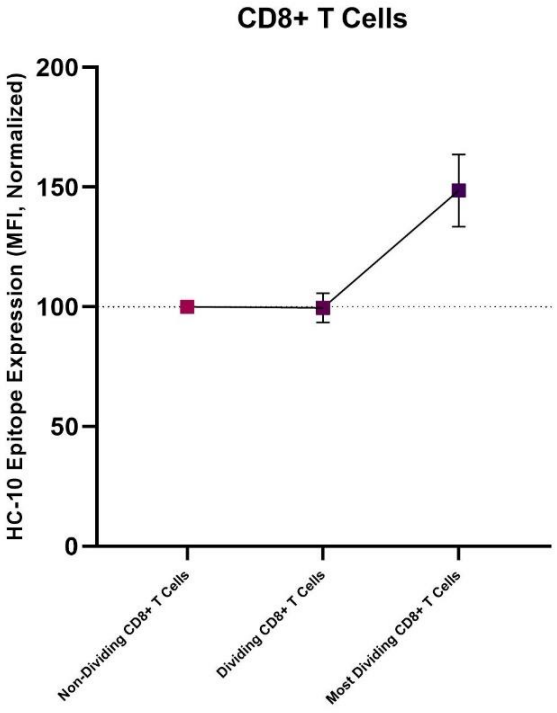

Figure 23. HC-10 epitope expression by non-dividing cells, total dividing cells and most dividing cells among CD8+ T cells after 12-day culture of PBL with IL-15. After culture in the presence of IL-15, PBL labelled with HC-10+GAM-PE, CD3 and CD8, acquired by flow cytometry, and MFI values calculated on gated CD8+ T cells. The graph shows HC-10 MFI values in non-dividing cells (Mean±SEM, n=4), total dividing cells (Mean±SEM, n=4) and most dividing cells CD8+ T cells (Mean±SEM, n=3). Data was normalized in accordance to formulas (3) and (4), indicated in the Materials & Methods chapter.

4.5 Effect of IL-10 and TGF-β on the Expression of the W6/32 and HC-10 Epitopes by Total Dividing and Most Dividing CD4+ and CD8+ T Cells

Once the levels of expression of the W6/32 and HC-10 epitopes on IL-15-activated CD4+ and CD8+ T cells were established, we proceeded to analyse the effect that the addition of IL-10 and TGF-β would have on the same cells.

The addition of IL-10 to the cultures had no statistically significant effect on the level of the HC-10 epitopes on the cell surface of total dividing CD4+ and CD8+ T cells (data not

shown). On the contrary, the expression of W6/32 epitopes was seen to increase when the same combination of IL-15 + IL-10 was added to the cell culture, with observable statistical significance (**Figure 24** and **Figure 25**).

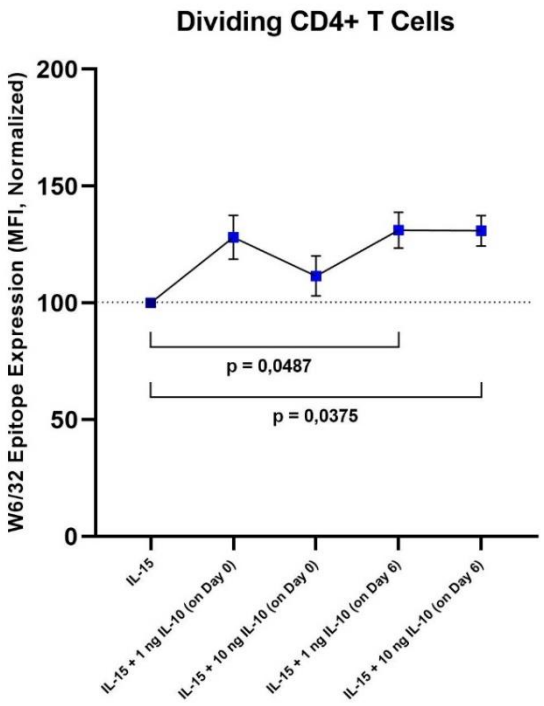

Figure 24. Effect of IL-10 on the expression of the W6/32 epitope by total dividing CD4+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the W6/32 MFI values on gated total dividing CD4+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and corresponding P values are indicated.

