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Characterization of TYK2 role in IL-7/IL-7R signaling in T-cell Acute Lymphoblastic Leukemia

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Authorship Statement

I declare to be the author of this work, which is original and unpublished. Authors and papers consulted are appropriately cited in the text and are listed in the included references.

Joana Margarida Bartolomeu Cavaco

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Resumo

A leucemia linfoblástica aguda (LLA) é uma neoplasia hematológica que pode ser subdividida de acordo com o tipo de linfócitos afetados. Assim sendo, denomina-se leucemia linfoblástica aguda de células T (LLA-T), quando existe a expansão e proliferação de linfócitos T imaturos, e leucemia linfoblástica aguda de células B (LLA-B), quando associada à proliferação de linfócitos B imaturos.

A LLA é o cancro pediátrico mais comum, sendo que dentro destes casos, aproximadamente 15% são de LLA-T. Após tratamento, 85% das crianças com LLA-T atingem remissão. No entanto, nos adultos esta percentagem é mais reduzida, ficando entre os 20-40%.

O tratamento da LLA-T consiste num programa intenso de quimioterapia, que está associado a vários efeitos secundários. Para além disso, apesar destes doentes terem um prognóstico favorável à data do diagnóstico, a taxa de recidiva é elevada e está associada a um pior prognóstico, uma vez que a doença persistente é geralmente resistente à quimioterapia. Como tal, os efeitos secundários associados à quimioterapia e a falta de tratamento alternativos após recidiva realça a necessidade urgente da descoberta de novas abordagens terapêuticas.

Este tipo de leucemia (LLA-T) está associada a diversas alterações genéticas, que resultam na desregulação do processo de desenvolvimento de células T, e por sua vez, levam à proliferação não controlada de células imaturas.

A via de sinalização da interleucina-7 (IL-7) desempenha um papel muito importante no processo de diferenciação das células T. Estudos realizados demonstram que a eliminação desta via resulta no bloqueio do desenvolvimento de linfócitos T e B, e consequentemente na produção de linfócitos T não funcionais e numa população de linfócitos B reduzida. A ativação desta via requer a participação da IL-7 e do seu recetor, o IL-7R. O recetor da IL-7 é composto por duas subunidades, nomeadamente a cadeia α (IL-7Rα) e a cadeia γ comum. Estas subunidades não possuem atividade catalítica intrínseca e como tal, para poderem fosforilar substratos e darem início à transdução de sinal necessitam da ação de outras proteínas, como as cinases *Janus* (JAK) JAK1 e

JAK3, que se ligam à cadeia IL-7Rα e à cadeia γ comum, respetivamente. Quando a interleucina se liga ao recetor, JAK1 e JAK3 fosforilam-se mutuamente, causando a sua ativação, o que resulta na fosforilação de tirosinas presentes nas cadeias do recetor, criando locais de ancoragem para a proteína STAT5. Esta proteína, ao ser ativada por JAK1 e JAK3, sofre dimerização e é transportada para o núcleo onde, atuando como um fator de transcrição, dá início à transcrição de genes envolvidos na proliferação, diferenciação e apoptose.

Vários estudos descrevem alterações na via da IL-7 no contexto da LLA-T. Por exemplo, foi reportado que 70% dos linfócitos T imaturos dos doentes com LLA-T expressam o IL-7R, sendo que 10% destes doentes apresentam mutações no recetor que levam à ativação constitutiva da via.

Uma compreensão detalhada dos processos fisiológicos permite perceber que alterações levam ao desenvolvimento de uma patologia e que estratégias terapêuticas poderão ser eficientes. Assim sendo, um dos objetivos do nosso laboratório é tentar caracterizar a via de transdução de sinal da IL-7 num contexto fisiológico para perceber de que modo alterações nesta via contribuem para a LLA-T. Recentemente, num estudo que visava caracterizar proteínas fosforiladas em resposta à estimulação com IL-7 em diferentes linhas celulares, verificámos que TYK2, uma *Janus* cinase, sofre fosforilação e ativação após estimulação com IL-7. TYK2, apesar de já ter sido associado a LLA-T, nunca foi associado à via da IL-7. Deste modo, este projeto tem como objetivo estudar a possível função de TYK2 na via de transdução de sinal da IL-7 e o seu papel na LLA-T dependente da IL-7.

Neste projeto começámos por validar os resultados preliminares e verificámos que TYK2 sofre fosforilação após estimulação com IL-7 em três linhas celulares diferentes. Nomeadamente numa linha celular derivada de um paciente com LLA-T dependente de IL-7 (TAIL7), numa linha derivada de timócitos murinos e transduzida com o recetor humano da IL-7 (D1 hWT/IL-7R) e numa linha murina de linfócitos pro-B transduzida com o recetor humano da IL-7 (BA/F3).

Seguidamente, tentámos perceber se a fosforilação de TYK2 ocorre como um produto final da ativação da via da IL-7, ou se tem um papel ativo durante a

transdução de sinal nesta via. Para tal, utilizámos uma abordagem de expressão ectópica, usando a linha celular HEK-293T, que com a exceção de JAK1, não expressam as proteínas envolvidas na ativação da via da IL-7. Este modelo permitiu-nos modular a presença das proteínas envolvidas nesta via para tentar perceber se TYK2, sendo uma *Janus* cinase, poderia exercer as funções de JAK1 e/ou JAK3. Para modular a presença de JAK1 criámos uma linha *knockdown* para JAK1. Os nossos resultados demonstraram que TYK2, quando sobrexpresso, aumenta a ativação de JAK1 e STAT5, independentemente dos níveis de JAK1 e de JAK3. Utilizando o mesmo modelo, verificámos igualmente que TYK2 está associado ao complexo do IL-7R.

Tentámos ainda compreender o papel de TYK2 no contexto da LLA-T dependente de IL-7. Para tal, utilizamos a linha celular TAIL7 que tratámos com um inibidor específico de TYK2 e avaliámos o seu impacto na viabilidade celular, na ativação celular e nos níveis de superfície do IL-7R após estimulação com IL-7. Apesar de não ter sido possível tirar conclusões no que diz respeito ao impacto de TYK2 da viabilidade celular, os resultados sugerem que ao inibir TYK2 existe uma tendência para uma diminuição da ativação destas células e dos níveis de superfície do recetor na presença de IL-7.

Estando ainda numa fase inicial, este projeto requer experiências adicionais para que se possa estabelecer o papel de TYK2 na via da IL-7 e o seu impacto na LLA-T dependente da IL-7. No entanto, conseguimos demonstrar que a sobreexpressão de TYK2 exacerba a ativação da via da IL-7, o que pode ser significativo na LLA-T onde mutações *gain-of-function* nesta proteína estão descritas. Ainda no que diz respeito à LLA-T, os nossos resultados sugerem que a inibição farmacológica de TYK2 diminui os níveis de IL-7R à superfície, o que em termos terapêuticos pode ser relevante.

Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is the most common childhood malignancy, characterized by the expansion of T-cell progenitors. Although the 5-year event-free survival rate has improved to 85% in children, it remains at 20-40% in adults. In addition, the relapse rate is high and is associated with a poor prognosis.

The IL-7/IL-7R signaling pathway plays a significant role in T-ALL onset and maintenance, and its inhibition displays therapeutic potential in the treatment of T-ALL. Our lab has recently found that TYK2, a Janus kinase (JAK) involved in cytokine receptor signaling, is activated in response to IL-7. Since this particular JAK has never been associated with the IL-7 signaling pathway, this project aimed to investigate TYK2's putative role in the IL-7 signaling pathway and its impact on IL-7-mediated T-ALL.

Using both a physiological and an ectopic model, we showed that IL-7 activates TYK2. Moreover, our results show that overexpression of TYK2 in the IL-7 signaling pathway ectopic model leads to the increased activation of JAK1 and STAT5, two major players of the IL-7 signaling cascade. Using the same model, we also found that TYK2 associates with the IL-7 receptor (IL-7R) complex.

In the context of IL-7-dependent T-ALL, our results suggest that inhibition of TYK2 may decrease IL-7R surface expression and cell activation.

Although a deeper characterization of TYK2's putative role in the IL-7 signaling pathway is necessary, our results suggest that it may have a role, in particular in the context of IL-7-dependent T-ALL.

Keywords: IL-7/IL-7R signaling pathway; TYK2; IL-7-dependent T-ALL; Deucravacitinib, Janus Kinases.

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Abbreviations

A

AKT/PKB: v-akt murine thymoma viral oncogene homolog/Protein kinase B

ALL: Acute lymphoblastic leukemia

APC: Antigen-presenting cell

B

B-ALL: B-cell acute lymphoblastic leukemia

BCR: B-cell receptor

BSA: Bovine Serum Albumin

C

CD3: Cluster of differentiation 3

CIS: Cytokine-inductively SH2-domain

CK2: Casein kinase 2

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

CT: comparative cycle

D

DMEM: Dulbecco's Modified Eagle Medium

DN: Double negative

DP: Double positive

E

EFS: Event-free survival

Epo: Erythropoietin

ETP-ALL: Early T-cell precursor T-cell acute lymphoblastic leukemia

F

FBS: Fetal Bovine serum

FDA: Food and Drug Administration

FERM: Four-point- one, ezrin, radixin, moesin

G

G-CSF: Granulocyte-colony stimulating factor

GH: Growth hormone

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GSI: Gamma secretase inhibitor

GTP: Guanosine triphosphate

H

HEK-293T: Human Embryonic Kidney 293 SV40 T-antigen cell

HRP: horseradish peroxidase

HSC: Hematopoietic stem cells

I

IFN: Interferon

IL-7: Interleukin 7

IL-7R: IL-7 receptor

J

JAK: Janus kinase

JH: Homology domains

L

LB: Lysogenia broth

LOF: Loss of function

M

MHC: Major Histocompatibility Complex

mTOR: mammalian target of rapamycin protein

N

NICD: NOTCH intracellular domain

NK: Natural Killer

O

ON: overnight

P

$p27^{kip1}$: Cyclin-dependent kinase inhibitor p27

PDX: Patient-derived-xenografts

PI3K: Phosphatidylinositol 3-kinase

PIAS: Inhibitor of activated STAT

PTB-1B: Protein tyrosine phosphatase 1B

PTEN: Phosphatase and tension homolog

PBS: Phosphate-buffered saline

Q

qPCR: Quantitative Real-Time PCR

R

RPPA: Reverse phase protein array

RT: Room temperature

RPMI: Roswell Park Memorial Institute

RPM: Rotations per minute

RAS: Small GTPase rat sarcoma virus

S

S.O.C: Super optimal broth

SCID: Severe combined immunodeficiency

SCR: Scramble

SH2: Src-homology 2

SHP-1: Src homology region 2 domain-containing phosphatase-1

shRNA: Short-hairpin RNA

SNP: Single nucleotide polymorphism

SOCS1: Suppressor of cytokine signaling 1

STAT: Signal transducer and activator of transcription

T

T-ALL: T-cell acute lymphoblastic leukemia

TBS-T: Tris-Buffered Saline with 0.1% Tween 20

TCF-1: T-cell factor 1

TCR: T-cell receptor

TNF: Tumor-necrosis factor

Tpo: Thrombopoietin

TpoR: Thrombopoietin receptor

TSLP: Thymic stromal lymphopoietin

TYK2: Tyrosine kinase 2

W

WB: Western Blot

X

X-SCID: X-linked severe combined immunodeficiency

1 Introduction

1.1 The Immune System

Our immune system plays a crucial role in safeguarding our bodies against a range of biological threats. It does so by detecting and removing harmful pathogens, substances, and cells that have deviated from their normal functions[1,2].

The immune system can be subdivided into the innate and the adaptive immune systems. The innate system is responsible for providing general defenses against all pathogens, and the adaptive system is highly specific in targeting the pathogens that overcome the innate response[1]. Despite exerting different functions, it is imperative that they work together and that they are correctly operating in order to protect the human body[2].

1.1.1 The Innate Immune Response

The innate immune system comprises physical barriers, such as the skin and mucosa, and different cell types, like macrophages, neutrophils, and eosinophils[2].

Physical barriers are the first line of defense and can prevent the entry of pathogens[1,3]. These barriers are covered in a layer of mucus composed of antimicrobial peptides, such as defensins, that can slow or inhibit the growth or even kill pathogens[3]. However, general immune responses intervene when pathogens can overcome these defenses[2].

When the cells responsible for the innate immune system, for example, mast cells, encounter a pathogen within the body, they elicit an inflammatory response, and a variety of molecules such as histamine, prostaglandins, cytokines, and growth factors will be released. Different cell types will be recruited into the environment due to positive chemotaxis[3]. Neutrophils and macrophages

will be called into action, release pro-inflammatory signals to elicit the chemotaxis further, and phagocyte the pathogen[4]. After the phagocytoses, neutrophils will release the pathogen's antigens to the bloodstream, and macrophages, as antigen-presenting cells (APCs), will present those antigens with the help of major histocompatibility complex (MHC) II to other cells, eliciting an adaptive immune response[4–6].

Other players can complement the innate immune response, such as the Complement system. The liver is responsible for producing more than 30 proteins that compose this system, and they can be subdivided according to their functional roles, such as binding to the antigen, activating enzymes, mediators of inflammation, membrane-attack proteins, among others[5]. When these complement proteins encounter a pathogen or antibodies connected to antigens, they will become activated and trigger several pathways, converging into the final goal of eliminating the pathogen[5].

1.1.2 Adaptive Immune Response

The adaptive immune response is responsible for destroying the pathogens that overcame the first line of defense and for maintaining long-lasting and precise protection against infectious agents that have previously entered the body. This task is performed by lymphocytes (B- and T-cells) and Natural Killer (NK) cells[7].

The adaptive immune response can be divided into an antibody response (also known as humoral response), carried out by B-cells, or a cell-mediated immune response, carried out by T-cells (Figure 1.1)[3].

The antibody response protects the extracellular spaces, such as the plasma, lymph, and interstitium, from extracellular threats[8]. For B-cells to become activated, they must endure two mechanisms. First, the B-cell receptor (BCR) present in the naïve B-cell must recognize the foreign antigen, induce its internalization, and display it in MHC-II complexes[9]. Th2 T-cells, previously subjected to the same antigen, will recognize the antigen in the MHC-II and induce B-cell activation[4,9]. The activated B-cell will suffer clonal expansion of two

subpopulations: plasma cells, which are antibody-producing cells, and memory B-cells that, after an encounter with the same antigen, will differentiate into plasma cells[4]. The antibodies formed and released by the plasma cells will induce neutralization and opsonization of the foreign antigen and elicit complement activation[4,10].

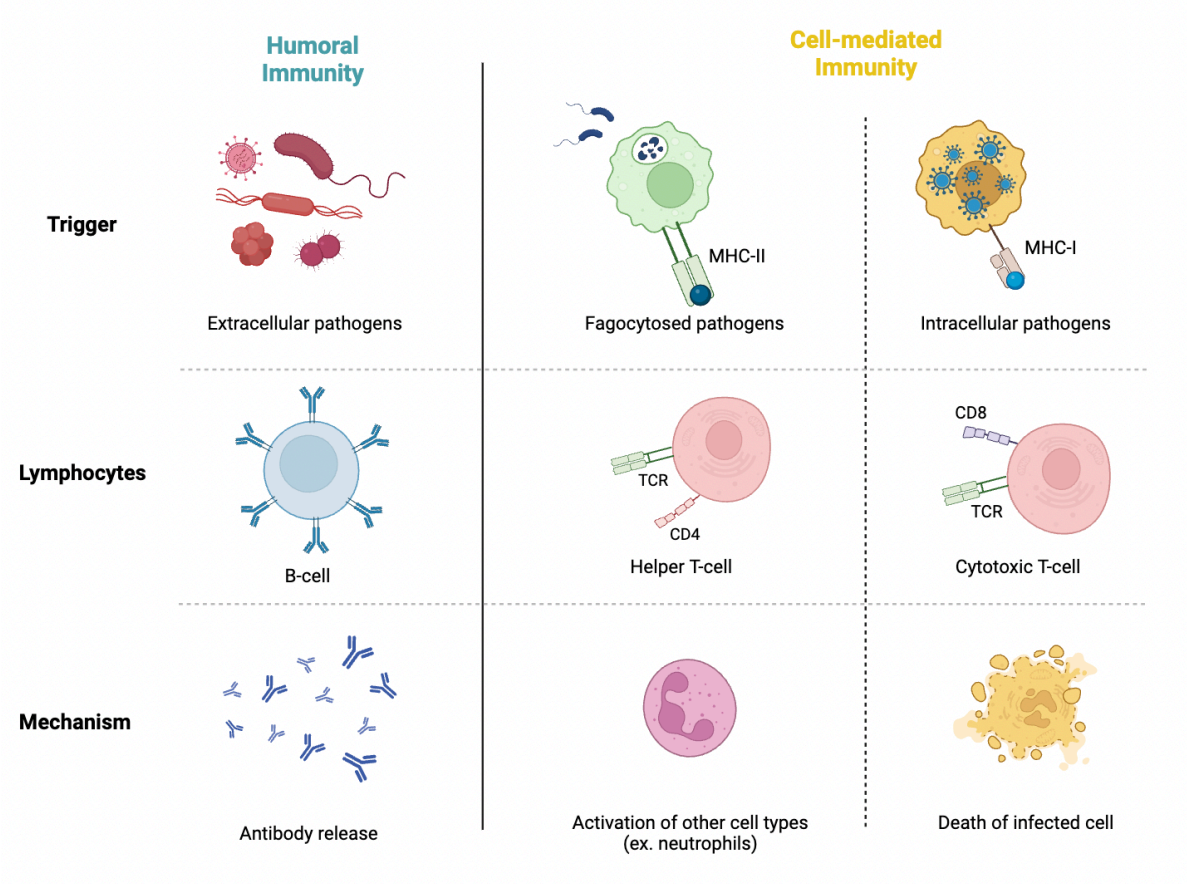

Figure 1.1. Graphical resume of the Adaptive Immune System. The adaptive immune system can be subdivided into the humoral and the cell-mediated immunity. These responses are elicited by different triggers, which result in the activation of different cell types and different response mechanisms. Humoral immunity is activated when extracellular pathogens, which display foreign antigens on their surface, activate B-cells, which will then release specific antibodies towards them. The cell-mediated response is triggered due to intracellular pathogens, resulting in the activation of helper T-cells, which will activate other cells to combat the pathogen, and cytotoxic T-cells, which will induce cell death of infected cells[10]. Figure created in BioRender.com

T-cells and APCs carry the cell-mediated response. T-cells can be divided into three types: cytotoxic T-cells ($CD8^+$), which destroy pathogen-infected cells; helper T-cells ($CD4^+$), which enhance the cell-mediated response, and the antibody response, which is subdivided into Th1 and Th2; and regulatory T-cells[10]. When an APC engulfs a pathogen, it will break down its antigens and present them in MHC-II and MHC-I molecules. Helper T-cells are able to recognize the non-self-antigen in the MHC-II and become activated. Th1 cells will enhance the activity of cytotoxic T-cells and macrophages, and Th2 cells will activate naïve B cells, as previously described. Naïve cytotoxic T-cells will interact with the MHC-I of the APC and become activated towards that specific non-self-antigen. The now-activated cytotoxic T-cell will destroy non-APC cells that display the same antigen on the MHC-I molecule[11].

As we can appreciate, T-cells play a crucial role in destroying infected cells and exacerbating various immune responses. To perform their duties, T-cells undergo a multi-step development process that ensures their proper functionality, which begins with hematopoiesis followed by T-cell development and maturation[12].

1.2 Hematopoiesis

Hematopoiesis refers to the process of production of all blood cells, starting from the embryonic phase, known as primitive hematopoiesis, and continuing throughout all lifetime, known as definitive hematopoiesis[13].

Definitive hematopoiesis begins in the bone marrow, where hematopoietic stem cells (HSCs) are present, although they can also be found in the peripheral blood. These cells are multipotent, as they can differentiate into all blood cell types through a multi-step process that involves different cellular mediators (Figure 1.2). HSCs can differentiate into either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP), which are oligopotent stem cells[14,15].

The CMP generates the myeloid lineage and can undergo four differentiation processes: thrombopoiesis, erythropoiesis, granulopoiesis, or

monocytopoiesis[16]. Each of these processes produces different cell types. For example, thrombopoiesis results in the creation of platelets, erythropoiesis produces red blood cells, granulopoiesis creates basophils, neutrophils, and eosinophils, and monocytopoiesis generates monocytes[17].