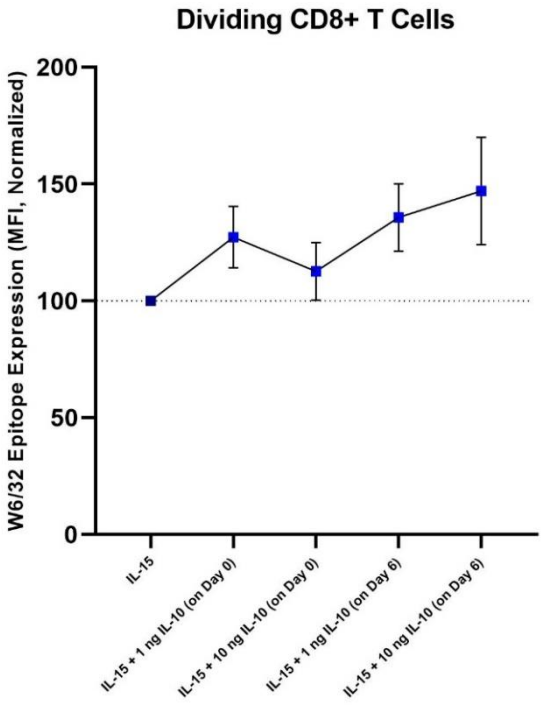

Figure 25. Effect of IL-10 on the expression of the W6/32 epitope by total dividing CD8+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the W6/32 MFI values on gated total dividing CD8+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter.

The addition of TGF-β was also seen to increase the expression of the W6/32 epitope both on total dividing CD4+ and CD8+ T cells (**Figure 26** and **Figure 27**). Despite these results, the only statistically significant increase was observed on total dividing CD4+ T cells when 10ng of TGF-β was added at day 0 (p=0,0040).

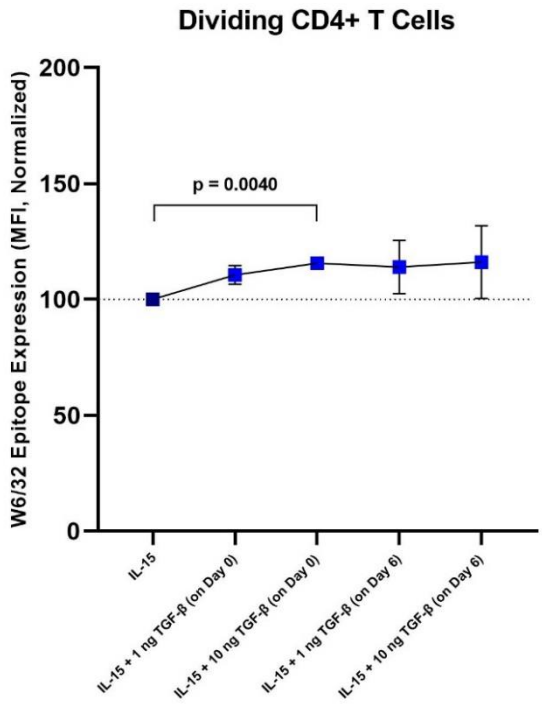

Figure 26. Effect of TGF-β on the expression of the W6/32 epitope by total dividing CD4+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the W6/32 MFI values on gated total dividing CD4+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and corresponding P values are indicated.