The lymphoid lineage starts with a CLP cell that has the ability to produce T-cells, B-cells, and NK cells[18]. The formation of mature cells from CLP cells is called lymphopoieses. Depending on different regulatory factors, CLPs can undergo B-cell development, which takes place in the bone marrow and leads to the production of B-cells, or T-cell development, which occurs in the thymus and results in the generation of T-cells[5].

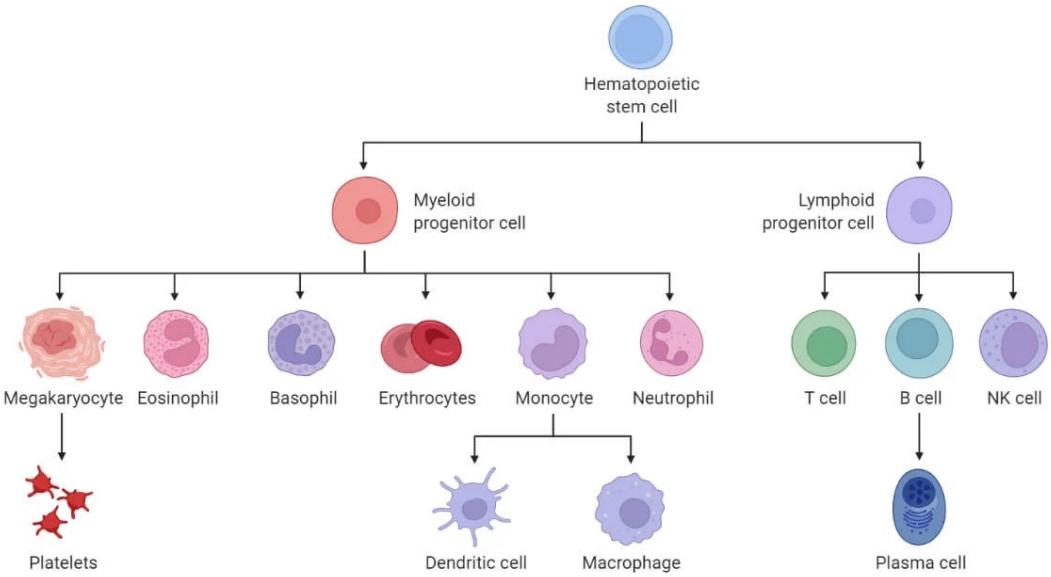

Figure 1.2. Schematic representation of Hematopoiesis. Hematopoietic stem cells are multipotent and can give rise to either the myeloid lineage, which comprises megakaryocytes, eosinophils, basophils, erythrocytes, monocytes, and neutrophils, or the lymphoid lineage, which comprises T-cells, B-cells, and Natural killer cells Adapted from Raza Y, Salman H, Luberto C. Sphingolipids in Hematopoiesis: Exploring Their Role in Lineage Commitment. Cells. 2021; 10(10):2507.

1.3 T-cell development

As previously stated, T-cell development begins in the thymus. This primary lymphoid organ exerts its maximum function during infancy and childhood and contains a vast number of developing T-cell precursors. As we age, the activity of the thymus begins to decrease and starts atrophying due to the formation of fibrous tissue[18,19]. It comprises the thymic cortex, an inner medulla, and the thymic stroma, creating the perfect environment for T-cell development[19]. The thymus secretes different chemotactic agents that attract the lymphoid progenitors, the CLPs, from the bone marrow to the thymus, where they will lose the potential for B-cell maturation and commit to the T-cell lineage[19].

The developmental stage of T-cells is characterized by the maturation status of different receptors present in their membrane: the T-cell receptor (TCR), which will suffer recombination throughout the maturation process; the cluster of differentiation 3 (CD3ε, CD3γ, CD3δ, and CD3ζ), also known as the CD3 complex; and the co-receptors CD4 and CD8, which will determine the final immunological function of the T-cell[12].

The TCR is a heterodimer composed of αTCR and βTCR chains or γTCR and δTCR chains, and mature T-cells can have only one of these combinations. This receptor is responsible for T-cell activation after the recognition of antigens present in the MHC complexes, and it is indispensable for proper T-cell function. The CD3 complex assists in the signal transduction initiated by the TCR, and it is necessary for TCR surface expression[20].

When CLPs arrive at the thymus, they become committed to the T-cell lineage due to Notch1 signaling[21]. These cells are called double negative (DN) thymocytes as they lack the expression of the co-receptors CD4 and CD8 and the TCR. The double-negative stage can be further divided into four other stages (DN1-DN4) depending on the presence of CD44 and CD25. DN1 is characterized by $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$[22]. This DN population will give rise to $\gamma\delta$ T cells and $\alpha\beta$ T cells, meaning they will have either an $\alpha\beta$ or $\gamma\delta$ TCR, which will endure different biological functions[18]. $\alpha\beta$ T-cells are responsible for mediating the cell-mediated

response of the adaptive immune system, while γδ T-cells have the ability to exert roles in both adaptive and innate systems[21, 22, 23].

In the DN2 and DN3 stages, cells start to experience gene segment rearrangements of the β-chain, γ chain, and δ chain mediated by the recombinases RAG1 and RAG2, a process that requires the interleukin 7 (IL-7) and Notch signaling pathways[24].

DN3 cells will suffer a triage, in which only cells with a robust β-chain capable of interacting with the pre-TCR α-chain and the CD3 complex and of forming the pre-TCR-complex will further maturate and proliferate, a process called β-selection[25]. Afterwards, the pre-TCR α chain suffers another set of rearrangements, generating the mature TCR. From now on, αβ cells have a functional αβ-TCR that will downregulate CD25 expression and allow the passage to the DN4 stage[18]. In the DN4 stage, cells start expressing CD8 and CD4 and are called double positive (DP) cells (Figure 1.3)[22].

Regarding the γδ T-cell lineage, only cells with a functional γδ TCR will be rescued from apoptosis at the DN3 stage. Afterwards, these cells will mature into interferon (IFN) γ producers (Tγδ1), which have roles in the eradication of intracellular pathogens and anti-tumor responses, or IL-17A producers (Tγδ17), displaying functions in the removal of extracellular pathogens[23].

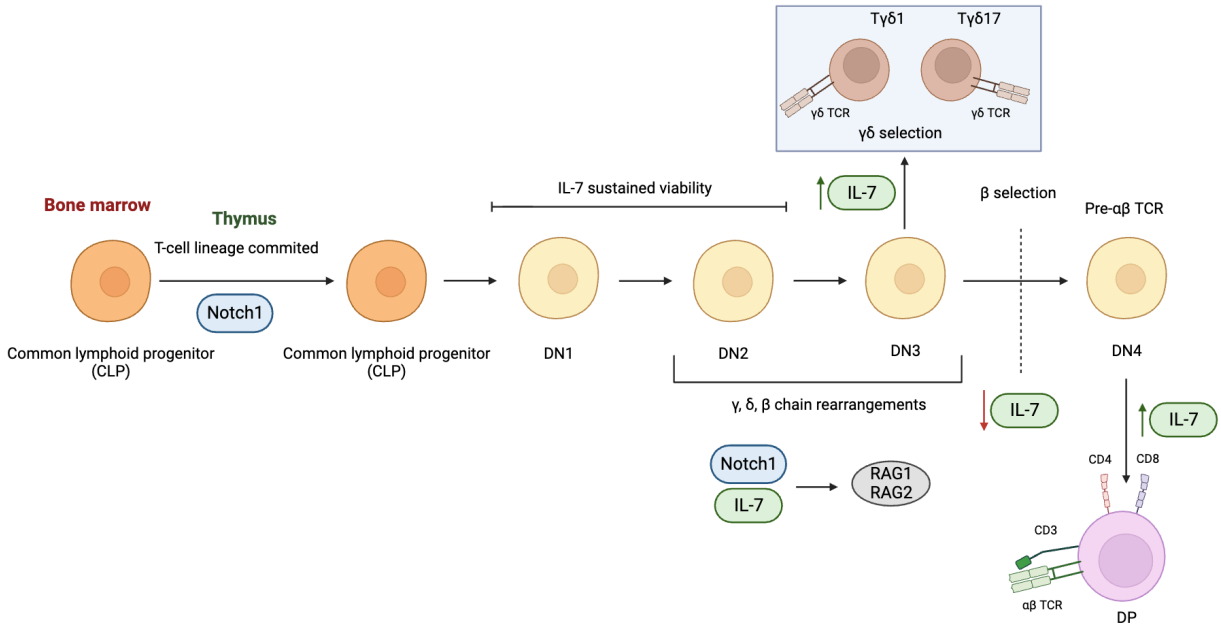

Figure 1.3. A schematic brief resume of T-cell development. T lymphocytes arise from a common lymphoid progenitor (CLP) in the bone marrow. Due to chemotaxis, this CLP enters the thymus and becomes committed to the T cell lineage in response to Notch1 signals. It enters the double-negative stage (DN) and goes through different TCR chain rearrangements due to RAG1 and RAG2 activity. At the DN3 stage, it either commits to the αβ lineage or γδ lineage, a process tightly regulated by IL-7. After the selection of correct TCR rearrangements, it further maturates. γδ T cells become either Tγδ1 or Tγδ17 cells. The αβ lineage goes into the double positive stage (DP), where they start to express the co-receptors CD4 and CD8[12]. The figure was created in BioRender.com.

In the DP stage, αβ cells pass through four processes: death by neglect, negative selection, positive selection, and lineage-specific development[22]. Death by neglect occurs when the TCR interacts poorly with the MHC molecules, reducing sustained viability intercellular signals. It is noteworthy that 90% of cells die at this stage. Negative selection induces apoptosis in cells whose TCR binds too strongly to self-ligands. Positive selection occurs when the TCR connects to the self-antigens with the suitable affinity - not too much or too little. This enables the cells to progress to their final stage of development, the lineage-specific development, and become either CD4+ (T-helper cells) or CD8+ (T-cytotoxic cells)[26]. Figure 1.4 resumes this process.

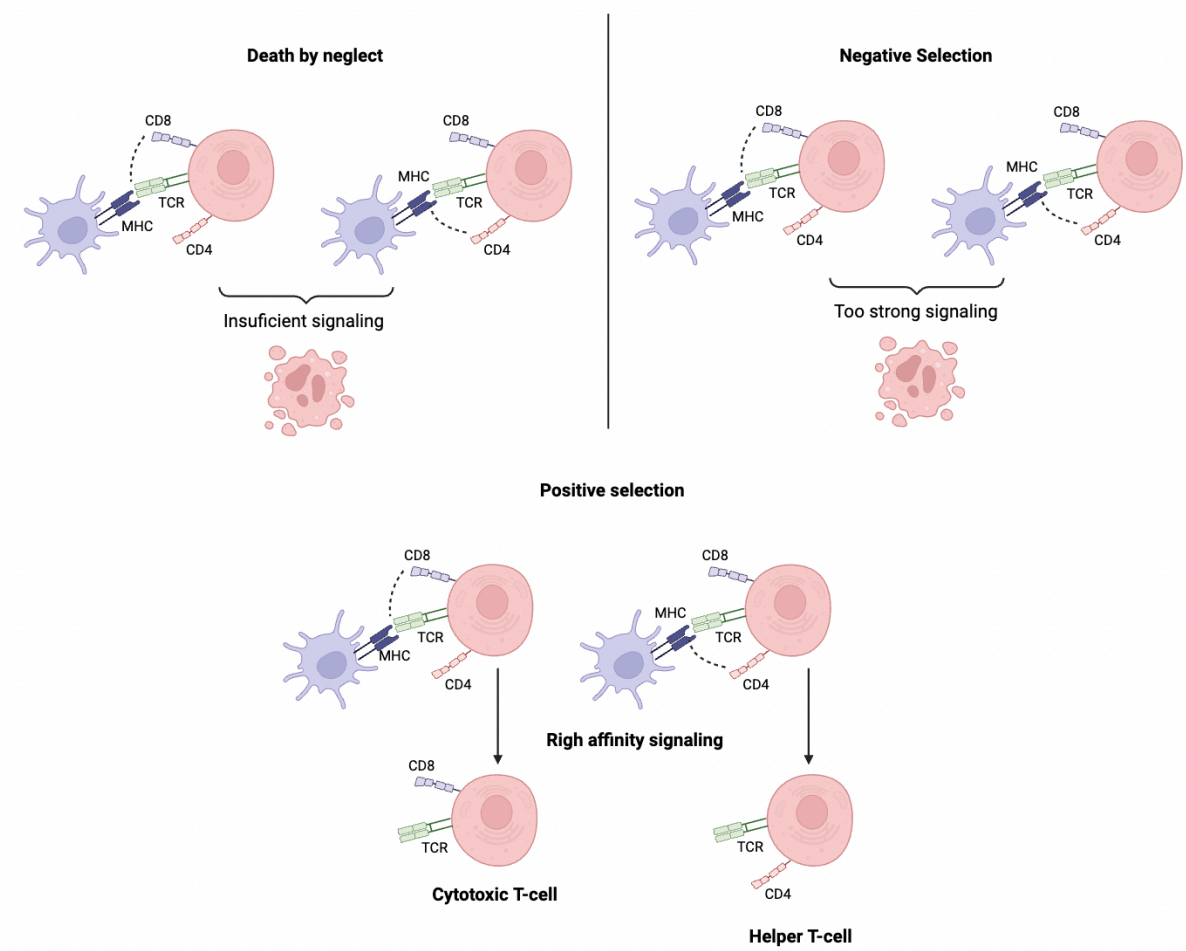

Figure 1.4. Schematic representation of double-positive cells maturation and selection process. Double-positive cells have to go through three main processes in order to become committed single-positive cells. With the assistance of cortical epithelial cells, the TCR and a co-receptor, either CD4 or CD8, will interact with the self-antigens present in the MHC complex. If the affinity is too strong, cells will die due to negative selection, and if the affinity is insufficient, cells will die due to insufficient signaling. However, when the right affinity is achieved, cells will finalize their maturation into CD4 or CD8 lineage commitment[12]. Image created in BioRender.

1.3.1 IL-7 in T-cell development

T-cell development is a tightly regulated process, which needs a careful balance between various cellular mediators, such as intrathymic cytokines. One of the most important players throughout T-cell development is the IL-7[26].

IL-7 is indispensable for the development of lymphoid cells and their maintenance, to such an extent that murine studies have shown that IL-7 deficiency results in a blockade of T- and B-cell development, characterized by the presence of non-functional and lower numbers of peripheral T- and B-cells, respectively [27].

The expression of IL-7 is found in different organs, such as the intestine, liver, skin, and lungs. However, its highest expression is in the lymphoid organs, such as the thymus and the lymph nodes, where it's produced by the stromal cells[28]. Moreover, patients with Severe Combined Immunodeficiency (SCID) lack T-cells, which can be attributed to alterations and mutations in the IL-7 pathway, such as those found in the IL-7 receptor (IL-7R) α or common γ chains[29].

At the DN2 stage, IL-7 is essential for cell proliferation and differentiation into the D3 stage[26]. Studies have demonstrated that cells deficient in IL-7 signaling are usually arrested at the β-selection stage, a checkpoint present at the DN3 stage[26]. Moreover, the differentiation into either $\gamma\delta$ or $\alpha\beta$ T cells, which occurs also at the DN3 stage, is dependent on the strength of the signals produced by the IL-7 pathway, with a greater impact on the development of $\gamma\delta$ T-cells[30]. Studies involving mice deficient in IL-7 at the DN3 stage have demonstrated a complete absence of $\gamma\delta$ T-cells[26,28,31]. In addition, IL-7 signaling was shown to inhibit the T cell factor-1 (TCF-1) transcription factor, known to be necessary for $\alpha\beta$ T cell lineage progression, and thus, it is not unsurprising that these cells require reduced IL-7 signaling in order to continue their development[26,32]

At the DN4 stage, the expression of the IL-7R is down-regulated by the suppressor of cytokine signaling 1 (SOCS1) protein. Later on, when DN4 cells differentiate into the DP stage and overcome the positive selection checkpoint, they re-express the IL-7R due to the downregulation of SOCS1, which will induce IL-7-dependent survival signals[26].

Studies have also reported that IL-7 plays a role in the CD4/CD8 lineage decision. For T-cells to differentiate into CD4+, they need persistent TCR signaling, which will override the signals induced by intrathymic cytokines. The presence of low TCR signals allows the cells to be signaled instead by intrathymic cytokines, including IL-7, that will induce $CD8^+$ differentiation. After T-cell development, IL-7 activity remains necessary for their long-term maintenance[33].

1.4 T-cell Acute Lymphoblastic Leukemia

Throughout Chapter 1.3, we can observe how T-cell development is a highly regulated multi-step process that requires the action of several cellular components. Any dysregulation that affects this differentiation process can potentially result in T-cell malignancies, such as leukemia[34]. One of the most common malignancies affecting the T-cell lineage is T-cell acute lymphoblastic leukemia (T-ALL)[34].

Leukemia is a malignant condition marked by the excessive production of abnormal or immature leukocytes, leading to a disruption in the production of healthy blood cells. This condition can be broadly classified into two categories based on the infiltration status of the leukemic cells in the peripheral blood and bone marrow: acute or chronic, with the former exhibiting the highest level of immature cell infiltration. Additionally, leukemia can also be characterized by the lineage of the leukemic cells, being accordantly either defined as myeloid or lymphoid leukemia[35].

Acute lymphoblastic leukemia (ALL) is a hematological neoplasm characterized by the malignant transformation and prompt proliferation of lymphoid progenitor cells due to a blockage in the lymphoid differentiation process[36]. ALL is considered the most prevalent childhood malignancy, representing about 80% of leukemia diagnoses in children and 20% of adults[34].

ALL can be subdivided into T-cell acute Lymphoblastic Leukemia or B-cell Acute Lymphoblastic Leukemia (B-ALL), depending on if it is derived from B- or T-lymphoid precursors[37]. T-ALL represents 15% of ALL cases in children and 25% in adults, and it is estimated that in 2023, 6540 people of all ages will be diagnosed with ALL just in the United States[38].

1.4.1 T-ALL classification and treatment

T-ALL can be subdivided into pro-T, pre-T/immature, cortical T, mature-T, and early T-cell precursor (ETP-ALL)[39]. This classification is established on immunophenotype, morphology, and genetic alterations[40]. ETP-ALL exhibits the

highest level of genomic instability and the lowest prognosis when treated with standard therapeutic approaches [40]. Additionally, this subtype is associated with significantly higher relapse rates, with a striking 72% relapse rate at the ten-year mark post-diagnosis, in contrast to the 10% seen for non-ETP subtypes[34]. Clinically, these patients present high levels of white blood cells, anemia, neutropenia, and thrombocytopenia. Moreover, they also suffer from mediastinal thymic masses of immature T-cells and meningeal infiltration of the central nervous system[41].

The survival rates without experiencing an event over five years, also known as event-free survival (EFS), have demonstrated a significant increase over the years [39]. This has been attributed to the advancements in therapeutic regimens, resulting in an EFS rate of 85% among children and 20-40% in adults, a noteworthy development in medicine[34]. However, these patients must be subject to multi-agent chemotherapeutic regimens, often coupled with corticosteroids for two to three years, which is a highly toxic therapeutic scheme[34,42]. Acute side effects include allergic reactions, pancreatitis, and neurologic impairment. Regarding chronic side effects, there can be neurocognitive deficits, obesity, osteoporosis, and others [43].

The multi-agent chemotherapeutic regimens are composed of different drug combinations, such as vincristine, a vinca alkaloid which can disrupt microtubule function, causing impairment in mitosis[44]; dexamethasone or prednisone, a type of steroid that interacts with the glucocorticoid receptor mediating anti-inflammatory and immunosuppressive responses[45]; and an anthracycline drug, which can disrupt the DNA due to their topoisomerase-poisoning properties leading to cell death[46].

The therapeutic scheme is divided into three phases, the first called induction, which aims at inducing leukemia remission. The second is the consolidation phase, which, making use of a more intense therapeutic approach, aims at reducing the persisting leukemia cells. Stem cell transplantation can also be performed at this phase and is coupled with radiation. The last phase is maintenance, where drugs like methotrexate, an antineoplastic drug able to inhibit nucleotide synthesis-responsible enzymes[47] and 6-mercaptopurine, are used for about two years[48].

One of the primary concerns related with T-ALL is the significantly high rates of relapse. Approximately 20% of pediatric patients and 40% of adult patients relapse after treatment [38,49]. Such patients tend to develop resistance to the chemotherapy and steroids used during treatment, leading to a dismal prognosis. Unfortunately, no therapies have proven effective in these cases [50]. Therefore, a deeper understanding of the genetic alterations of this malignancy is essential to serve these patients better.

1.4.2 Genetic alterations in T-ALL

T-ALL, like other types of cancer, is caused by genetic alterations, which have been extensively studied and documented. The pathways most frequently affected by genetic alterations include the NOTCH, Phosphatidylinositol 3-kinase (PI3K), and the small GTPase rat sarcoma virus (RAS) pathways, which results in hyperactivated signaling[34].

1.4.2.1 Notch pathway

One of the most acknowledged genetic alterations in T-ALL are *NOTCH1* activating mutations, which are present in 60% of T-ALL patients[51]. As discussed above, Notch signaling is very important throughout T-cell development for proliferation and survival[51]. These genetic alterations result in conformational changes that facilitate the ligand-independent proteolysis of the receptor's intracellular domain (NICD), a transcriptional factor, or impair its degradation, promoting constitutive activation of the pathway and gene transcription[52].

Characterization of these alterations allowed the identification of several NOTCH1-related genes capable of inducing the proliferation and malignant transformation of T-cells or their precursors, such as *MYC* and the *IL7R* [52].

There is also an important crosstalk between the Notch1 and IL-7 signaling pathways, namely, Notch1 can transcriptionally upregulate the expression of IL-7Rα, a subunit of the IL-7R. Moreover, the involvement of IL-7 in Notch-mediated

leukemia cell maintenance has also been identified[24]. More precisely, Notch1 promotes IL-7R transcription by binding to his gene promotor[53].

As Notch pathway alterations are amongst the most common genetic abnormalities in T-ALL, several studies and clinical trials have evaluated the capacity to target this pathway. One of the most studied approaches is the use of γ secretase inhibitors (GSIs), a protein complex responsible for NOTCH receptor cleavage, which induces pathway activation[40]. These molecules have demonstrated the ability to reduce endogenous NICD levels, cell growth, and cause cell cycle arrest in T-ALL cell lines [54]. While promising in *in vitro* and *in vivo* models, clinical trials have revealed high gastrointestinal toxicity[55]. Nonetheless, these molecules are still under investigation with lower dosages and with intermittent therapeutic schemes, as a study demonstrated that this new approach was able to reduce toxicity[56]. Moreover, the capacity to use these inhibitors in combination with other drugs, such as glucocorticoids, is also under investigation[40,57].

1.4.2.2 PI3K/AKT/mTOR pathway

PI3K pathway is also frequently mutated in T-ALL. In fact, about 85% of T-ALL patients have this pathway activated[58]. In a significant number of patients, precisely 5-30%, non-sense or frame-shift mutations are present in the phosphatase and tensin homolog (PTEN), a vital inhibitor of this pathway. Furthermore, it was also shown that the protein casein kinase 2 (CK2) is frequently overexpressed in T-ALL and can induce PTEN inhibition[59]. These genetic alterations cause hyperactivation of the pathway, which sustains leukemic cell maintenance[41].

In T-ALL, resistance to glucocorticoids is also primarily associated with the activation of the PI3K pathway. More precisely, the v-akt murine thymoma viral oncogene homolog/Protein kinase B (AKT/PKB) can phosphorylate the glucocorticoid receptor, reducing nuclear localization and decreasing target-gene activation. Additionally, the mammalian target of rapamycin protein

(mTOR) can upregulate the expression of the anti-apoptotic protein Mcl-1, thus inhibiting glucocorticoid-induced apoptosis[41].

PI3K pathway inhibitors have been tested in T-ALL. Rapamycin is a mTOR inhibitor that has been extensively studied in the context of T-ALL[60]. Research indicates that rapamycin is able to decrease T-ALL proliferation and cell cycle progression[60]. Despite the promising results for rapamycin, studies are trying to focus on dual PI3K/AKT/mTOR pathway inhibitors, such as PKI-587, as targeting more than one player might be more advantageous. This inhibitor was demonstrated to be the most selective drug in inducing T-ALL apoptosis in a panel of 88 drugs tested in both *in vitro* and *in vivo* models[58].

1.4.2.3 RAS/MEK/ERK pathway

The RAS protein from the RAS/MEK/ERK pathway requires conformational changes to be activated. In T-ALL, *HRAS* mutations frequently result in the protein being arrested in its active state, translating into a hyperactivated pathway commonly associated with the PI3K/AKT/mTOR[59]. Approximately 50% of T-ALL cases exhibit increased RAS/MEK/ERK signaling, which is more pronounced in relapsed patients and those resistant to steroids[61].

Although there have been numerous studies on RAS-targeted therapies for various types of cancer, there are currently no Food and Drug Administration (FDA)-approved drugs available. Inhibiting the RAS protein is highly challenging due to the substantial amount of its substrate, guanosine triphosphate (GTP), and the strong affinity between these two proteins[62]. This has limited the number of studies evaluating the therapeutic potential of targeting the RAS/MEK/ERK pathway in T-ALL.

1.4.3 IL-7/IL-7R pathway

As discussed above, the IL-7 signaling pathway has significant roles during T-cell development, and its proper function is indispensable for maintaining immune system homeostasis[26,28]. Thus, it is not surprising that

deregulation of this pathway also plays a significant role in T-ALL, being among the most commonly altered pathways[41].

1.4.3.1 IL-7/IL-7R signaling cascade

IL-7 accomplishes its biological role via its receptor. The IL-7R comprises two subunits: the IL-7Rα chain and the common γ chain. These receptor chains require the presence of Janus kinases (JAKs) for activating their molecular targets since they don't have intrinsic catalytic activity[63]. The IL-7Rα chain couples with JAK1, and the common γ chain couples with JAK3[28]. These receptor chains heterodimerize upon the binding of the interleukin, causing the auto-phosphorylation of tyrosine residues in JAK1 and JAK3, leading to their activation[24]. JAKs will then phosphorylate tyrosine residues within the intercellular portion of the corresponding chains of the receptor, creating docking sites for different proteins, such as signal transducer and activator of transcription (STAT) 5 and PI3K[28].

Upon binding to the receptor chains, STAT5 is phosphorylated and activated by the JAKs, which results in homodimerization. STAT5, as a transcriptional factor, will then enter the nucleus and induce transcription of different genes involved in survival, proliferation, and differentiation[26]. For instance, different proteins of the anti-apoptotic protein family of BCL-2 are upregulated, and pro-apoptotic proteins such as BAX and BAD are downregulated (Figure 1.5)[64].

As with any other signaling pathway, IL-7 has the ability to activate different pathways, generating various types of crosstalk. Through its IL-7R/JAK1/JAK3 complex, it can phosphorylate the p85 subunit of PI3K and consequently activate this pathway, which collaborates with AKT, converging in cell cycle progression[65]. There is also a crosstalk between IL-7 and the MAPK pathway (Figure 1.6)[28].

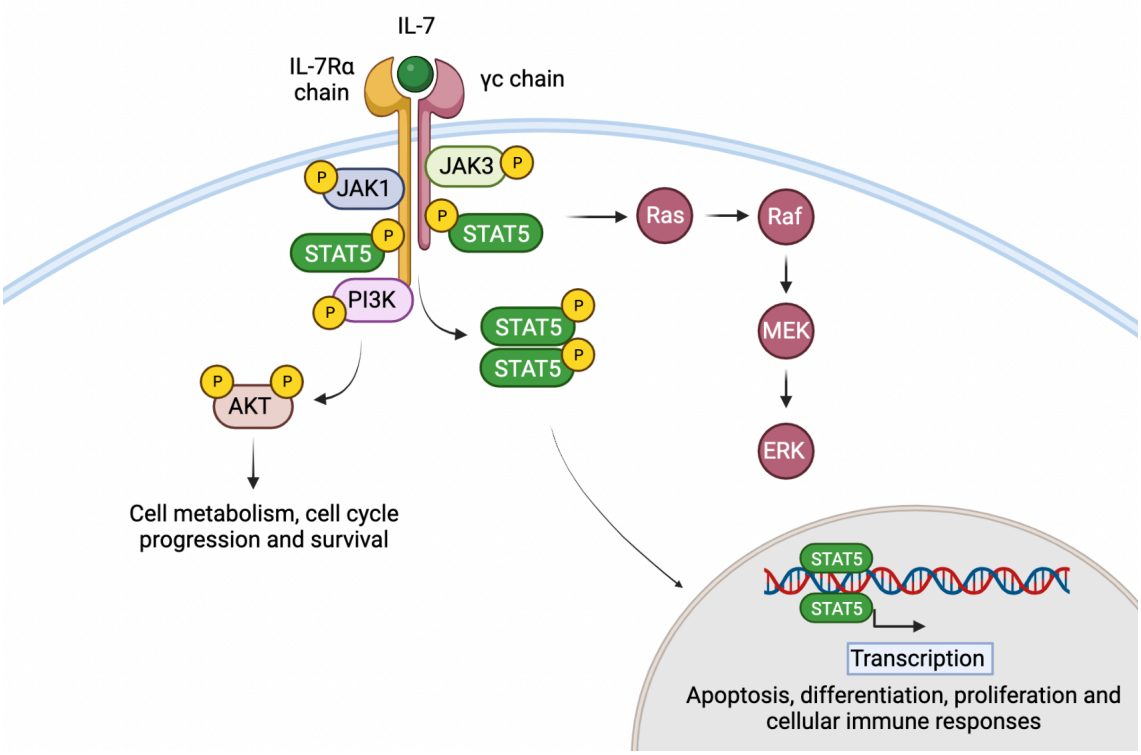

Figure 1.5. IL-7/IL-7R signaling cascade. IL-7 induces receptor heterodimerization via the IL-7Rα and common γ chain. JAK1, coupled to the IL-7Rα chain, and JAK3, coupled to the common γ chain, auto-phosphorylate each other and the respective receptor chains. The phosphorylated tyrosines in the receptor create docking sites for STAT5. The JAKs activate STAT5 by inducing the phosphorylation of tyrosine residues, leading to its homodimerization and translocation into the nucleus, where it will induce gene transcription. The phosphorylated tyrosine residues in the receptor are also docking sites for PI3K, which will result in the activation of the PI3K/AKT/mTOR pathway[24]. Image created in BioRender.com

Activation of the IL-7 signaling pathway induces receptor internalization and decreases expression of the *IL7R* gene[66]. Like other cytokine receptors (including IL-2R and IL-5R), the internalization of the IL-7Rα chain is required for effective signal transduction[67]. According to the "altruistic model", T-cells internalize the IL-7R and reduce the transcript expression to make the interleukin available to other cells that have not benefited from it[24]. This occurs in order to maximize the number of T-cells that benefit from the IL-7-induced survival signals[68]. The preferred internalization route utilized is via clathrin-dependent endocytosis, and a considerable portion of the internalized receptors are degraded by lysosomes and proteosomes in a JAK3-dependent manner[66]. JAK3 inhibition impairs IL-7-induced IL-7Rα degradation but not the internalization process[66]. Curiously, it has also been shown that even though the receptor is internalized, the overall surface levels remain constant, suggesting a recycling route[66].

1.4.3.2 IL-7/IL-7R signaling in T-ALL

Studies show that more than 70% of T-ALL patients have IL-7R^{+} blasts, which correlates to their capacity to respond to IL-7 *in vitro*.[24] Moreover, approximately 10% of T-ALL patients have a gain-of-function mutation in the *IL7R* gene, which is considered a dominant oncogene[69].

There are three types of mutations that affect the IL-7Rα chain in T-ALL (Figure 1.6). The first type, 1a, involves the homodimerization of the chains due to the formation of disulfide bonds between cysteine residues. This leads to constant pathway activation. The second type, 1b, involves the formation of a hydrogen bond between two serine and two glycine residues, resulting in the homodimerization of IL-7Rα chains and constant signaling. The third type, 1c, involves the interaction between a negatively charged residue and a positively charged one, facilitating the heterodimerization of the IL-7Rα and common γ chain, increasing IL-7 sensitivity[28].

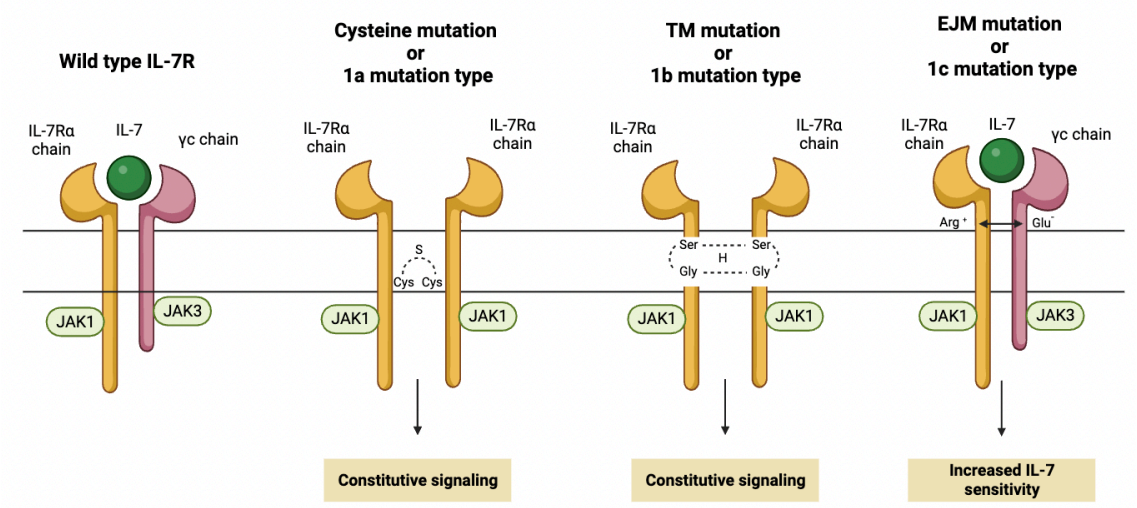

Figure 1.6. Representative scheme of the IL-7R mutations described in T-ALL. IL-7R mutations can be subdivided into three types. The cysteine mutation occurs when two unpaired cysteine residues create disulfide bonds in two IL-7Rα chains, resulting in homodimerization and constitutive pathway activation without the need for the interleukin. The transmembrane (TM) mutation occurs when there is a hydrogen bond between two serine and two glycine residues, which results in homodimerization of the IL-7Rα chains and constitutive signaling without the requirement of IL-7. The extracellular juxtamembrane (EJM) mutations occur when a positively charged residue and a negatively charged one interact and facilitate the heterodimerization of the receptor, resulting in increased IL-7 sensitivity[71]. Image created in BioRender.com

Besides IL-7Rα mutations in T-ALL, JAK1, JAK3, and STAT5 alterations have also been described as affecting IL-7 signaling.

JAK1 mutations are somatic and usually of gain-of-function type. In adults, they are present in about 18% of the cases and 2% in children. These mutations are found within all the different domains of the protein and converge in inducing STAT5 activation. Moreover, these alterations are more prevalent in older patients with poor response to therapy[70].

JAK3 mutations are present in 8% of pediatric and 12% of adult cases and are usually associated with JAK1 mutations[72]. One of the most acknowledged alterations is the M511I mutation. A study showed that transplanting T-ALL cells with this mutation in mice caused lymphoproliferative disease and accumulation of CD8+ immature T cells in the blood and hematopoietic tissues. Curiously, when transplanted into a secondary recipient mouse, these cells demonstrated new mutations in *Notch1, Pten, Kras*, and other genes[73].

STAT5 mutations have also been associated with T-ALL, including the most common STAT5 alteration (N642H) that alters the binding pocket of the SH2 domain, which leads to constitutive STAT5B phosphorylation[59,74].

Our lab and others have demonstrated how this pathway can affect and contribute to T-ALL in several ways. As previously noted, IL-7R mutations can lead to constitutive pathway activation and IL-7 hypersensitivity. However, even the wild-type IL-7/IL-7R signaling can be oncogenic. More precisely, studies conducted in transgenic mice showed that overexpression of the receptor results in increased proliferation of T-cell precursors, ultimately leading to leukemia's development[75]. Of note, several genetic alterations can also upregulate IL-7R expression, such as Notch mutations and ZEB2 translocations, leading to IL-7 hypersensitivity[24].

IL-7 by itself has been shown to stimulate T-ALL cell proliferation and survival *in vitro*, including when produced by bone marrow or thymic stroma[24,28.] Moreover, studies *in vivo* using xenotransplant models of T-ALL have also demonstrated that IL-7 promotes T-ALL development[69]. This increase in proliferation and survival induced by IL-7 in T-ALL cells results from the activation of the PI3K/AKT/mTOR pathway downstream of IL-7 signaling. The crosstalk

between these two pathways leads to the up-regulation of Bcl-2 expression and to the downregulation of the cyclin-dependent kinase inhibitor p27 ($p27^{Kip1}$), which is STAT5 independent[76]. Curiously, IL-7 activation and subsequent PI3K/AKT/mTOR pathway activation in healthy T-cells does not regulate Bcl-2 expression or cell survival (Figure 1.7). This is interesting because while PI3K or mTOR inhibitors have a cytostatic effect on healthy T-cells, they have a cytotoxic effect on T-ALL cells by impairing their viability[24].

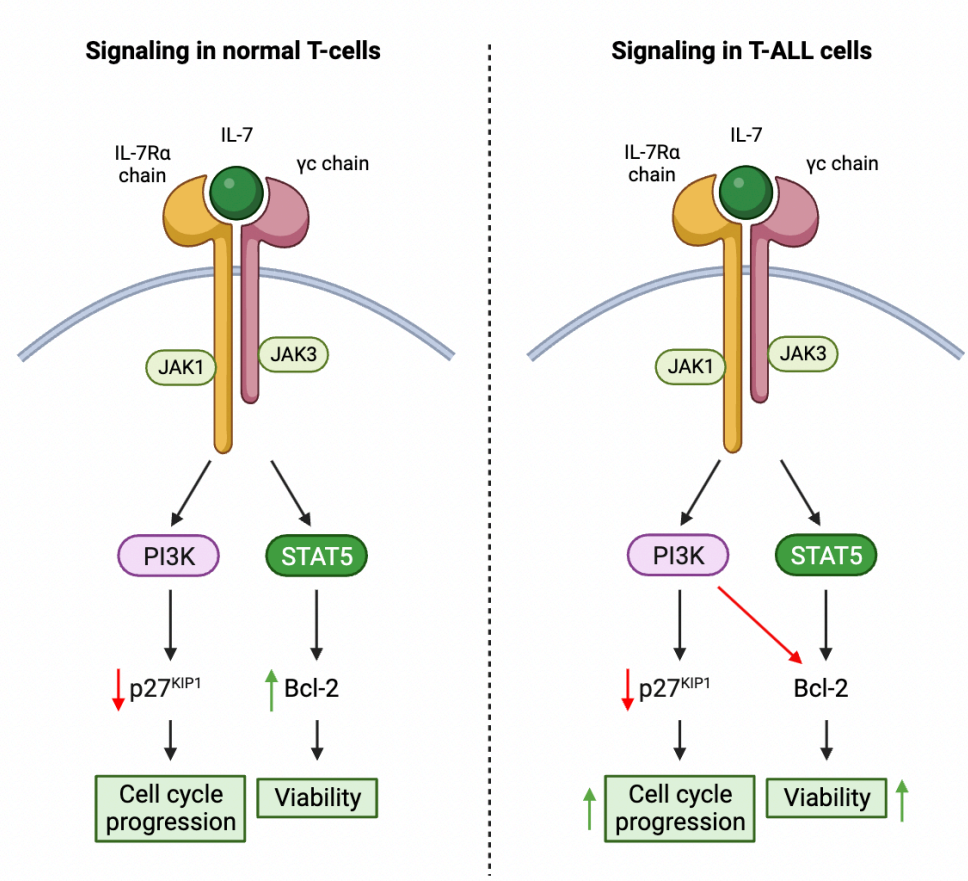

Figure 1.7. IL-7 signaling in normal T-cells versus IL-7 signaling in T-ALL cells. IL-7 induces the activation of STAT5, which increases the expression of the anti-apoptotic protein Bcl-2, increasing viability. In physiological conditions, IL-7 also inhibits the cell cycle inhibitory protein $p27^{Kip1}$ via PI3K pathway activation, ultimately leading to cell cycle progression. In T-ALL cells, IL-7 induces the same signaling processes, however, the PI3K pathway can also induce Bcl-2 activity, which leads to exacerbated viability and cell cycle progression[28].

In order to improve our understanding regarding the IL-7/IL-7R pathway, our lab has been trying to establishing the IL-7/IL-7R signaling landscape in health and in malignancy. Using Reverse Phase Protein Array (RPPA) to

characterize IL-7-induced changes in the phosphorylation status of proteins in T-ALL, we have been able to identified changes in proteins not yet associated with the IL-7/IL-7R signaling cascade. Interestingly, one of these belongs to the Janus kinase family, and it is known as Tyrosine Kinase 2 (TYK2). The next chapter will address the different interactions between Janus Kinases and their properties, including TYK2.