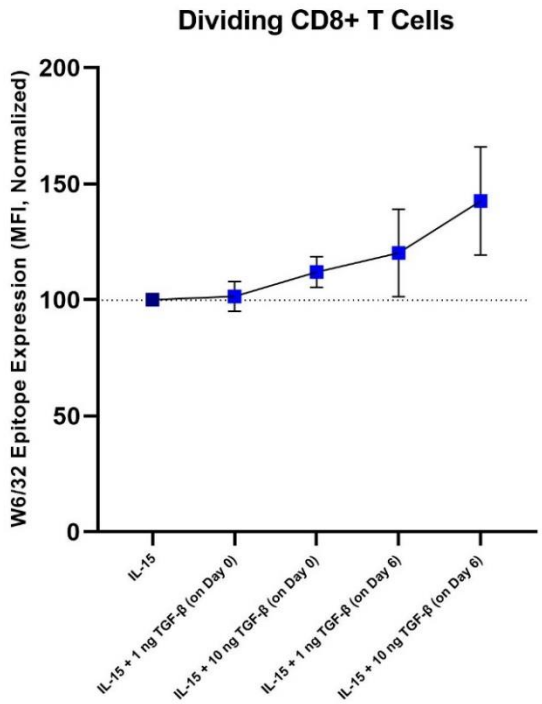

Figure 27. Effect of TGF-β on the expression of the W6/32 epitope by total dividing CD8+ T cells. After the 12-day culture, PBL were labelled with W6/32+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the W6/32 MFI values on gated total dividing CD8+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter.

Contrary to what happened with the expression of W6/32, the expression of HC-10 decreased on total dividing CD4+ and CD8+ T cells when PBL were cultured in the presence of IL-15 and TGF-β, in comparison to IL-15 alone (**Figure 28** and **Figure 29**). The decreases in HC-10 epitope expression were statistically significant for both CD4+ (p=0,0273) and CD8+ (p=0,0341) T cells, for the addition of 10ng of TGF-β to the PBL cultures on day 6.

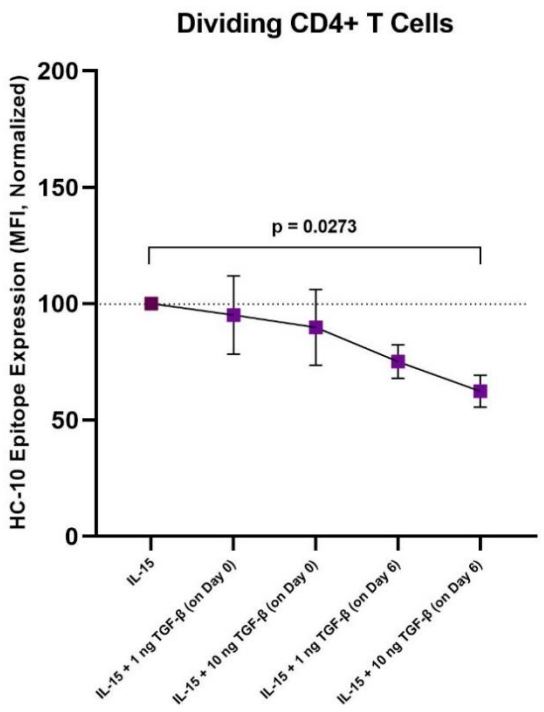

Figure 28. Effect of TGF-β on the expression of the HC-10 epitope by total dividing CD4+ T cells. After the 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the HC-10 MFI values on gated total dividing CD4+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and corresponding P values are indicated.

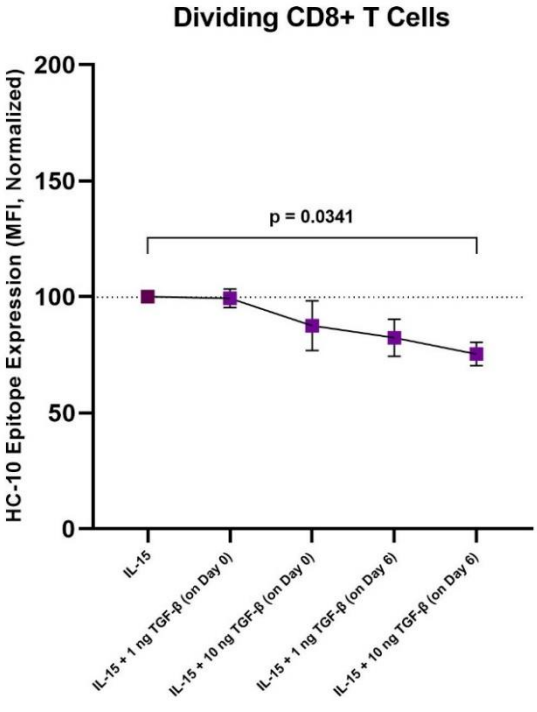

Figure 29. Effect of TGF-β on the expression of the HC-10 epitope by total dividing CD8+ T cells. After the 12-day culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies and analysed by flow cytometry. The graph shows the HC-10 MFI values on gated total dividing CD8+ T cells (Mean±SEM, n=4), in the different culture conditions. Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and corresponding P values are indicated.