1.5 Janus Kinases

Janus Kinases are key players in several signaling pathways with important roles in T-cell development and T-cell functions, including in the IL-7 signaling pathway. JAKs are the first players to start signal transduction in JAK/STAT pathways [63,77].

JAKs are tyrosine kinases that are non-covalently associated with the cytoplasmatic domain of Type I and Type II cytokine receptors since these lack intrinsic catalytic activity[78]. These kinases work with the associated receptors to induce the signal transduction from different cytokine superfamily proteins, including interleukins, chemokines, growth factors, IFN, and tumor necrosis factor (TNF) family members, as shown in Figure 1.9[79]. These messengers play a crucial role in differentiation, growth, and development. Additionally, interleukins and cytokines activate both immune and non-immune cells, making them essential for the proper functioning of the immune system[80].

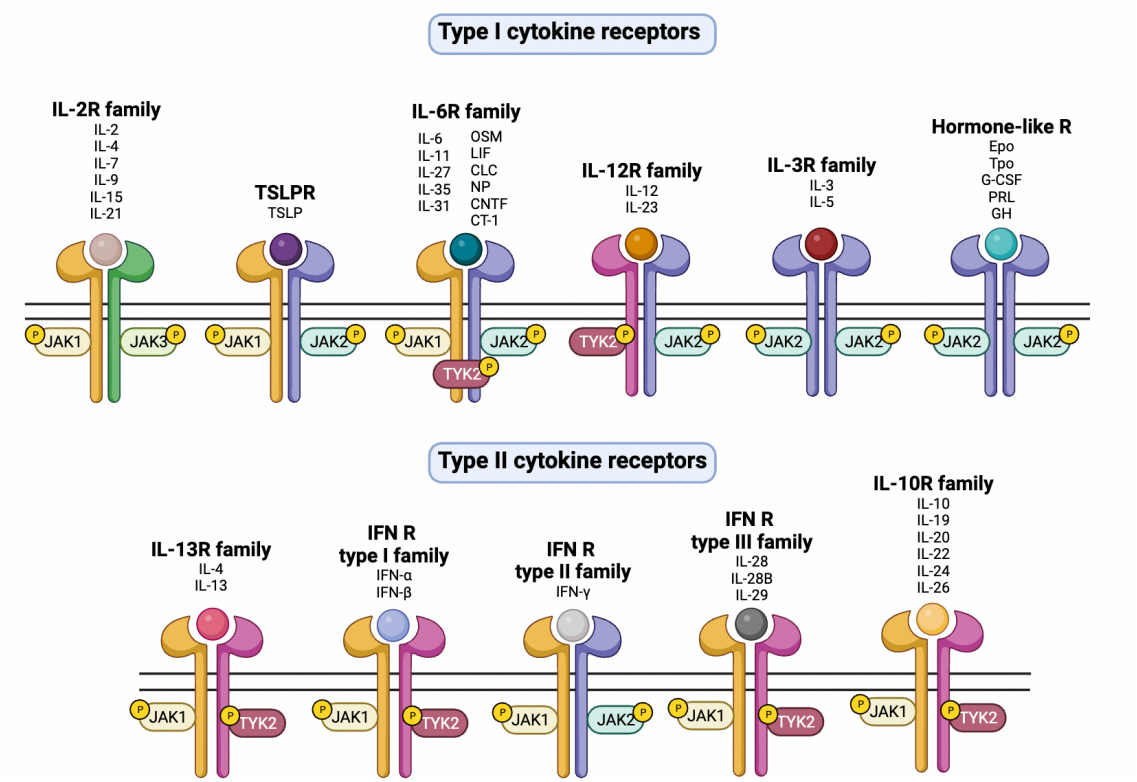

Figure 1.8. Type I and type II cytokine receptors and their associated JAKs. The different cytokine families rely on different combinations of receptor chains and their associated Janus Kinases in order to perform their biological activities[63].
Figure created in BioRender.com

In mammals, there are four known JAKs: JAK1, JAK2, and TYK2, which are ubiquitously expressed, and JAK3, primarily present in the lymphatic system, bone marrow, vascular smooth cells, and endothelial cells[72]. These proteins are composed of seven homology domains (JH) distributed in four different main domains represented in Figure 1.9, and their functional properties are explained in Table 1.1[77].

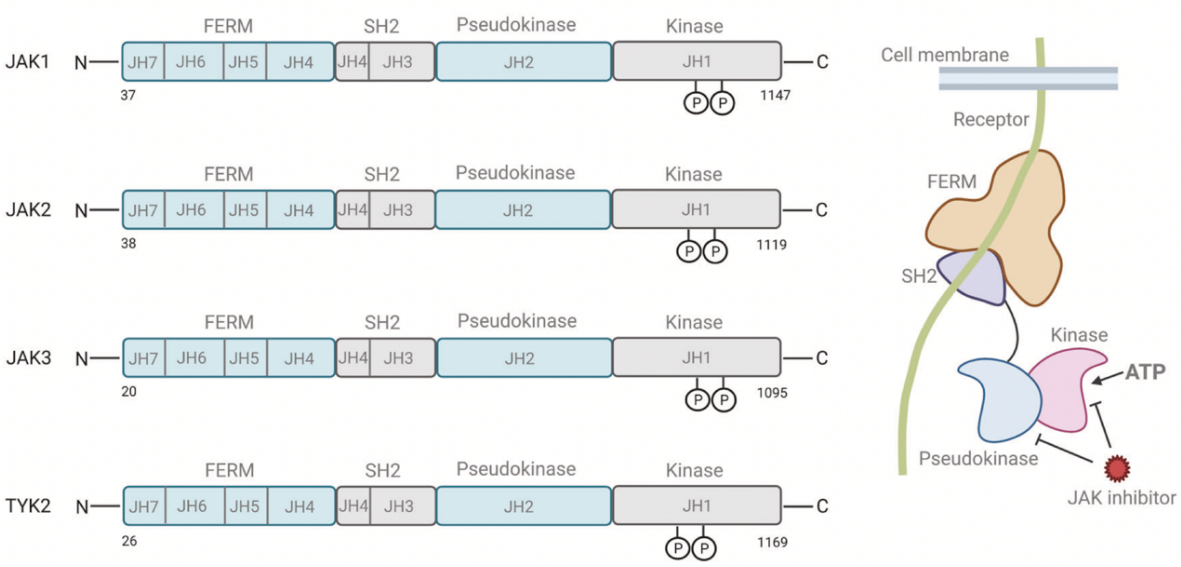

Figure 1.9. Structure of the different JAK family members. Janus kinases have a highly similar structure, comprising four different domains: the FERM domain, the SH2 domain, the pseudokinase domain and kinase domain. Within these main domains JAKs are further subdivided into seven different homology domains. JAKs catalytic activity resides in their pseudokinase and kinase domains.
Adapted from Hu, X., Li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. Signal transduction and targeted therapy, 6(1), 402.

Table 1.1. The different domains and functional properties of the JAK family members.

Domain	Homology Domain	Function
Four-point- one, ezrin, radixin, moesin (FERM)	JH7-JH4	Regulates the connection to the receptor;
Src-homology 2 (SH2)	JH4, JH3	
Pseudokinase	JH2	Regulates the activity of the kinase domain;
Kinase	JH1	Protein kinase functionally capable of phosphorylating a substrate.

JAK/STAT signaling pathways are activated all in the same fashion. Upon the binding of a cytokine to its respective receptor, the associated chains are brought into close proximity and undergo reorientation, allowing for JAKs to trans- or autophosphorylate one another. This process ultimately leads to the activation of their kinase domain[81]. Following activation, JAKs proceed to phosphorylate tyrosine residues located within the C-terminal tails of the receptor chains. This

phosphorylation event facilitates the binding of STAT proteins, including STAT1-4, STAT5a/STAT5b, and STAT6, by means of their SH2 domain[81]. Finally, JAKs will phosphorylate the corresponding STAT within their C-terminal and activate them, allowing the STATs to form hetero- or homodimers due to the connection of the phospho-tyrosines [81].

In order to provide tight regulation of these signaling pathways, STATs induce the expression of different negative regulators of the pathway. For instance, SOCS proteins (SOCS1-SOCS7) contain a kinase-inhibitory region that can directly inhibit JAKs, leading to their deactivation[82]. Another example is the cytokine-inducible SH2-domain (CIS) proteins, which can physically block the binding of STAT proteins to the SH2 domain[63]. Proteasomal degradation can be induced by both types of proteins. Moreover, phosphatases like Src homology region 2 domain-containing phosphatase-1 (SHP-1), CD45, and Protein tyrosine phosphatase 1B (PTB-1B) are also in the regulatory process. Regarding STAT inactivation, these can be sumoylated by the protein inhibitor of activated STAT (PIAS), leading to their degradation[82] .

Besides their kinase function, JAKs are also able to display other roles, such as stabilize each other and their corresponding receptors in the cell membrane[83–86].

Growing evidence shows that the deregulation of JAKs' functions can potentially lead to hematological malignancies [72,87]. These alterations can arise from different mechanisms, such as aberrant cytokine signaling, activating point mutations, and gene translocations[50]. According to the literature, approximately 140 JAK mutations are associated with different types of leukemia[72]**.** Interestingly, one of the main mutational hotspots for activating mutations is the JH2 domain, also known as the regulatory region for protein kinase activity [72]. Studies have also shown that the FERM domain can inhibit JAKs' functions, and, unsurprisingly, mutations within this domain leading to its defective activity have also been associated with leukemia[72]. The JH1 domain has also been reported to suffer different activating mutations[72].

1.5.1 JAK1

JAK1 participates in signaling transduction for Type I and Type II cytokine receptors and can pair with JAK2, JAK3, and TYK2, resulting in significant roles in immunity, inflammation, and lymphoid cell maturation[79].

Within the Type I cytokine receptors that require JAK1 are the IL-2 receptor family and the IL-6 family[79]. The IL-2 family comprises IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and works with the common gamma chain, restrictedly associated with JAK3[63]. Most of these cytokines have roles in lymphocyte survival, differentiation, proliferation, and activation[79]. JAK1 then allies with the corresponding alpha chain subunit, IL-2Rα, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, and IL-21R, respectively[88]. JAK1 is also present in the Thymic stromal lymphopoietin (TSLP) signaling, where it associates with the IL-7Rα and JAK2 with the TSLP receptor[78].

The IL-6 family, differently from the IL-2 family, works with the gp130 subunit associated with JAK1 and the corresponding α chain with JAK2. These include IL-6, L-31, IL-11, IL-27, IL-35, and different growth factors[79]. TYK2 is also associated with these complexes. However, its biological role is still uncertain[79].

Within the type II cytokine receptors that utilize JAK1 is the IL-10R that couples with TYK2, and it is used by the IL-10 family, comprising IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26[78]. These play significant roles in tissue homeostasis during inflammation and infection[89]. In the same category as Type II cytokine receptors are Type I (IFN-α/β) and III IFNs (IL-28, IL-28B, and IL-29), which are responsible for anti-viral and immune responses, both receptor types coupling with TYK2[63]. The final receptor is the II IFN (IFN-γ) receptor, which couples with JAK2 and exerts anti-tumoral responses [79].

As we can appreciate, JAK1 has multiple roles regarding cytokine signaling, so it is not surprising that biological alterations can lead to pathogenesis. Studies have shown that JAK1 is crucial for embryonic development since JAK1 knockdown in mice results in perinatal lethality, reduced numbers of thymocytes, and of mature T- and B-cells[79,90]. Moreover, JAK1 also exerts major roles in protection against infections, microbial responses, and tumor surveillance[91].

1.5.2 JAK2

JAK2 exerts its role within the type I and II cytokine receptors. Regarding type I, JAK2 couples with TYK2 in the IL-12R family and in the IL-3R family.[79] The interaction between JAK2 and JAK1 in type II cytokine receptors has been described in Chapter 1.5.1.

The IL-12R family, composed of IL-12, IL-23, and granulocyte-macrophage colony-stimulating factor (GM-CSF), displays roles in the regulation of effector cells and the development/ function of hematopoietic cells[92]. The IL-3R family (IL-3 and IL-5) exerts roles in inflammatory and immune responses [93].

JAK2 is also able to homodimerize in Hormone-like receptors, which are used by erythropoietin (Epo), thyroid peroxidase, granulocyte-colony stimulating factor (G-CSF), prolactin and growth hormone (GH) [79,94].

Studies with mice revealed that JAK2 knockout impairs erythropoiesis and leads to embryonic death [95, 91]. Although functional or deletional mutations have not been described in humans[95], JAK2 has been associated with hematological malignancies as a result of gain-of-function mutations and gene translocations[72]. JAK2 fusion proteins such as PCM1-JAK2, BCR-JAK2 and PAX5-JAK2 have been described in acute leukemia, promoting JAK2 dimerization and constitutive activation[87]. Moreover, JAK2 activation mutations are also correlated with myeloproliferative neoplasms[72].

JAK2 has also been proven to have a scaffold function. In the IFN-γ pathway, JAK2, using JAK1 as a cooperator, acts as a scaffold for the heteromeric IFNGR cellular localization[97], and this function of JAK2 is independent of its kinase domain [96]. Moreover, in the thrombopoietin receptor (TpoR), JAK2 in cooperation with TYK2, promotes cell surface expression and stabilization[97].

1.5.3 JAK3

As mentioned above, JAK3 is associated with the common γ chain and operates in conjunction with JAK1 in the IL-2R family[63]. Given that JAK3 is primarily expressed in the hematological lineages, it is not unexpected that defects in its activity can lead to immunodeficiency, such as X-linked severe combined immunodeficiency (X-SCID), which has the same phenotype as X-SCID linked to common γ chain mutations[79].

The relationship between JAK3 and the common γ chain has been well established. Besides their co-role in cytokine signaling, studies showed that when JAK3 is overexpressed, it is able to upregulate the membrane levels of the common γ chain [84]. Moreover, JAK3 has also been reported to have more than a kinase role in the IL-7R complex. More specifically, JAK3 has been shown to be necessary for IL-7R degradation [66].

1.5.4 TYK2

TYK2 can pair with JAK1 or JAK2 and activate all STAT proteins to mediate signals required for hematopoietic and immune cells and inflammatory responses [98]. TYK2 can be found in class I (IL-10R2, IL-12Rβ1, IL-13Rα1, IL-6Rα and gp130) and class II (IFNAR1) cytokine receptors, as schematically represented in Figure 1.10, exerting different roles induced by variety of cytokines such as IL-12, IL-23, IL-10, IL-22, IL-6, and IFN-α, IFN-β, IFN-λ, CG-CSF, OSM, LIF and CNTF [82,99,100].

The physiological functions that TYK2 exerts are varied. For instance, through IFN-α and -β it can potentiate anti-viral and anti-cancer immune responses[99]. It can also mediate the signal transduction of type III IFNs (IFN-λ), which are responsible for initial host responses against minor infections and epithelial layer damage[101]. By cooperating with the IL-10 family members, which have immunosuppressive actions,[100] can induce epithelial homeostasis and barrier function upon infection. TYK2 also interacts with IL-12 family members

(IL-12 and IL-23) which are imperative in cell-mediated immunity and inflammation, respectively[100].

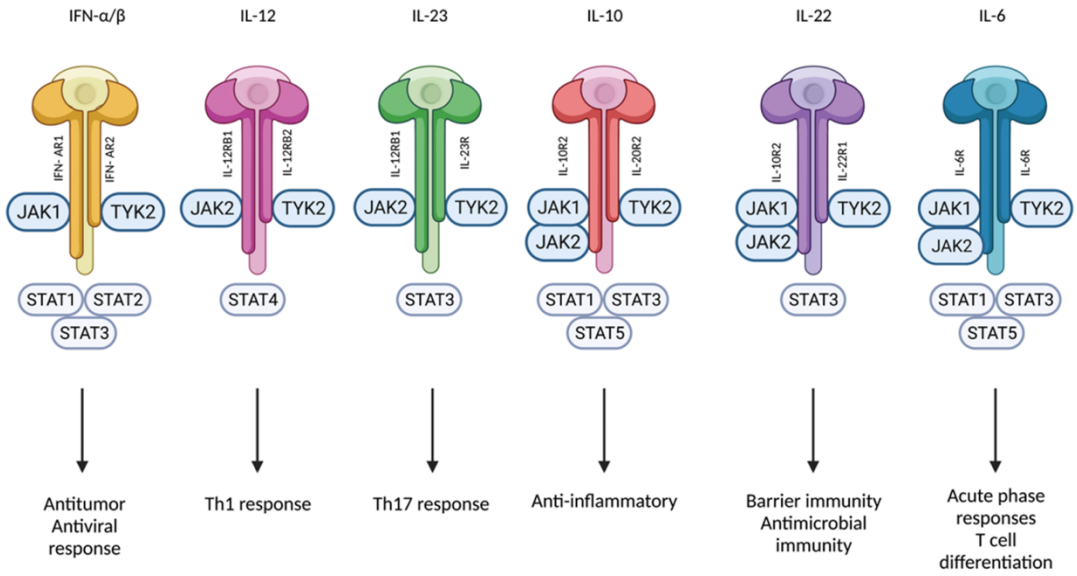

Figure 1.10. TYK2 mediated signaling. TYK2 has different biological roles depending on the pathway activated, and on the combination of JAK/receptor associated with. It can couple with JAK1 and JAK2 and activate all STAT proteins. TYK2 has roles in anti-tumoral, anti-viral and anti-inflammatory responses; it can mediate Th1 and Th17 responses; potentiate barrier and antimicrobial immunity; and T-cell differentiation.
Adapted from Rusiñol, L., & Puig, L. (2023). Tyk2 Targeting in Immune-Mediated Inflammatory Diseases. International journal of molecular sciences, 24(4), 3391.

There is emerging evidence regarding TYK2 potential roles in different pathologies. For instance, patients with a non-functional TYK2 protein are associated with immune and inflammatory diseases, such as atopic dermatitis, inflammatory bowel disease, systemic lupus, rheumatoid arthritis, augmented susceptibility to viruses, fungi, and mycobacterial-induced infections[82]. These are due to alterations in IL-12, IL-6, IL-10, IL-23, IFN-α and β signaling pathways. In these patients, naïve $CD4^+$ cell differentiation is compromised, in particular, the Th1 subpopulation[82]. Moreover, reports of murine studies also demonstrate that TYK2 defects increase Th2 cytokine production [102].

Regarding cancer, TYK2 has been described as a “bona fide oncogene” as it has been reported to have several roles in tumorigenesis[98], which are briefly explained in Table 1.2 and represented in Figure 1.11.

Table 1.2. TYK2 roles in cancer. Adapted from Leitner, N. R., Witalisz-Siepracka, A., Strobl, B., & Müller, M. (2017). Tyrosine kinase 2 - Surveillant of tumors and bona fide oncogene. Cytokine, 89, 209–218.

Characteristics	**Cell extrinsic effects**
Avoid immune destruction	TYK2 loss of function (LOF) in mice leads to impaired surveillance of B- and T-cell tumors and of transplantable tumors
Tumor promoting inflammation	TYK2 is detrimental in several mouse models of auto-inflammatory diseases; also linked to and causative for several human auto- inflammatory diseases
Angiogenesis	TYK2 is essential in uPA-uPAR signaling
Characteristics	**Cell intrinsic effects**
Invasion and metastasis	Enhances epithelial-mesenchymal transition and matrix metalloproteinase expression in various cancer types
Cellular metabolism	Involved in Warburg Effect
Resisting cell death	Induces pro-apoptotic factors and anti-apoptotic genes
Genome instability and mutations	TYK2 mutations and fusions are oncogenic

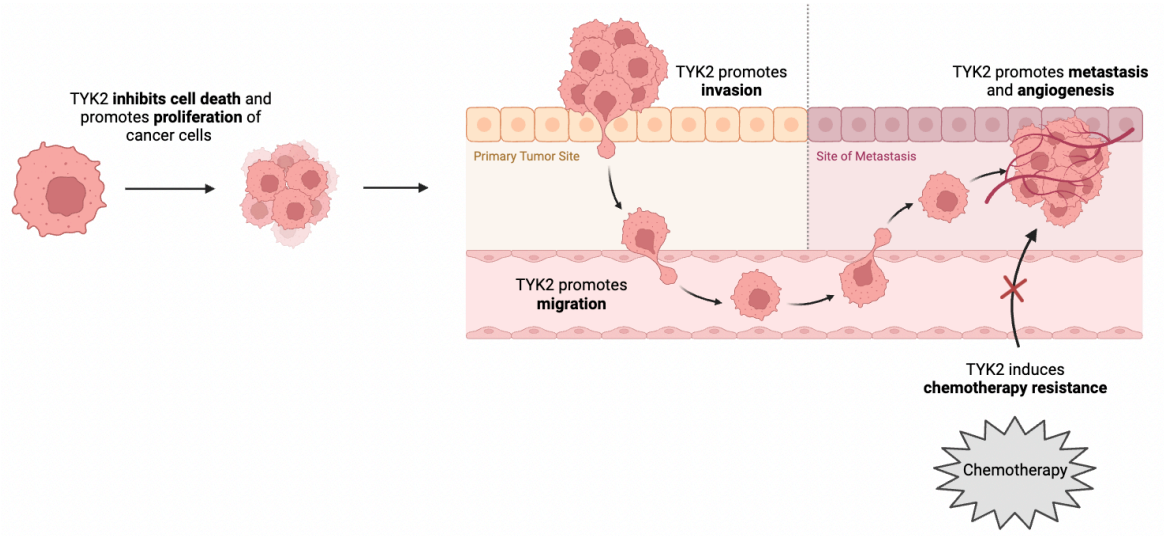

Figure 1.11. Role of TYK2 in cancer. TYK2, via upregulation of anti-apoptotic proteins (Bcl-2, MclL-1), inhibits cell death and promotes proliferation of cancer cells via STAT1 and STAT3 activation, as well as upregulation of c-myc, cyclin D, and heat-shock protein 90. TYK2 is also able to induce invasion and migration through the activation of the epithelial-mesenchymal transition and metalloproteinase expression. After metastasis assembly, TYK2 induces angiogenesis via upregulation of the uPA signaling. Additionally, TYK2 is able to induce chemotherapy resistance via FGF-2 signaling[82]. Image created in BioRender.com

TYK2 has recently been associated with human T-ALL. Using whole-exosome sequencing of five patients with a secondary ALL, a study revealed that two patients had TYK2 germline mutations affecting the pseudokinase domain (p.Pro760Leu and p.Gly761Val), which resulted in TYK2 autophosphorylation and enhanced STAT1, 3 and 5 phosphorylation. These genetic alterations were found in patients who suffered multiple *de novo* ALL[103]. Moreover, the P760L mutations demonstrated transformation capacity in hematopoietic cells and induced leukemia formation in transplanted mice[103]. Activating point mutations, also found in these patients, induced the activation of oncogenic pathways, such as PI3K/AKT/mTOR and RAS[104].

A recent study conducted on primary leukemic cells from a pediatric patient has shown that leukemia cells rely on TYK2 for survival. In this study, 14 out of 16 T-ALL cell lines analyzed had their growth inhibited after TYK2 silencing, an effect attributed to the inhibition of the TYK2-STAT1 pathway[105]. In addition, Patient-Derived-Xenografts (PDX) have also demonstrated sensitivity to TYK2 silencing. Interestingly, it was found that the anti-apoptotic protein Bcl-2 was downregulated after TYK2 silencing, suggesting that these TYK2-dependent leukemia cells rely on the activation of Bcl-2[106]. Another study showed that in 50

T-ALL patient samples, 21 had TYK2 single nucleotide-polymorphisms (SNPs), and in T-ALL cell lines (CCRF-CEM, MOLT-16, MOLT-4 and RPMI-8402) 7 variants were detected[105].

Table 1.3. TYK2 genetic alterations described in T-ALL

TYK2 alterations in T-ALL	
TYK2 status	Activated STAT proteins
Activating somatic mutations	
TYK2-G36D; S47N	STAT1, 3
TYK2-731I	STAT1, 3, 4
TYK2-E957D	STAT1, 3, 5
TYK2-R1027H	STAT1, 3
Activating germline mutations	
TYK2-G761V	STAT1, 3, 5

1.5.5 JAK inhibitors T-ALL

As discussed above, JAKs play significant roles in regulating various signaling pathways, including the IL-7 signaling pathway, thereby establishing them as promising therapeutic targets. Furthermore, given their involvement in the initial stages of signaling cascades, impairing the activity of JAK kinases can potentially halt the pathway's activation at an earlier stage. In this regard, several studies have evaluated the use of JAK inhibitors in the context of T-ALL.

A recent *in vitro* study found that combining Ruxolitinib, a JAK1/JAK2 inhibitor, and dexamethasone increases cell death in both ETP and non-ETP IL-7-dependent T-ALLs. The JAK inhibitor effectively balanced the steroid response in steroid-resistant cells, resulting in a behavior similar to cells that respond well to steroids. Additionally, while a JAK3 inhibitor was used with dexamethasone, its effect was less significant than Ruxolitinib[107]. In *in vivo* studies, mice transplanted with ETP-ALL cells and treated with Ruxolitinib had reduced blasts in peripheral blood and spleen. Moreover, although the ETP-ALLs analyzed had

different genetic alterations, the study demonstrated that even in the presence of JAK3 or JAK1 mutations, Ruxolitinib maintained the same ability to reduce peripheral blood blasts[108].

Another study showed that the most frequent T-ALL JAK3 mutation, M511I, located in its pseudokinase domain, can trigger T-ALL in mouse models. These mice, when treated with Tofacitinib, a JAK3 inhibitor, presented decreased white blood cell counts, high apoptotic cell levels, and a reduction in the size of the spleen and thymus.[109] Another illustration of the impact of JAK3 inhibition in T-ALL can be seen through the use of the IL-7-dependent TAIL7 cell line. Inhibition of JAK3 in this cell line, using the JAK3 specific inhibitor WHI-P131, results in decreased IL-7-induced leukemia cell proliferation and survival[65].

TYK2 inhibition has also been evaluated in the context of T-ALL. Deucravacitinib is a specific TYK2 inhibitor that acts through the blockade of the protein's JH1 domain (pseudokinase domain), impeding the activation of the kinase domain. This drug has already been approved by the FDA for the treatment of psoriasis[110] A study with this pharmacological inhibitor demonstrated that TYK2 P760L-transformed cells had their metabolic activity decreased. These results were also coupled with the abrogation of TYK2, STAT1 and STAT3 phosphorylation upon treatment with Deucravacitinib[103].

2 Research Aims

Although TYK2 has never been associated with the IL-7/IL-7R pathway, considering its well-documented role in T-ALL and our recent RPPA results, we asked whether TYK2 could play a role in IL-7-mediated T-ALL.

Thus, the main goal of this project is to understand and characterize a potential role for TYK2 in IL-7/IL-7R signaling in T-cell Acute Lymphoblastic Leukemia.

The first step aims at validating our lab preliminary results by evaluating IL-7-mediated phosphorylation of TYK2 in hWT IL-7R D1, hWT BA/F3 and TAIL7 cells via Western Blot (WB).

The second part of this project aims at understanding the mechanistic role of TYK2 in the IL-7 signaling pathway. For this purpose, the different elements of the IL-7 signaling (JAK3, STAT5, IL-7R α chain and common γ chain) and TYK2 will be transfected in the HEK-293T cell line that does not express them endogenously, except for JAK1, which is expressed by these cells. For signaling evaluation, WB will be performed.

The third part of this study aims at analyzing the putative binding of TYK2 to the IL-7R complex in transfected HEK-293T cells and TAIL7 cells via Immunoprecipitaiton.

Lastly, and to characterize the functional role of TYK2 in T-ALL cells, we will use a specific TYK2 inhibitor (Deucravacitinib) to treat TAIL7 cells stimulated with IL-7 and analyze its impact on T-ALL cell viability, activation and IL-7R surface levels.

In its all, this study has the potential to expand our understanding regarding the IL-7 signaling pathway and its critical elements in the context of T-ALL. The successful achievement of our aims has the potential to identify a new therapeutic approach in the management of IL-7-dependent T-ALL.

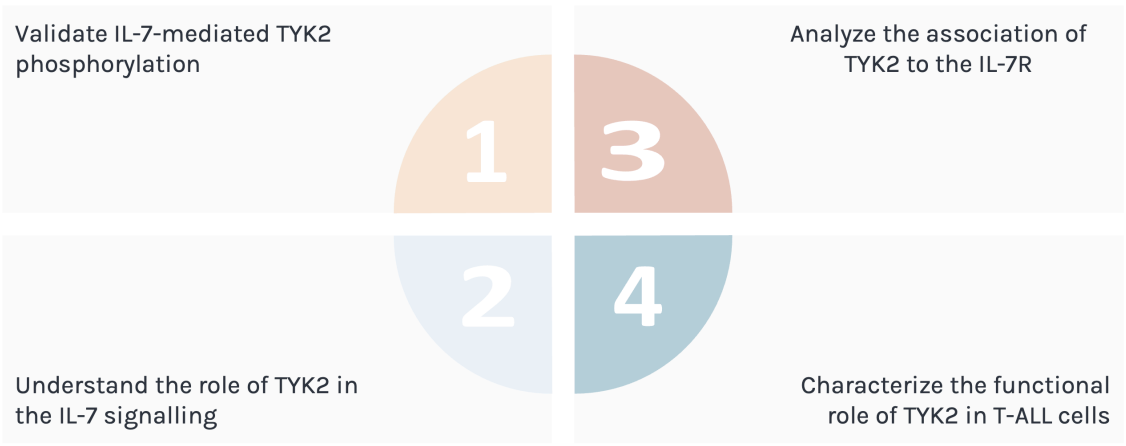

Figure 2.1. Schematic illustration of the leading research aims of this project.

3 Materials and methods

3.1 Cell culture

Human TAIL7, a primary-like IL-7-dependent T-ALL cell line, murine BA/F3 hWT IL-7R, an IL-7-dependent pro-B-cell line stably transduced with the human IL-7R, and D1 hWT IL-7R, an IL-7-dependent murine thymocyte cell line stably transduced with the human IL-7R, were used in this project[111]. All the cell lines were cultured at 37ºC in 5% CO_2. The cells were cultured in Roswell Park Memorial Institute (RPMI) – 1640 medium supplemented with 1% L-glutamine (Gibco), 10% of Fetal Bovine serum (FBS) (BioWest), 1% Penicillin/Streptomycin (Gibco) and 1% N-2-Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid (HEPES), henceforward referred to as R10. The culture conditions for TAIL7, D1, and BA/F3 were $2x10^6$ cells/mL with 25 ng/mL of IL-7, $0.5x10^6$ cells/mL with 25 ng/mL of IL-7 and 0.5 $x10^6$ cells/mL with 50 ng/mL of IL-7, respectively.

Human Embryonic Kidney 293 cell line containing the SV40 T-antigen (HEK-293T) was used and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% L-Glutamine (Gibco), 10% FBS, and 1% Penicillin/Streptomycin, hereafter referred to as D10. To detach the cells, Trypsin-EDTA 1x (Gibco) was utilized and deactivated with the serum-supplemented medium, D10.

3.2 JM109 Competent Cells transformation

JM109 Competent Cells 10^8 cfu/ug (VWR, Promega) were transformed with the following plasmids: pMig hWT IL-7R, pcDNA 3.1+ γc, pcDNA 3.1+ JAK3, pcDNA 3.1+ STAT5, pcMV6 XL5 TYK2, pMig empty, pcDNA 3.1+ empty and pcMV6 empty.

The following protocol was performed at the flame in aseptic conditions. For each plasmid, 1µL was added to 50 µL of JM109 competent cells and incubated for 30 minutes on ice, followed by a heat shock at 42ºC for 45 seconds

and 5 minutes on ice. The bacteria were supplemented with 450 µL of Super Optimal Broth (S.O.C) medium (ThermoFisher) and incubated at 37ºC, shaking at 220 rotations per minute (RPM) for 45 minutes for maximal transformation efficiency. From the cell suspension, 30 µL was spread into a petri dish with Lysogenia Broth (LB) agar supplemented with ampicillin and left overnight at 37ºC.

The next day, a colony from the LB agar plate was picked and transferred to a 15 mL tube with LB medium supplement with ampicillin and incubated at 37ºC, shaking at 220 RPM for eight hours. Afterwards, 30 µL of the inoculum was transferred to a 2L erlenmeyer with 300 mL of LB medium supplemented with ampicillin and left overnight at 37ºC shaking at 220 RPM. The bacterial suspension was centrifuged at 4ºC and at 4000 RPM for 10 minutes, and the supernatant was discarded. Afterwards, the cell pellet was frozen at -20ºC or DNA extraction was followed.

3.3 Maxi-Prep for high copy number plasmids

A Genopure Plasmid Maxi Kit High Copy Number (Roche) was used to extract the DNA of interest from the transformed bacteria.

The cell pellet was resuspended in 12 mL of Suspension Buffer + RNAse A followed by adding 12 mL of Lysis Buffer, after which the suspension was inverted 6-8 times and incubated for 2-3 minutes at room temperature (RT). Twelve mL of Neutralization Buffer at 4ºC was added to the suspension, and the mixture was homogenized by inverting the tube 6-8 times and incubating for 5 minutes on ice. Subsequently, the precipitate was cleared with a filter (240 mm diameter) placed in a 50 mL Falcon tube, previously moistened with Equilibration Buffer, and collected. A NucleoBond AX 500 column was assembled into a new Falcon and equilibrated with 6mL of Equilibration Buffer, and the flow through was discarded. The cleared lysate was loaded into the column, and the flow through was removed. Afterwards, the column was washed twice with 16 mL of Wash buffer, and the flow through was discarded. The column was transferred into a new 50 mL Falcon, and 15 mL of pre-warmed (56ºC) Elution Buffer was

added. The collected flow through containing the plasmid was eluted with 11 mL of isopropanol and centrifuged for 45 minutes at 3900 RPM at 10ºC. The supernatant was carefully discarded, and the plasmid was washed with 4 mL of chilled (4ºC) 70% ethanol and centrifuged at 3900 RPM for 10 minutes at 10ºC. The ethanol was carefully removed, and the plasmid was left to air dry for 10 minutes. The plasmid was resuspended in 100-500 µL of UltraPure™ Distilled Water DNAse/ RNAse Free (Invitrogen) and stored at 4ºC until use.

3.4 Protein extraction

Cells used for protein analysis were centrifuged at 1500 RPM at 4ºC, and the supernatant was discarded. For protein extraction per se, the cell pellet was resuspended thoroughly in a mix of lysis buffer (50 mM Tris-Base pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM Sodium Pyrophosphatase, 1% v/v NP-40) supplemented with the protease inhibitor cocktail Complete Mini (Roche) and the protease inhibitor AEBSF hydrochloride 1 mM (Fisher Scientific), followed by centrifugation at 13000 RPM during twenty minutes at 4ºC. Afterwards, the protein extract (supernatant) was recovered and stored at -20ºC for later use. The Bradford assay (Bio-Rad) was performed for protein quantification with the GeneQuant Pro (Amersham Biosciences) spectrophotometer at 595 nm.

3.5 Western blot

Samples were prepared with equal protein amounts (50 µg), resuspended in 6X Sample Loading Buffer [375 mM TRIS-HCl (pH 6.8), 9% SDS, 50% glycerol, and 0.03% bromophenol blue], and denatured at 96ºC for five minutes. The samples were loaded onto a SDS-PAGE gel (12% or 10% of acrylamide) and ran in an electrophoresis chamber (Bio-Rad) with Running Buffer (Tris-glycine-SDS buffer) at 60 volts for thirty minutes and 120 volts for one hour and a half or two hours, depending on the acrylamide percentage of the gel and the protein separation required.

The proteins were transferred in a Transfer Buffer (Tris-Glycine buffer, 20% Methanol) to a nitrocellulose membrane (BioVision) for 90 minutes at 400 mA at 4°C. Ponceau S solution (Sigma) was used for transference validation, which also assisted in cutting the membranes. Subsequently, the membranes were blocked for 1 hour with slow agitation with 3% w/v skimmed milk diluted in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) buffer at RT and washed three times with TBS-T buffer for 10 minutes with mild agitation.

Immunoblotting was followed at 4ºC overnight (ON) with slow agitation with the respective primary antibodies. On the next day, membranes were washed three times with TBS-T buffer for 15 minutes each wash and immunoblotted with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies anti-mouse (Promega), anti-rabbit (Promega) with slow agitation at RT for one hour, followed by three more washes of 15 minutes each with TBS-T.

For immunodetection, membranes were treated with Pierce ECL Plus Western Blotting Substract (ThermoFisher Scientific) for five minutes in the dark, and chemiluminescence was performed using Curix60 (AGFA HealthCare).

3.5.1 Primary antibodies for Immunoblotting

The following primary antibodies were used: JAK1 (#50996, Cell Signaling), p-JAK1 (Tyr1022, 1023) (#3331 Cell Signaling), JAK3 (#8863 Cell Signaling), p-JAK3 (Tyr980, 981) (#5031, Cell Signaling), TYK2 (#9312, Cell Signaling), p-TYK2 (Tyr1054/1055) (#9321, Cell Signaling), STAT5 (#94205, Cell Signaling), p-STAT5 (Tyr694) (#9351, Cell Signaling), β-Actin (ab8224, Abcam), AKT (#9272, Cell Signaling), p-AKT (Ser473) (#4060, Cell Signaling). All antibodies were diluted in TBS-T 1:1000, except for beta-actin, which was used at a 1:10 000 dilution.

Antibodies against JAK3, p-JAK3 and p-JAK1 were optimized as demonstrated in Table 3.1.

Table 3.1. Antibody optimization protocols used for p-JAK1 (Tyr1022, 1023) (#3331, Cell Signaling) and p-JAK3 (Tyr980, 981) (#5031, Cell Signaling), and JAK3 (#8863 Cell Signaling).

<table>
<tr><th>Blocking solution</th><th>Primary antibody</th><th>Secondary antibody</th></tr>
<tr><td rowspan="5">TBS-T 3% milk</td><td>Dilution: 1:1000 TSB-T
Incubation: ON at 4ºC</td><td rowspan="7">Dilution:
1:5000 TBS-T 3% milk
Incubation:
1h at RT</td></tr>
<tr><td>Dilution: 1:1000 TBS-T 3% milk
Incubation: ON at 4ºC</td></tr>
<tr><td>Dilution: 1:500 TBS-T
Incubation: ON at 4ºC</td></tr>
<tr><td>Dilution: 1:500 TBS-T 3% milk
Incubation: ON at 4ºC</td></tr>
<tr><td>Dilution: 1:500 TBS-T
Incubation: 2h at RT</td></tr>
<tr><td rowspan="2">TBS-T 5% Bovine Serum Albumin (BSA)</td><td>Dilution: 1:1000 TBS-T 5% BSA
Incubation: ON at 4ºC</td></tr>
<tr><td>Dilution: 1:500 TBS-T 5% BSA
Incubation: 2h at RT</td></tr>
</table>

3.5.2 Membrane stripping

Membrane stripping was performed to allow the re-blot of the membranes. A Stripping Buffer [15 nM Tris Base, 100 nM 2-β-Mercaptoethanol (Sigma), 2% SDS, pH 6,7] supplemented with 1:1000 v/v dilution of 2-β-Mercaptoethanol was used to submerge the membranes for thirty minutes in a water bath at 56ºC. The membranes were thoroughly washed with running water and then with TBS-T buffer for 10 minutes three times with agitation.

3.6 Lentiviruses production and transduction in HEK-293T cells

HEK-293T cells were transduced with short-hairpin RNA (shRNA) Clones set against Human Janus Kinase 1 (shRNA hJAK1 A, B and C) and a scramble sequence (shRNA SCR) used as control (NM_002227.3, Tebubio).

Briefly, VSV-G-pseudotyped lentiviruses were produced by transient transfection of HEK-293T cells at 70% confluence in 10 cm plates, using the Lipofectamine™ 3000 Transfection Reagent and following the transfection reagent guidelines. The plasmid vectors and DNA amount used for viral production are described in Table 3.2. Lentiviruses were collected 48h and 72h after transfection, snap-frozen and stored at -80 ºC until used.

Table 3.2. Plasmids used for lentiviruses

Plasmid	Quantity used for transfection
Target vector	6.6 µg
pPAX2 packaging vector	6 µg
pMD2.G VSV-G envelope vector	3 µg

For cell transduction, HEK-293T cells were incubated with lentiviruses and 10 µg/ml of Polybrene (Sigma) for 24 hours at 37ºC. shRNA SCR, hJAK1 A, hJAK1 B, and hJAK1 C transduced cells were collected 24 hours, 48 hours, and 72 hours after transduction. Cells were centrifuged at 1500 RPM at 4ºC for 5 minutes, the cell pellet was homogenized in TRIzol (Ambion) or snap-frozen and stored at -80ºC until further use.