Subsequently, the same analysis was conducted, this time focusing exclusively on the population of cells that divided more than 5 times, i.e., the most dividing cells. In **Figure 30**, an example of the region that was selected to study the effect of IL-10 and TGF-β on the expression of the W6/32 and HC-10 epitopes can be observed.

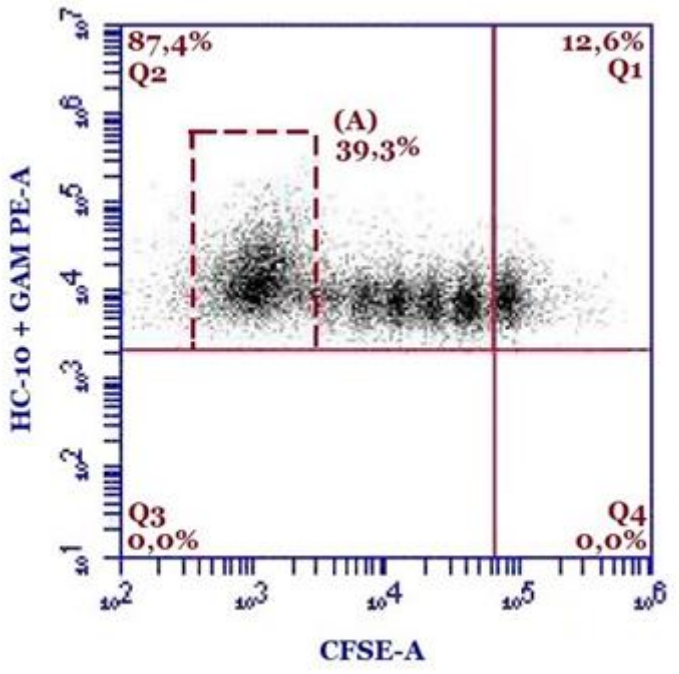

Figure 30. Expression of open conformers (HC-10 epitope) by the most dividing CD4+ T cells. After the culture in the presence of IL-15 and TGF-β, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 and acquired by flow cytometry. Dot-plot shows CFSE versus HC-10 expression on gated CD4+ T cell blast. Three populations can be distinguished: Non-Dividing Cells in Q1, Total Dividing Cells in Q2, and Most Dividing Cells in gate A, within the Q2 quadrant. HC-10 (this dot-plot) and W6/32 (dot-plot not shown) MFI values were obtained and compared to the MFI values obtained in cultures of IL-15 alone.

The analysis of the effect of IL-10 and TGF-β on the MFI values for HC-10 on the most dividing CD4+ and CD8+ T cells gave similar, albeit not equally significant, results. The expression of the HC-10 epitope was remarkably reduced on the most dividing CD4+ and CD8+ T cells when IL-10 was added to the IL-15 cultures, with great statistical significance (**Figure 31** and **Figure 32**). In contrast, and even though the addition of TGF-β to the IL-15 cultures induced a similar decrease in the expression of the HC-10 epitopes in most dividing CD4+ and CD8+ T cells, the decrease was not statistically significant (data not shown).