3.7 RNA extraction

For RNA isolation, the protocol from Ambion was followed with certain alterations. The homogenized sample was incubated at RT for five minutes, then 200 µL of chloroform were added and the sample vortexed. An incubation of 2-3 minutes at RT was performed until the two-phase separation was observed. Afterwards, 500 µL of isopropanol was added, and samples were inverted 2-3 times and incubated for 15 minutes on ice, followed by centrifugation at 13000 RPM for 15 minutes at 4ºC. The supernatant was removed, and the RNA pellet was washed with 1 mL of 75% ethanol. Later, the ethanol was removed, and the RNA pellet was left to air dry for 5 minutes. The RNA pellet was resuspended in 50 µL of DNase/RNase-free water and incubated in a dry bath at 55-60ºC for 10

minutes to help resuspension. The RNA was quantified using NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific) and stored at -80ºC until further use.

3.8 cDNA synthesis

NZY First-Strand cDNA Synthesis Kit was used for cDNA synthesis with 500 ng of RNA resuspended in 8 µL of UltraPure™ Distilled Water DNAse/ RNAse Free (Invitrogen) for each sample. All the following steps were performed on ice.

A mix of UltraPure™ Distilled Water DNAse/ RNAse Free (Invitrogen) with NZYRT 2X Master Mix and the respective primers was prepared according to kit guidelines, from which 12 µL was distributed onto the nuclease-free microcentrifuge tubes, and 8 µL of RNA was loaded. The samples were gently mixed and incubated at 25ºC for 10 minutes, followed by 30 minutes at 50ºC. Next, the reactions were incubated for 5 minutes at 85ºC and placed in ice to cease the reaction. To eliminate the RNA template, 1 µL of NZY RNase H was added, and the samples were incubated at 37ºC for 20 minutes. The cDNA was stored at -20ºC until further use.

3.9 Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed to evaluate the knockdown efficiency of human JAK1 variant 1 in HEK-293T transduced with the specific shRNAs.

Power SYBR™ Green Master Mix (ThermoFisher Scientific) was used for qPCR according to the manufacturer's directions. The reaction was performed in the Viia™ 7 Real-Time PCR system (ThermoFisher Scientific) instrument and with the primers listed in Table 3.3. The comparative cycle (CT) method 2-ΔΔCT was used to calculate the relative gene expression. The gene 18S was used as the endogenous control. Samples were then equated with the shRNA hJAK1 SCR condition.

Table 3.3. List of primers used in RT-qPCR

Species	Gene	Strand	Primer sequence (5'-3')	Concentration
Human	JAK1 Variant 1	Forward	TGGCTGTCATGGTCCAATCT	10 pmol/μL
		Reverse	CAAATGTTGCTTGTCTGGTG	10 pmol/μL

3.10 HEK-293T transfection

Lipofectamine 3000 (Invitrogen) was used as the transfection reagent in all experiments requiring cell transfection, and manufacturers' guidelines were followed. The plasmids used are listed in Table 3.4. Twenty-four hours before the transfection procedure, $0.25x10^6$ HEK-293T cells were plated in a 6-well plate or $2 x10^6$ cells in a 10 cm plate, depending on the experiment.

On the day of the transfection, the D10 medium was changed. The quantities of the reagents p3000, lipofectamine, OPTI-MEM – Reduced Serum Medium (Gibco), DNAs, and the plate's size were followed and optimized according to the manufacturer's instructions. On the third day, the cells were stimulated with IL-7 at 50 ng/mL for 15 minutes and retrieved for protein extraction, or for snap-freeze of the cell pellet, depending on the experiment to be performed.

Table 3.4. Plasmids used for co-transfections in HEK-293T cell line

Plasmid	Quantity used for transfection
pMig WT hIL-7R	0,8 μg
pcDNA 3.1+ γc	0,4 μg
pcDNA 3.1+ JAK3	0,4 μg
pcDNA 3.1+ STAT5	0,1 μg
pcMV6 XL5 TYK2	0,1 μg

3.11 Immunoprecipitation protocol

For IL-7Rα and TYK2 pull-down, immunoprecipitation was executed. For this protocol, Dynabeads Protein G (ThermoFisher), Co-IP NP40 buffer (50 mM Tris HCl, 150 nM NaCl, 2 nM EDTA, 1% NP40), and a wash buffer [0.1% BSA; 2 mM EDTA in Phosphate-buffered saline solution (PBS)] were used. Every step of this protocol was done on ice with the solutions at 4ºC.

After each experiment, the cell pellet was snap-frozen and stored at -80ºC, and the following day protein extraction was performed with Co-IP buffer. Samples were prepared for 10 µg and 30 µg (input) in a total volume of 40 µL Co-IP buffer plus 20 µL of 3X Laemmli Buffer supplemented with denaturing agent. For the protein precipitation per se, 420 µg – 500 µg of protein in 150 µL Co-IP buffer for each experimental condition was used, as well for the control (IgG).

On day 1, the main goal is to bind the beads to the respective antibodies. A pre-clearing step was performed, where 20 µL of beads were added to each sample and incubated for one hour at 4ºC in the rotator. Afterwards, in two microcentrifuge tubes, one for the antibody and the other for the respective IgG, 50 µL of beads per sample were added. The beads were washed with 1 mL of wash buffer, inverted 30 times, and placed into the magnetic holder to attach to the microcentrifuge tubes' wall to remove the wash buffer. This washing step was performed three times. After, the beads were resuspended in 500 µL of wash buffer, and the quantity of the respective antibodies, IL-7Rα (sc-514445, Santa Cruz Biotechnology), TYK2 (sc-5271, Santa Cruz Biotechnology), and mouse mAb IgG2a isotype control (#61656, Cell Signaling), was added accordingly to the manufacturer's instructions. The beads were then incubated overnight at 4ºC in the rotator.

On day 2, the main goal is to bind the protein extracts to the beads previously incubated with the antibodies. After incubating ON, the beads were washed three times with wash buffer and then diluted in 50 µL times the number of samples. Next, 50µL of this mixture was added to the protein extracts, and 300 µL of Co-IP buffer was added to reach a final volume of 500 µL. The prepared samples with the beads were incubated for 2 hours at 4ºC in the rotator. After the

incubation, the microcentrifuge tubes were placed onto the magnetic column, and the supernatant was removed. Then, the beads were washed five times with Co-IP buffer. Before the last washing step, the beads were transferred to a new microcentrifuge tube, and the supernatant was removed. 60 uL of Co-IP buffer + Laemmli Buffer 3X (without a denaturing agent) was added and homogenized gently, followed by an incubation of 10 minutes at 70ºC. The tubes were placed in the magnetic holder, and the supernatant was collected into a new microcentrifuge tube. The samples were frozen at -20ºC, and on the day of the WB, 0.88 µL of the reducing agent 2-β-Mercaptoethanol (Sigma) was added, and the samples boiled at 96ºC for 5 minutes.

3.12 Flow cytometry analysis in TAIL7 cells

Flow cytometry evaluation via the equipment BD Accuri C6 Plus (BD Biosciences) was performed to assess cell viability, activation and IL-7Rα surface expression using a Human IL-7R alpha/CD127 PE-conjugated antibody (#FAB306P, R&D Systems) in TAIL7 cells treated with increasing concentrations of Deucravacitinib (BMS-986165) (Selleckchem).

Cells were directly washed with FACs buffer (2% Serum PBS) at 4ºC in the 96-well plate, centrifuged for 3 minutes at 2000 RPM at 10ºC, and the supernatant was discarded. IL-7Rα staining was performed for 30 minutes at RT and protected from light, as indicated by the manufacturer. Afterwards, another wash cycle with FACs buffer was performed. Finally, the cells were resuspended in 100 uL of FACs buffer and retrieved to microcentrifuge tubes for analysis.

4 Results

4.1 Validation of TYK2 phosphorylation in TAIL7, D1 hWT IL-7R and BA/F3 hWT IL-7R cell lines.

In an effort to establish the IL-7/IL-7R signaling landscape in health and in malignancy, our lab has been characterizing IL-7-induced protein phosphorylation in different cell models. For this a T-ALL cell line (TAIL7), that endogenously expresses the IL-7R, and two other cell lines, the D1 murine thymocytes hWT IL-7R and the BA/F3 murine pro-B hWT IL-7R, which express the IL-7R ectopically, were stimulated with IL-7 and the cells collected at different time points after stimulation for RPPA analysis.

The phosphorylation status of 91 proteins was evaluated via RPPA. From this phosphoproteomic approach, proteins known to be associated with the IL-7 signaling, such as STAT5 (Tyr694), were found phosphorylated, attesting for the validity of the experimental approach (Figure 4.1). More interestingly, this phosphoproteomic approach allowed us to identify the IL-7-induced phosphorylation of proteins not yet associated with IL-7 signaling, such as TYK2.

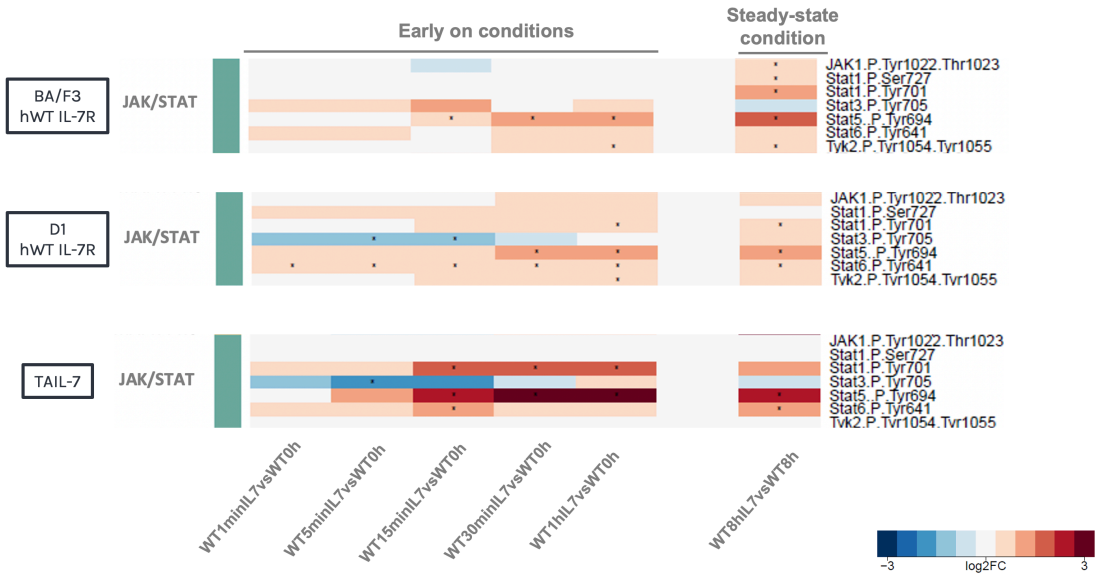

Figure 4.1. Heatmap of proteins phosphorylated in response to IL-7 in the different cell lines. Reverse Phase Protein Array (RPPA) demonstrates JAK/STAT pathway players' phosphorylation status upon IL-7 stimulation in BA/F3 hWT IL-7R, D1 hWT-IL-7R and TAIL7 cells. * Statistical significant.

As part of the project's initial phase, we started by validating the RPPA results, which demonstrated TYK2 phosphorylation in response to IL-7 stimulation.

To accomplish this, we stimulated the same cell lines (TAIL7, D1 hWT IL-7R, and BA/F3 hWT IL-7R) with IL-7 and collected the cells at increasing time points after stimulation (0h, 30 seconds, 1 minute, 5 minutes, 15 minutes, 30 minutes, and 8 hours) to evaluate TYK2 phosphorylation by WB analysis.

As expected and shown in Figure 4.2, stimulation of the three cell lines with IL-7 results in the phosphorylation of STAT5. AKT activation can also be observed in D1 hWT IL-7R and TAIL7 cells, with AKT phosphorylation levels increasing overtime. However, in BA/F3 hWT IL-7R cells, AKT activation is not observed.

In agreement with our RPPA results, stimulation of D1 hWT IL-7R and of TAIL7 cells with IL-7 induced TYK2 phosphorylation (Figure 4.2. A, B). In both cell lines, TYK2 phosphorylation was detected as early as 30 seconds upon stimulation with IL-7 and remained induced up to 8 hours after stimulation.

As for the BA/F3 hWT IL-7R cell line, TYK2 phosphorylation was only observed at the 8 hour time-point, and the TYK2 total protein levels varied across the different time points. Of note, STAT5 phosphorylation was only detected 5 minutes upon IL-7 stimulation and so considerably later than what was expected and observed for the other two cell lines (Figure 4.2. C).

These results confirm our RPPA data, indicating that TYK2 is phosphorylated after IL-7 stimulation in the three cell lines tested.

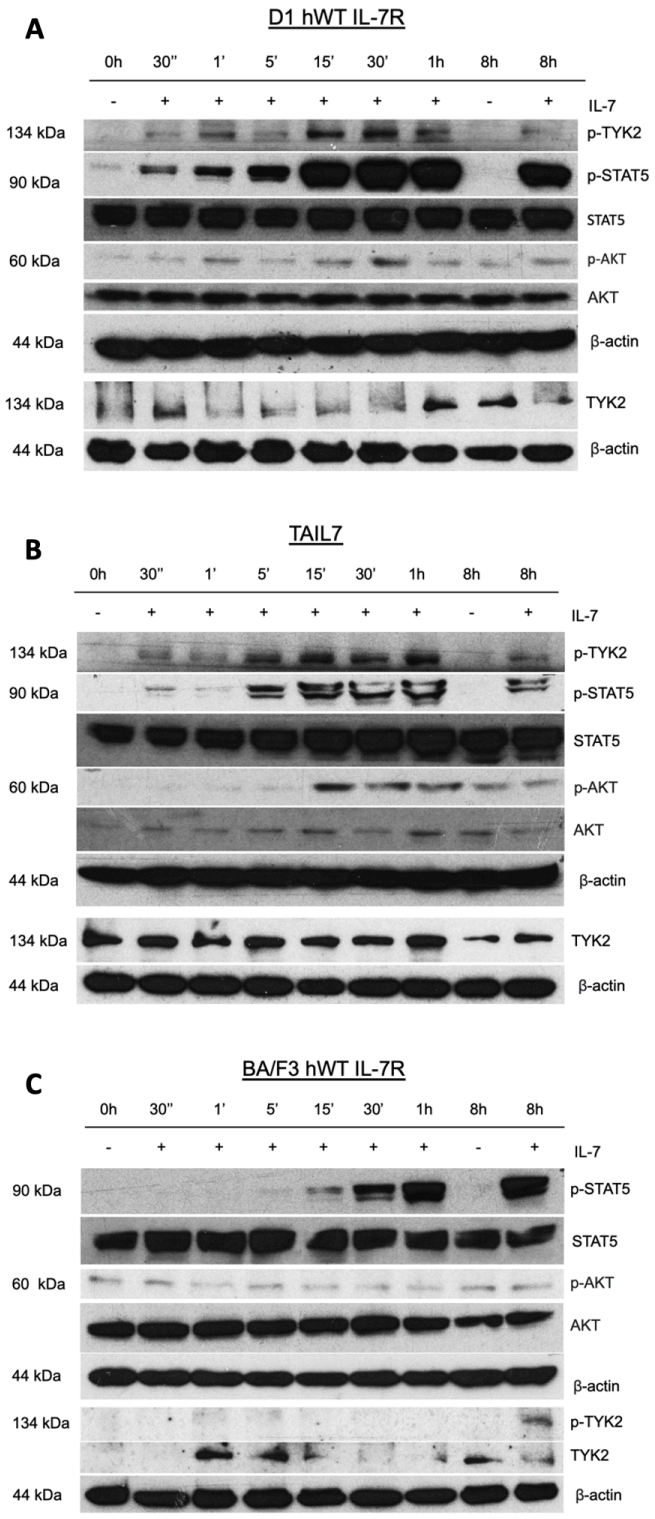

Figure 4.2. TYK2 IL-7-dependent phosphorylation in D1 hWT IL-7R, TAIL7 and BA/F3 hWTIL-7R cell lines. A) D1 hWT IL-7R cells were deprived of IL-7 for 16 hours and subjected to IL-7 stimulation at 25 ng/mL for increasing time points. IL-7 stimulation induces TYK2 phosphorylation from 30 seconds up to 8h; B) TAIL7 cells were deprived of IL-7 for 24 hours and stimulated with IL-7 at 25 ng/mL in increasing time points. IL-7 stimulation induces TYK2 phosphorylation from 30 seconds up to 8h; C) BA/F3 hWT IL-7R cells were depleted of IL-7 for 24 hours and stimulated with IL-7 at 100 ng/mL in increasing time points. IL-7 induces TYK2 phosphorylation after 8h. Western Blot analysis was performed in order to evaluate the phosphorylation status of TYK2, and STAT5 and AKT phosphorylation as outputs for the activation of the IL-7 signaling.

4.2 Optimization of an IL-7/IL-7R signaling pathway ectopic model in HEK-293T cells.

We next sought to evaluate a putative role for TYK2 in the IL-7/IL-7R signaling pathway using as approach an ectopic model of IL-7/IL-7R signaling pathway in HEK-293T cells, already developed at the JBarata Lab[93]. This cell line doesn't express the IL-7 pathway players, except for JAK1, which allow us to modulate the pathway players in order to understand the role of TYK2 in the signaling cascade.

We started by optimizing the amount of TYK2 plasmid DNA to be transfected in the HEK-293T cell line in order to be detected by WB. We tested the following amounts of pCMV6 XL5 TYK2 plasmid DNA: 0,05 μg, 0,1 μg, 0,2 μg and 0,4 μg.

As observed in Figure 4.3, 0,1μg of plasmid DNA was sufficient to detect TYK2 phosphorylation and TYK2 total protein levels by WB. Of note, these cells were not stimulated with IL-7 or co-transfected with the IL-7 pathway players. Since the 0,1μg condition allowed for the visualization of TYK2 total protein by WB, we proceeded with this condition for the co-transfections.

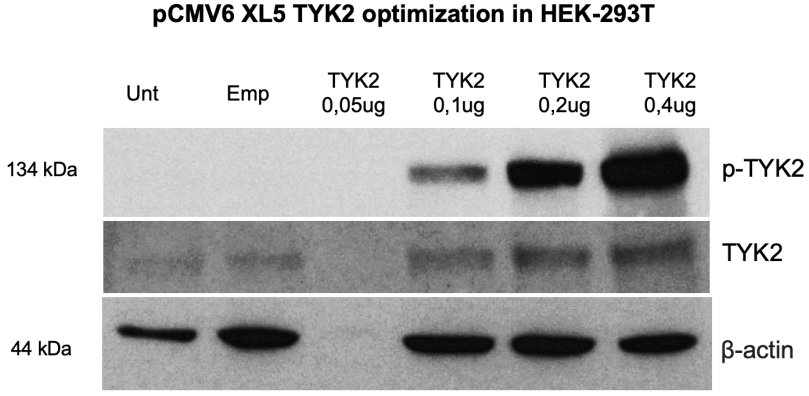

Figure 4.3. pCMV6 XL5 TYK2 plasmid DNA optimization for HEK-293T cells transfection. HEK-293T cells were transfected with increasing concentrations of the construct TYK2 pCMV6 XL5 in order to optimize its quantity for future transfections. Unt – Untransfected; Emp – Empty constructs.

To evaluate the impact of TYK2 on IL-7 signaling pathway using this model, we co-transfected HEK-293T cells with the different IL-7 signaling players (IL-7Rα chain, common γ chain, JAK3, and STAT5, not endogenously expressed by these cells) and, in some experimental conditions, with TYK2. JAK1, known to be expressed by these, was not co-transfected[93]. All conditions were stimulated with IL-7 at a concentration of 50 ng/mL for fifteen minutes.

As shown in Figure 4.4, the correct operation of the system can be observed through the detection of p-STAT5 upon IL-7 stimulation. Noteworthy, co-transfection with TYK2 results in STAT5 phosphorylation in the absence of IL-7 stimulation. These phosphorylation levels are nonetheless further increased upon IL-7 stimulation.

Regarding TYK2, the protein displays phosphorylation prior to stimulation, as previously observed in the optimization process (Figure 4.3). However, the phosphorylation levels increase upon stimulation with IL-7 (Figure 4.4).

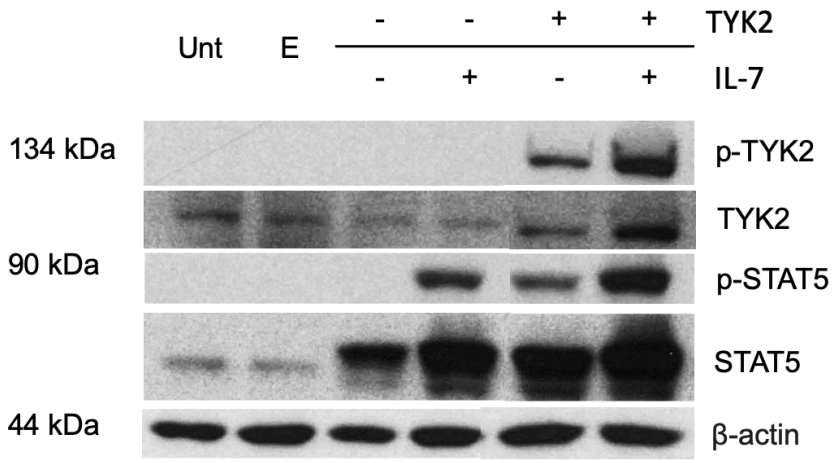

Figure 4.4. Evaluation of TYK2 in HEK-293T IL-7/IL-7R signaling pathway ectopic model. HEK-293T cells were co-transfected with the different IL-7 pathway players and TYK2 and stimulated with IL-7 for fifteen minutes at 50 ng/mL. L-7 induces TYK2 phosphorylation in HEK-293T IL-7/IL-7R signaling pathway ectopic model.
Unt –Untransfected E –Empty constructs

We attempted to visualize the phosphorylation status of JAK1 and JAK3 but failed to obtain reliable results. Precise WB bands were unobservable, and despite different conditions were tested (shown in Table 3.1 in the Methods section), the outcome was still unsatisfactory (data not shown). Nonetheless, with

this experiment, we were able to demonstrate that TYK2 is responsive to IL-7 stimulation in this ectopic model.

4.2.1 Evaluation of JAK1 and JAK3 activation in the HEK-293T IL-7/IL-7R signaling pathway ectopic model

We were puzzled by the inability to detect the phosphorylation status of JAK1 and JAK3 and questioned if the 15 minute time point at which the phosphorylation levels were being analyzed was indeed the best one to observe the activation of these proteins. Thus, we conducted a kinetic experiment in which the HEK-293T cells were stimulated with IL-7 and cells retrieved at increasing time points.

As shown in Figure 4.5, although STAT5 activation was detected within 5 minutes after stimulation (with clearer results at 15 minutes), phosphorylation of JAK1 and JAK3 was not observed (Figure 4.6).

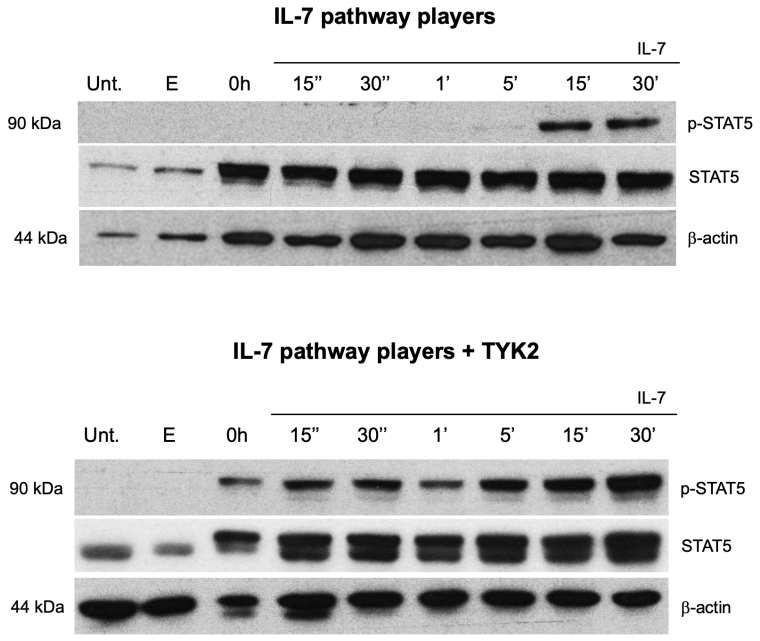

Figure 4.5. STAT5 phosphorylation in kinetic experiment using HEK-293T IL-7/IL-7R signaling pathway ectopic model. HEK-293T cells were transfected with IL-7 pathway players (IL-7Rα, common γ chain, JAK3, and STAT5) and co-transfected with TYK2. Cells were stimulated with IL-7 at 50 ng/mL at increasing time points. Western blot analysis of the phosphorylation status of STAT5 was performed. TYK2 overexpression in HEK-293T induces earlier STAT5 phosphorylation.
Unt –Untransfected; E –Empty constructs.

These results were unexpected. Since these proteins interact directly and very closely in time in this signaling cascade, we would expect their activation to occur (and be detected) at approximate time points.

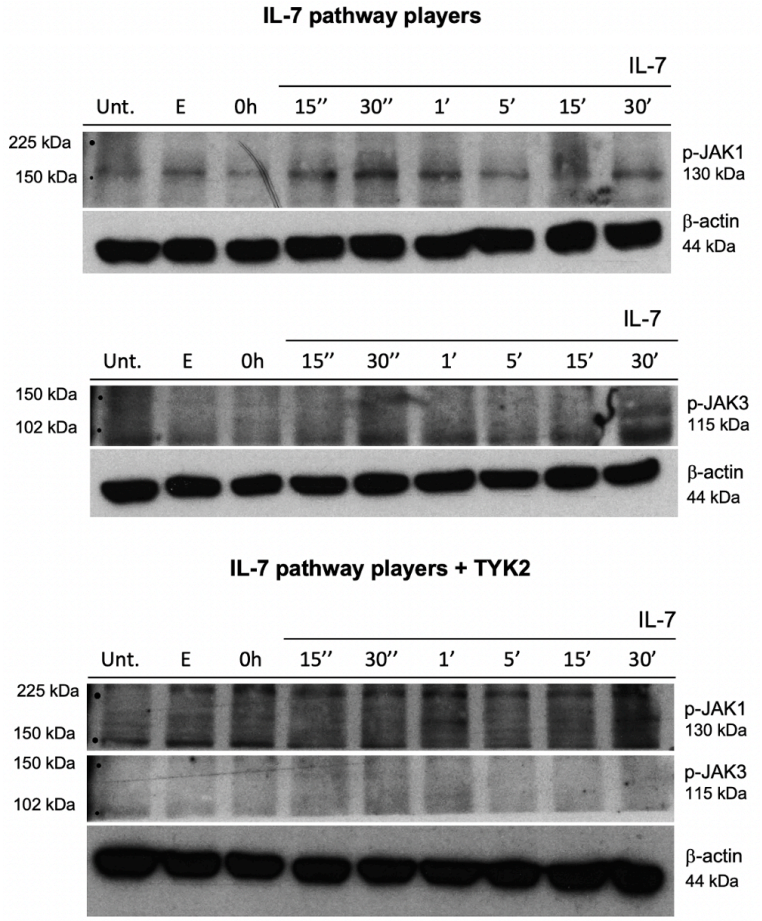

Figure 4.6. JAK1 and JAK3 phosphorylation kinetic experiment using HEK-293T IL-7/IL-7R signaling pathway ectopic model. HEK-293T cells were co-transfected with IL-7 pathway players (IL-7Rα, common γ chain, JAK3, and STAT5) and with TYK2. Cells were stimulated with IL-7 at 50 ng/mL at increasing time points. Western blot analysis of the phosphorylation status of JAK1 and JAK3 was performed. JAK1 and JAK3 phosphorylation was not detected at any of the time-points tested and with TYK2 overexpression.
Unt – Untransfected; E –Empty constructs.

Noteworthy, co-transfection with TYK2 impacted the kinetic of STAT5 phosphorylation, inducing faster and increased levels upon IL-7 stimulation (Figure 4.5). However, we still couldn't observe the phosphorylation of JAK1 or JAK3 in the TYK2 overexpression condition (Figure 4.6).

With this experiment, we concluded that the lack of detectable levels of phosphorylated JAK1 and JAK3 was not related to the time point at which cells were being analyzed. Thus, since STAT5, our readout for the activation of the IL-7 signaling pathway, was clearly phosphorylated at the 15 minute time point in both conditions with and without TYK2, we kept using this time point for the following experiments.

4.3 Evaluation of TYK2 role in the IL-7 signaling pathway

Since TYK2 is a Janus kinase, like JAK1 and JAK3, we wondered if TYK2 could have a role in the IL-7 signaling pathway similar to the one performed by JAK1 and/or JAK3, which would result in its phosphorylation downstream of IL-7 stimulation. To address this, we sought of using the HEK-293T IL-7/IL-7R signaling pathway ectopic model validated above, to test the impact of TYK2 overexpression when combined with either JAK1 or JAK3. More precisely, to test the impact of TYK2 in the IL-7 signaling pathway when combined: with JAK1 in the absence of JAK3; with JAK3 in the absence of JAK1; and in the absence of both JAK1 and JAK3.

In order to proceed with our experiment, we set out to generate a HEK-293T cell line knockdown for JAK1 since it is expressed endogenously by these cells. As for JAK3, since it is not expressed by HEK-293T cells, it could be simply removed from the co-transfections of the IL-7 signaling players.

4.3.1 Generation of a HEK-293T cell line knockdown for hJAK1

To create a HEK-293T knockdown for JAK1, we used shRNAs against the human JAK1 variant 1 gene. A shRNA scramble sequence (shSCR) was used as control.

HEK-293T cells were transduced with a shSCR and 3 different shRNAs against the hJAK1 variant 1 gene (shJAK1 A, shJAK1 B, and shJAK1 C). The cells were then expanded and sorted for mCherry, the reporter gene for positively

transduced cells (Figure 4.7), in order to obtain a pure population of mCherry positive cells.

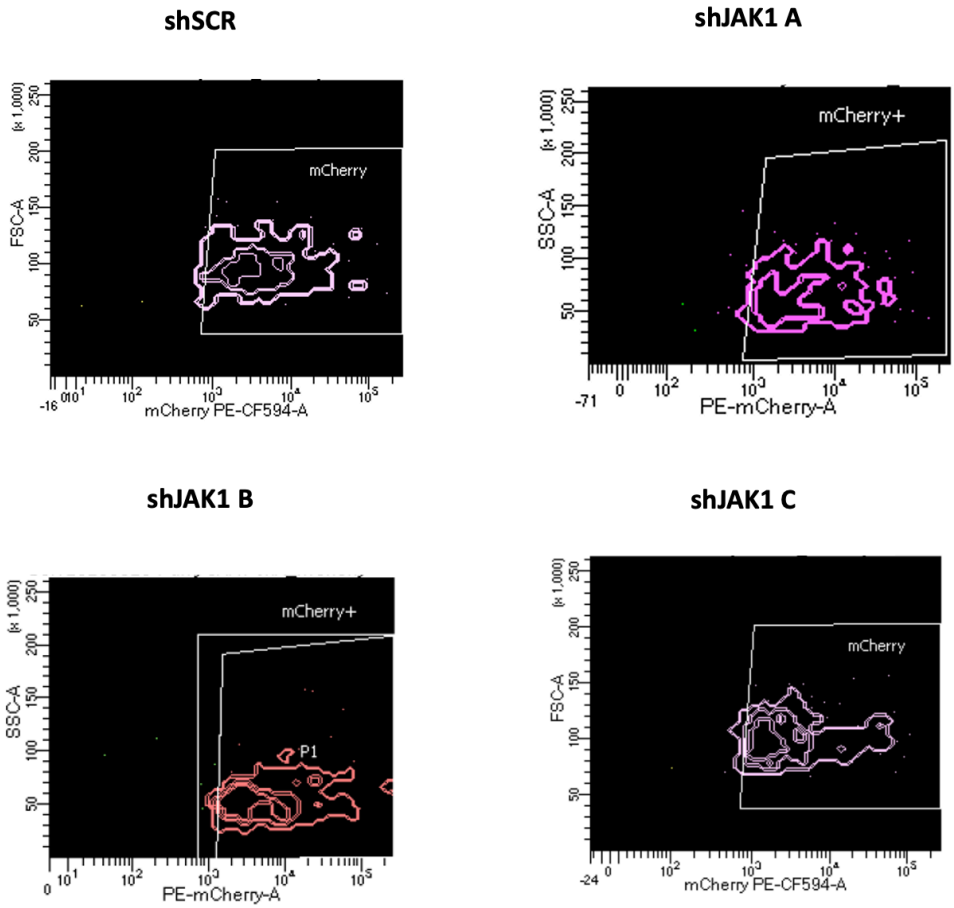

Figure 4.7. Gating strategy for mCherry sorting of HEK-293T cells transduced with sh RNAs for hJAK1. Cells were sorted for mCherry via FACSAria Fusion.

In order to verify the efficiency of knockdown by the shRNAs, we evaluated JAK1 mRNA levels by RT-qPCR and JAK1 protein levels by WB.

As demonstrated in Figure 4.8, all shRNAs induced a knockdown of at least 74% at the mRNA level, with shJAK1 B being the most efficient shRNA, inducing a knockdown of approximately 84%. In what regarded JAK1 protein levels, shJAK1 C showed the lowest levels.

Thus, we decided to proceed with our experiments using both shJAK1 B and shJAK1 C cell lines together with shSCR cell line as the control.

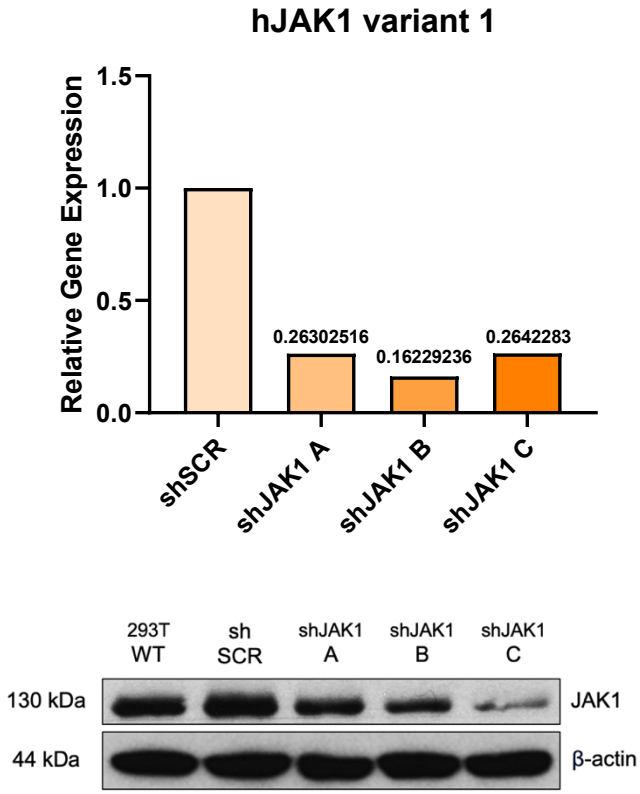

Figure 4.8. Relative gene expression and protein levels of JAK1 in HEK-293T cells transduced with shRNAs for hJAK1. Relative gene expression was evaluated by RT-PCR and normalized to shSCR condition. Protein expression was evaluated via Western Blot.
293T WT - HEK-293T cells untransduced.

4.3.2 Evaluation of TYK2 role in the IL-7 signaling pathway in the absence of JAK1, JAK3 or both

After establishing the new cell lines, we transfected the HEK-293T shSCR, HEK-293T shJAK1 B, and HEK-293T shJAK1 C with the conditions depicted in Figure 4.9. This experiment had three main goals:

1) To evaluate if TYK2 can replace JAK1 in the IL-7 signaling. The three cell lines were transfected with the different IL-7 pathway players and co-transfected with TYK2.
2) To evaluate if TYK2 can replace JAK3 in the IL-7 signaling. The three cell lines were transfected with the different IL-7 pathway players except for JAK3 and co-transfected with TYK2.

3) To evaluate if TYK2 can replace both JAK1 and JAK3. The three cell lines were transfected with the different IL-7 pathway players except for JAK3 and co-transfected with TYK2.

All of the experimental conditions were stimulated with IL-7 at 50 ng/mL for 15 minutes. WB analysis of IL-7 signaling downstream effectors was performed, and all blots were exposed simultaneously and equally in time to be comparable among experimental conditions. Some blots were exposed for more time to visualize the signal better.

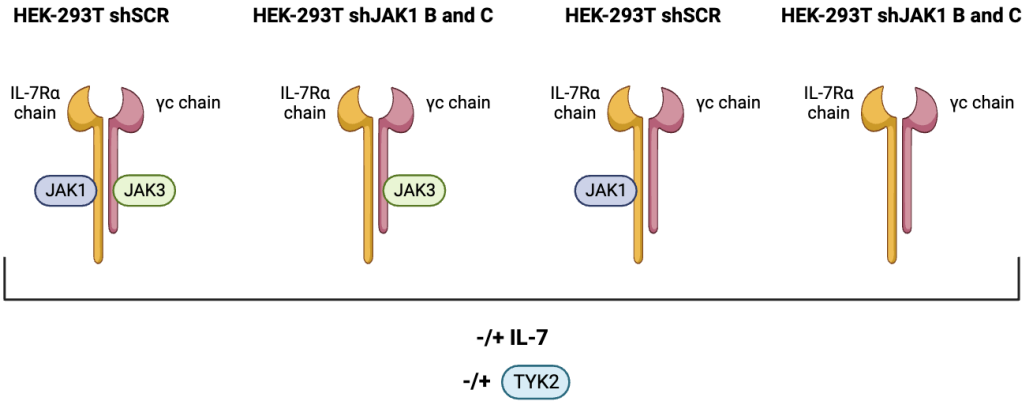

Figure 4.9. Schematic illustration of the conditions used in HEK-293T shSCR, shJAK1 B and shJAK1 C transfections to understand the role of TYK2 in the IL-7 signaling pathway. In all of the conditions, TYK2 was co-transfected to understand if it can replace the "missing" JAK. All of the conditions were unstimulated and stimulated with IL-7.
Image was created in BioRender.com.

As shown in Figure 4.10 A, IL-7 stimulation of shSCR cells transfected with all the IL-7 pathway players elicits an increase in STAT5 phosphorylation, indicating successful signaling (see higher exposure blot). Although a higher exposure blot is necessary to observe p-STAT5 in the control condition (shSCR) there is an increase of about 2.65 fold after stimulation (Figure 4.10 B).

It is worth noting that in shJAK1 B and shJAK1 C cells, despite the low JAK1 protein levels (Figure 4.10 A), IL-7 stimulation results in the phosphorylation of STAT5, although at lower levels in comparison with shSCR (Figure 4.10 B). The cell line shJAK1 B has an IL-7-induction fold of 1.42 and the shJAK1 C of 1.16. This result is consistent with IL-7 signaling being compromised in the absence of JAK1. Curiously, we further observed that JAK1 knockdown results in increased basal p-STAT5 levels (Figure 4.10 A and Figure 4.14).