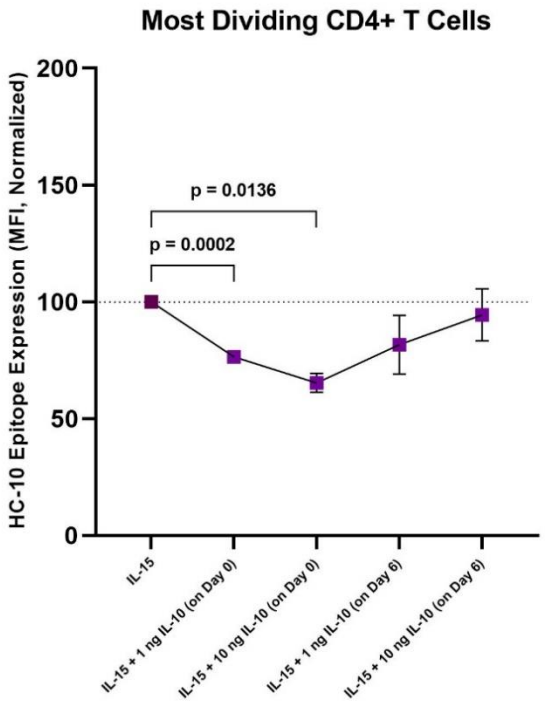

Figure 31. Effect of IL-10 on HC-10 epitope expression by the most dividing CD4+ T cells after 12-day culture of PBL with IL-15. After culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies, acquired by flow cytometry, and MFI values calculated after gating on the most dividing CD4+ T cells. The graph shows HC-10 MFI values in the different culture conditions (Mean±SEM, n=3). Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and the corresponding P values are indicated.

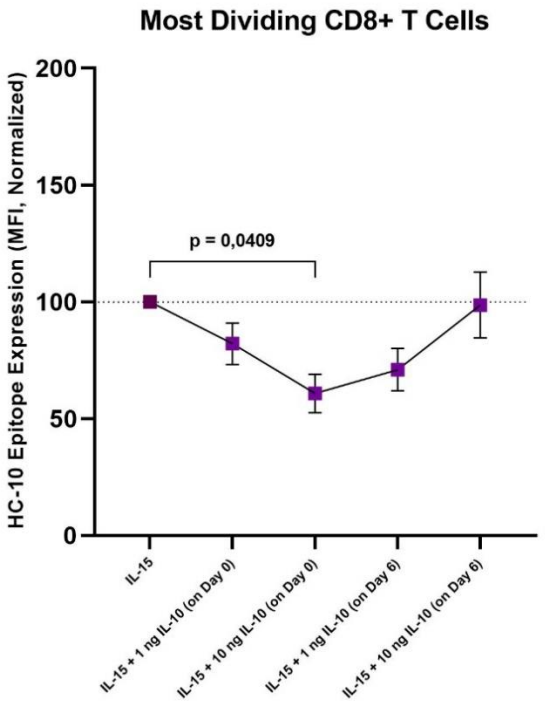

Figure 32. Effect of IL-10 on HC-10 epitope expression by the most dividing CD8+ T cells after 12-day culture of PBL with IL-15. After culture, PBL were labelled with HC-10+GAM-PE, CD3 and CD8 antibodies, acquired by flow cytometry, and MFI values calculated after gating on the most dividing CD8+ T cells. The graph shows HC-10 MFI values in the different culture conditions (Mean±SEM, n=3). Data was normalized in accordance to formula (5), indicated in the Materials & Methods chapter. Statistically significant differences between conditions and the corresponding P values are indicated.

Chapter V – Discussion

Chapter V – Discussion

To elucidate the impact of IL-10 and TGF-β on the IL-15-driven activation of T cells, and to answer the pivotal questions posed along this study, cells from healthy individuals were cultured *in vitro* for 12 days and exposed to different stimuli. The culture was always conducted in the presence of IL-15 alone and with the presence of IL-10 and TGF-β, added at days 0 and 6. As previously shown by our group and by several other studies [3,19,56–63], IL-15 is involved in the activation and proliferation of human T cells, therefore an increase in the proliferation of T cells due to the influence of IL-15 was to be expected. However, the effect of IL-10 and TGF-β on T cells is broader, at times contradicting, and highly dependent on the specific culture conditions [22,27,30–38,43,45–47,49,50]. Remarkably, the results of this study show significant increase in the proliferation of T cells when the presence of IL-15 is accompanied by the presence of IL-10 and TGF-β. This increase seems to be mostly noticeable when a higher concentration of the secondary cytokine (meaning IL-10 or TGF-β) is added to the cell culture. With this in mind, it can be inferred that both IL-10 and TGF-β do, in fact, have considerable influence on the IL-15-driven activation of CD4+ and CD8+ T cells, with an enhanced cell proliferation, now uncovered, being achieved as a result. Given that IL-10 and TGF-β are immunosuppressive cytokines and considering their predominantly negative influence on the proliferation of T cells, this discovery certainly presents a surprising revelation, particularly in regard to TGF-β, best known for influencing T cell differentiation in combination with other cytokines [43,45,46], but not necessarily their proliferation. IL-10, on the other hand, is known for inhibiting or stimulating T cell proliferation depending on the cell status at the time of exposure and whether an APC is present or not. When in combination with IL-2, and in the absence of an APC, IL-10 can in fact induce the proliferation of T cells [27,35]. Considering that professional APC (e.g., dendritic cells) or monocytes were not present or were residual in this study, and taking into account the similarities between IL-2 and IL-15, namely in their structure and signaling, perhaps a similar mechanism was at play when, in this study, T cells proliferated in the presence of a combination of IL-15 and IL-10. Bearing in mind that IL-15 is known to bind to the IL-15Rα receptor expressed on the surface of T cells, and that this binding can lead to the activation of downstream signaling pathways ultimately resulting in the proliferation of T cells, one could suggest that a similar scenario could also take place for the influence of IL-10 and TGF-β on T cells, with these cytokines acting by binding to specific receptors on the same cells. It can additionally be hypothesised that IL-15 facilitates an increase in the expression of IL-10 and TGF-β receptors at the plasma membrane, allowing for a more pronounced effect of these cytokines on T cells. Further

studies would, however, be required in order to disclose the exact mechanisms behind this interplay.

Even though the presence of IL-10 and TGF-β potentiated the effect of IL-15 in inducing an increase in the percentage of CD4+ and CD8+ T cells, they did not have a significant influence on the total percentage of IL-15-induced blast cells. A plausible interpretation for this result could be that these cytokines do not markedly influence the effect of IL-15 in the induction of blast cells, which include mostly CD3+ T cells (analysed in this study) and CD3- NK cells (not analysed). However, their effect is observed when particular lymphocyte populations, namely CD4+ and CD8+ T cells, are analysed. Alternatively, they could, for example, inhibit the proliferation of some lymphocytes while promoting the proliferation of others, resulting in a net effect where the total % of IL-15-induced blast cells remain relatively unchanged. Also, it is possible that, with the conditions used in this study, IL-10 and TGF-β had distinct effects on the activation and proliferation of B and NK cells, contributing to the observed results. Even so, further experiments would be needed to confirm this hypothesis.

While studying the impact of IL-10 and TGF-β on the percentage of CD4+ and CD8+ T cell blasts after culture with IL-15, a certain lack of statistical significance when cells were labelled with HC-10 antibodies was noted, whilst in graphs of cells labelled with W6/32 antibodies, statistical significance was observable. This occurred due to a greater disparity in the values of the different experiments in regard to cells labelled with HC-10 antibodies. Even though data shows that the results are similar for HC-10 and W6/32-labelled cells, this disparity resulted in a higher SEM, and thus in the absence of statistical significance.

Another main objective of this study was to investigate the effects of IL-15, IL-10 and TGF-β on the expression of open and closed MHC-I conformers. It is known that these conformers, having a multitude of immunological and non-immunological functions, are expressed at the cell surface of both activated CD4+ and CD8+ T cells [78–80]. While the closed conformers mainly have a role related to the immune response [83], the open conformers have been proposed to regulate biological processes related with the interaction of hormones and other growth factors with their receptors and therefore regulate cell activation, proliferation, and differentiation [81,83,96–99,100–102]. Moreover, the level of open conformers at the cell surface is known to increase in antigen stimulated T cells both *in vivo* and *in vitro*. However, to our knowledge, the effect that cytokines, either alone or in combination, have on the expression of these open conformers is not known. The effect of IL-15 in inducing T cell proliferation is evident, but the simultaneous effect on the expression of open MHC-I conformers on the same cells remains thus far unknown. What

is known, however, is that upon cellular activation and growth, the expression of open MHC-I conformers tends to increase proportionally to the level of proliferation [81,103]. The present study has allowed us to demonstrate a clear increase in the expression of HC-10 epitopes when cells are proliferating in the presence of IL-15, with a higher expression noticed in cells that divided the most (i.e., more than five cycles of cell division). To the best of our knowledge, this is the first study demonstrating that IL-15 induces the formation of open MHC-I conformers (HC-10 reactive) at the plasma membrane of dividing T cells. Both IL-10 and TGF-β have also been shown to be associated to modulations in the expression of MHC-I molecules on certain cell types [27,86,87,89]. Nevertheless, to this day, evidence remains to be found on their impact on open/closed MHC-I conformers present on T cells. In the present study, a correlation between the presence of these anti-inflammatory cytokines on the cell culture environment and a decrease in the expression of HC-10 epitopes induced by IL-15 was found. Hence, IL-10 and TGF-β were seen to reduce the increase in the expression of IL-15-induced HC-10 epitopes. Furthermore, the decrease in the expression of open MHC-I conformers (HC-10 reactive) was paralleled by an increase in the expression of closed MHC-I conformers (W6/32 reactive). This observation is not unexpected considering that there is a physiological equilibrium between open and closed conformers at the cell surface of dividing T cells [5,83].

Taking into consideration the findings gathered in this study, it becomes evident that IL-15 is able to increase the expression of open MHC-I conformers on T cells, and that this increase is significantly counterbalanced by the presence of IL-10 and TGF-β. The "inhibitory" effect of these cytokines on the expression of open MHC-I conformers induced by IL-15 could be related to the fact that IL-10 and TGF-β have multiple immunosuppressive properties. Nonetheless, the molecular mechanisms and possible functional consequences for this decrease will remain unknown until further studies are carried out. In any case, these results are novel in the field of immunology and could prove to be relevant considering the multitude of roles that MHC-I molecules are known for, as well as their impact in certain clinical and biomedical settings.

Chapter VI – Conclusion

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During the course of this study we were able to successfully obtain answers to all the questions raised beforehand regarding the effect of IL-10 and TGF-β on the IL-15-driven activation of T cells, and on the expression of open and closed MHC-I conformers. Through experiments, it was found that the already known effect of IL-15 in inducing T cell proliferation was greatly increased by the presence of the anti-inflammatory cytokines IL-10 and TGF-β. This was more significant when higher concentrations of IL-10 or TGF-β were present. This study has also demonstrated that, in addition to being capable of increasing T cell proliferation, IL-15 also causes an increase in the expression of open MHC-I conformers present on the same cells. Furthermore, IL-10 and TGF-β were seen to decrease the expression of open MHC-I conformers, counterbalancing the effect of the presence of IL-15, while simultaneously increasing the expression of closed MHC-I conformers.

Conducting additional experiments under the same conditions to those described in this study would notably enhance the statistical significance of the results. Nonetheless, it can be confidently stated that this study is a step in the right direction toward better understanding the influence of these cytokines on the human immunological system, providing novel findings that can bear relevance in various disease contexts.

Chapter VII – Future Perspectives

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The findings of this study provide a valuable insight into the intricate interplay taking place in the human immune system between IL-10, TGF-β, IL-15, T Cells and the MHC-I conformers present on these cells. Additional studies would be welcomed to further validate the results and to test different conditions. It would be interesting, for example, to combine both cytokines, IL-10 and TGF-β, and to observe their effect on the same IL-15-driven activation of T cells. Other variables, such as different concentrations added to the cell culture, or changes to the days in which such concentrations would be added, could also be of great interest to better understand the implications of these cytokines.

Based on these results, several intriguing avenues for future research emerge. Further investigation would be needed to unravel the underlying mechanisms through which IL-10 and TGF-β enhance T Cell activation induced by IL-15, while simultaneously reducing the expression of open MHC-I conformers. An understanding of these mechanisms would shed light on the complex regulatory pathways involved in T cell activation, and on how these cytokines can modulate T cell responses. Furthermore, a deeper knowledge on how the modulation of open MHC-I conformers is achieved, whether by endocytosis and recycling of closed MHC-I conformers, a simple induction in the shift from closed conformer to open conformer, the shedding of open MHC-I conformers from the surface to the extracellular milieu, an increase in cis-associations or even a decrease in their transport from the ER to the plasma membrane, could contribute to a better understanding of numerous diseases, particularly regarding the non-immunological functions of these conformers. Assessing the implications of IL-10 and TGF-β modulation in disease contexts, such as cancer, autoimmune disorders and chronic inflammatory disorders, could prove to be a promising pathway for future research. It would be important to evaluate whether a manipulation in IL-10 and TGF-β signaling could impact T cell responses in specific pathological conditions.

Additional investigations could also be conducted to assess whether IL-10 and TGF-β would have the same effects on different but equally important cells of the immune environment, as they did in this study with T cells.

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