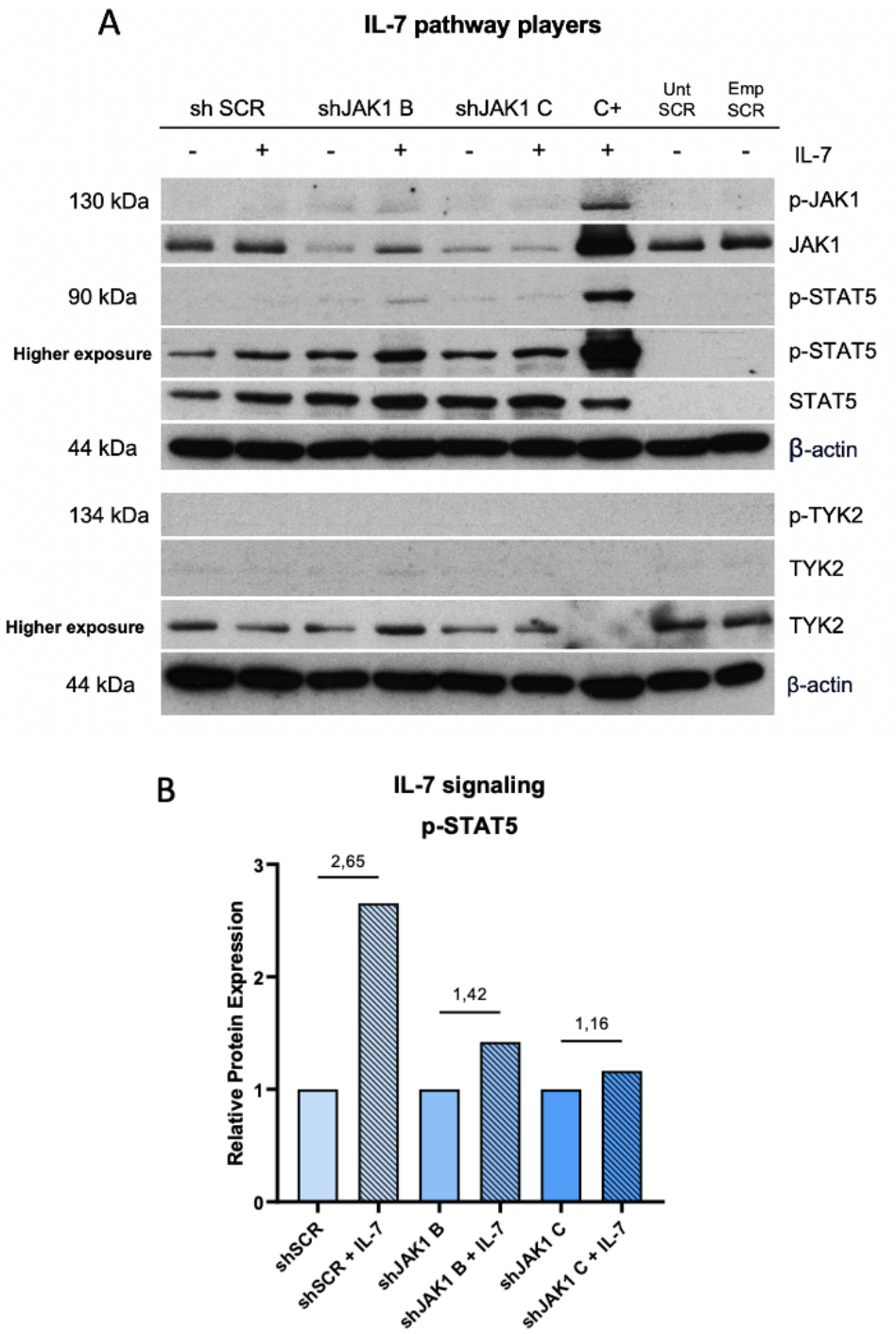

Figure 4.10. IL-7 signaling in the new HEK-293T cell lines with knockdown for JAK1. (A) Western blot analysis of cells transfected with the IL-7 pathway players (IL-7Rα chain, common γ chain, JAK3, and STAT5) and stimulated with IL-7 at 50 ng/mL for fifteen minutes. In order to better visualize p-STAT5 and TYK2, a higher exposure time was done. **(B)** Protein quantification analysis of p-STAT5 levels via ImageJ. p-STAT5 levels were normalized to total protein levels and then for β-actin. All conditions were normalized to each unstimulated condition. IL-7 stimulation induces STAT5 activation in JAK1 knockdown HEK-293T cells.
C+ - Positive Control for p-JAK1 (HPB-ALL cells stimulated with IL-7 at 50 ng/mL for fifteen minutes); Unt SCR – Untransfected HEK-293T shSCR cells; Emp SCR – HEK-293T shSCR cells transfected with empty constructs.

The introduction of TYK2 into the system, as depicted in Figure 4.11 A, results in an enhancement of STAT5 basal phosphorylation in the three cell lines (Figure 4.14). Moreover, in the cell lines HEK-293T shSCR and shJAK1 B, STAT5 activation increased upon IL-7 stimulation, with a fold change of 1.30 and 1.19, respectively. Regarding the shJAK1 C cell line, there isn't a push when the cytokine is introduced, as observed in Figure 4.11 A and B.

Furthermore, as shown in Figure 4.11 A and C, TYK2 overexpression leads to increased basal levels of p-JAK1 and an increase in the phosphorylation levels of JAK1 in response to IL-7 in all three cell lines. However, in the shSCR cell line, the push is more prominent. The reduced response to IL-7 in the cell lines shJAK1 B and shJAK1 C may be due to the high levels of basal p-JAK1 (Figure 4.15).

TYK2 co-transfection (Figure 4.11 A) results in its basal phosphorylation in shSCR and shJAK1 B cell lines, in which stimulation with IL-7 induces a push. In the shJAK1 C cell line, TYK2 basal phosphorylation is also enhanced, but it doesn't increase with IL-7.

Although the low STAT5 activation in the shJAK1 B cell line and the absence of it in shJAK1 C could suggest that TYK2 can't replace JAK1 in the IL-7 signaling pathway, our results are difficult to interpret and to make a final conclusion regarding TYK2's ability to perform JAK1's role. First, because we observe that TYK2 leads to an exacerbated activation of the remaining levels of JAK1 in the knockdown cell lines, which can mask our results in what concerns the exact role of TYK2. Second, the reduction of JAK1 endogenous levels increases STAT5 basal phosphorylation, which can result in reduced STAT5 IL-7-dependent activation. Taken together these considerations, it would be necessary in future experiments to do a knockout of JAK1 in this model. Nonetheless, our results show that TYK2 overexpression leads to increased p-STAT5 basal levels and JAK1 activation, independently of JAK1 levels.

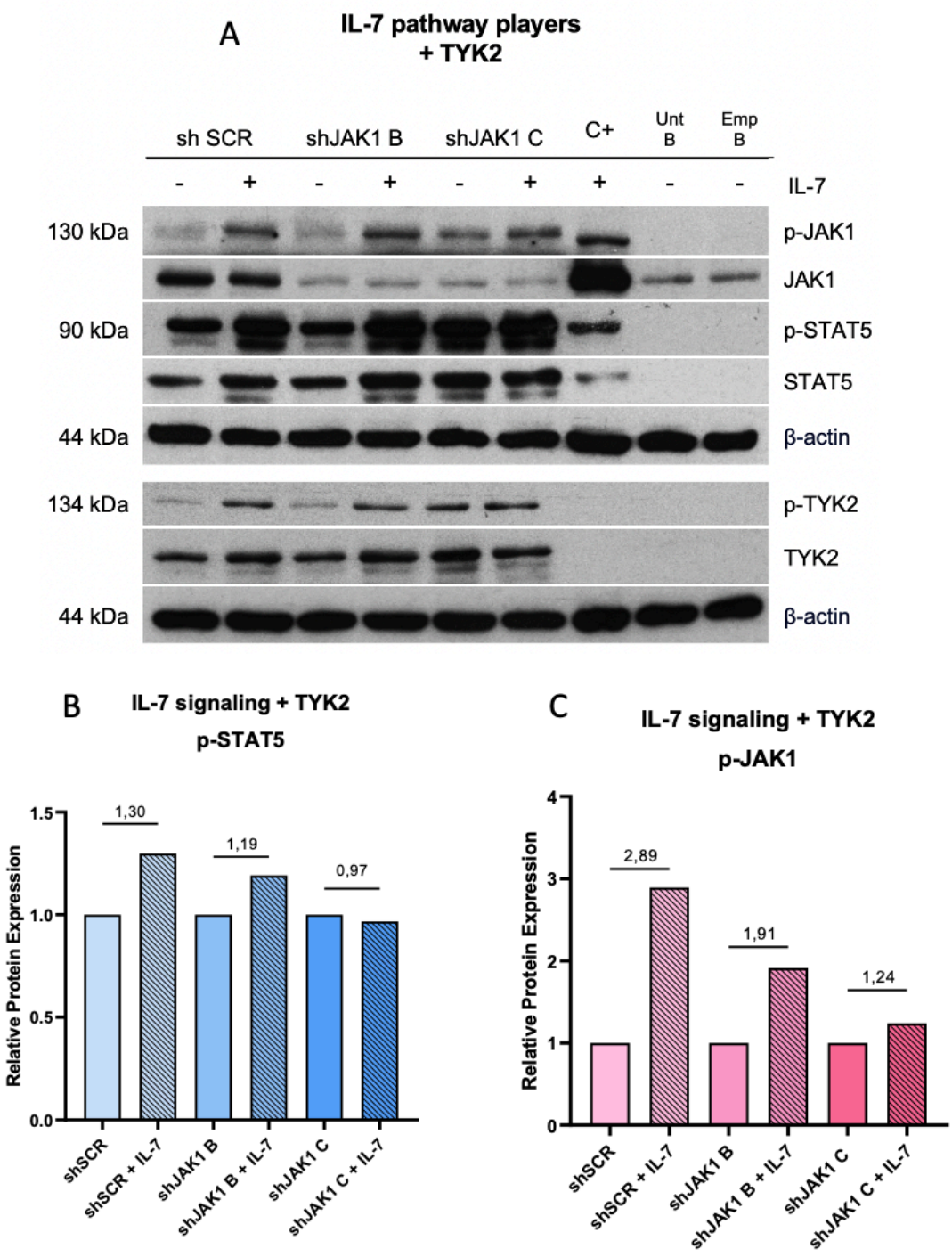

Figure 4.11. IL-7 signaling with TYK2 overexpression in the new HEK-293T cell lines with knockdown for JAK1. (A) Western blot analysis of cells transfected with the IL-7 pathway players (IL-7Rα chain, common γ chain, JAK3, and STAT5) and TYK2 and stimulated with IL-7 at 50 ng/mL for fifteen minutes. **(B)** Protein quantification analysis of p-STAT5 levels via ImageJ. p-STAT5 levels were normalized to total protein levels and then for β-actin. All conditions were normalized to each unstimulated condition. **(C)** Protein quantification analysis of p-JAK1 levels via ImageJ. p-JAK1 levels were normalized for β-actin. All conditions were normalized to each unstimulated condition. TYK2 increases basal STAT5 phosphorylation and JAK1 activation in response to IL-7 in the presence of low levels of JAK1.
C+ - Positive Control for p-JAK1 (HPB-ALL cells stimulated with IL-7 at 50 ng/mL for fifteen minutes); Unt B – Untransfected HEK-293T shJAK1 B cells; Emp SCR – HEK-293T shJAK1 B cells transfected with empty constructs.

Surprisingly, when JAK3 is not introduced into this system, it's possible to observe STAT5 activation in the shSCR condition, where JAK1 is fully present (Figure 4.12, higher exposure blot). This result is intriguing because the IL-7 pathway requires JAK3 to induce signaling[66], and according to the Human Protein Atlas, HEK-293 cells don't have JAK3 mRNA transcripts[112]. Nonetheless, in the absence of ectopic expression of JAK3 and in the presence of low levels of JAK1, as expected and observed in Figure 4.12, in shJAK1 B and shJAK1 C conditions, there is no response to IL-7 in what regards STAT5 and JAK1 activation.

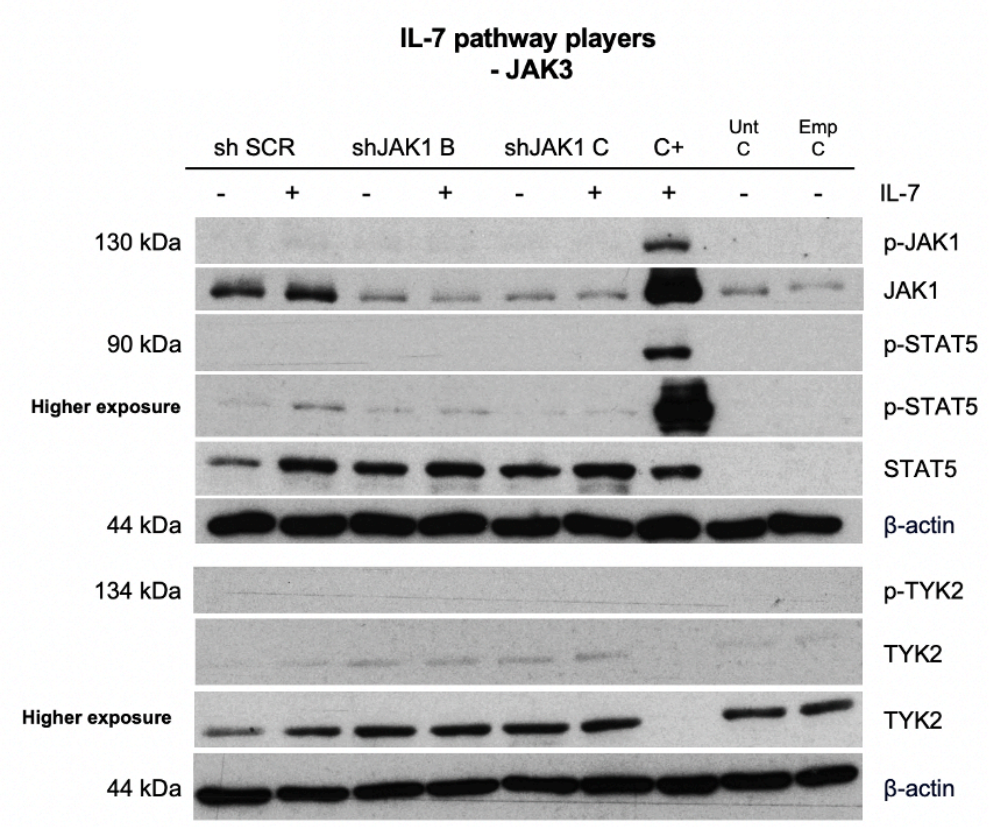

Figure 4.12. IL-7 signaling in the absence of JAK3 in the new HEK-293T cell lines with knockdown for JAK1. Western blot analysis of cells transfected with the IL-7 pathway players (IL-7Rα chain, common γ chain, and STAT5) and stimulated with IL-7 at 50 ng/mL for fifteen minutes. In order to better visualize p-STAT5, a higher exposure time was done. Reduced p-STAT5 levels in the absence of JAK3 in response to IL-7 in the HEK-293T shSCR cells.
C+ - Positive Control for p-JAK1 (HPB-ALL cells stimulated with IL-7 at 50 ng/mL for fifteen minutes); Unt SCR – Untransfected HEK-293T shJAK1 C cells; Emp SCR – HEK-293T shJAK1 C cells transfected with empty constructs.

Adding TYK2 to this system without JAK3 ectopic expression leads to an increase in basal p-STAT5 levels both in shSCR and shJAK1 B and C cells (Figure 4.13 A and Figure 4.14). Moreover, p-STAT5 levels are further increased upon IL-7 stimulation. A 1.25 fold increase in the shSCR cell line and a 1.33 fold increase in the shJAK1 B and C cell lines (Figure 4.13 B) can be observed in response to IL-7. However, with JAK1 normal levels (shSCR cell line), STAT5 activation happens at a lower extent when compared to the control condition (Figure 4.14 shSCR IL-7 signaling) since the basal levels are enhanced.

Furthermore, co-transfection of TYK2 in the absence of JAK3, also exacerbated JAK1 activation in response to IL-7, in the three cell lines, and p-JAK1 basal levels in the shJAK1 C cell line (Figure 4.13). Moreover, TYK2 activation in the presence of IL-7 also occurred, but only in the shSCR and shJAK1 B cell lines. In the shJAK1 C cell line, this absence of activation may be due to the high basal phosphorylation of TYK2.

Taken together, our results could suggest that in the absence of JAK3, TYK2 co-transfection restores IL-7 signaling, even if lower than the control condition (Figure 1.14), indicating that TYK2 is able to replace JAK3 in the IL-7 signaling pathway. However, the major drawback is the fact that even in the absence of JAK3, we observed STAT5 activation and, as such, we can't determine if TYK2 is the only factor inducing signaling activation.

Regarding TYK2's ability to perform JAK1 and JAK3's function simultaneously, we also can't make any conclusions. Even though co-transfecting TYK2 induces signaling, determined by STAT5 activation, in the cell lines shJAK1 B and C without JAK3 ectopic expression (Figure 4.12 A and B), we still have JAK1 endogenous levels, which activation is exacerbated by TYK2 co-transfection. As such, we can't confirm if the activation of the signaling is solely due to the presence of TYK2, or due to the remaining levels of JAK1.

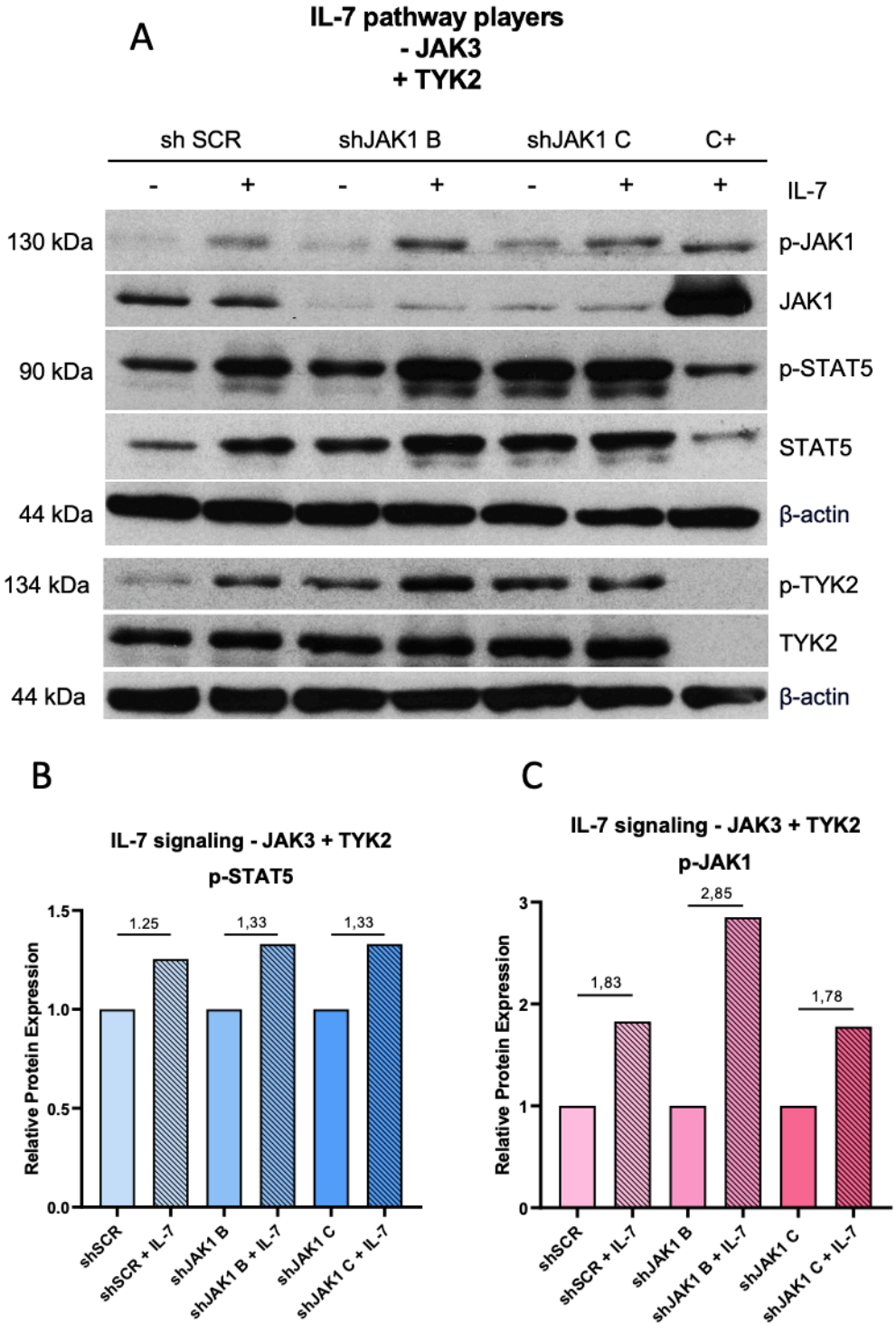

Figure 4.13. IL-7 signaling in the absence of JAK3 and with TYK2 ectopic expression in the new HEK-293T cell lines with knockdown for JAK1. (A) Western blot analysis of cells transfected with the IL-7 pathway players (IL-7Rα chain, common γ chain, and STAT5) and TYK2 and stimulated with IL-7 at 50 ng/mL for fifteen minutes. **(B)** Protein quantification analysis of p-STAT5 levels via ImageJ. p-STAT5 levels were normalized to total protein levels and then for β-actin. All conditions were normalized to each unstimulated condition**. (C)** Protein quantification analysis of p-JAK1 levels via ImageJ. p-JAK1 levels were normalized for β-actin. All conditions were normalized to each unstimulated condition. TYK2 overexpression in HEK-293T shJAK1 cells in the absence of JAK3 leads to higher basal p-STAT5 and JAK1 activation.
C+ - Positive Control for p-JAK1 (HPB-ALL cells stimulated with IL-7 at 50 ng/mL for fifteen minutes.

Although the experiments above do not allow us to conclude if TYK2 can replace JAK1 or JAK3 in the IL-7 signaling pathway, we showed nonetheless that TYK2 can exacerbate the activation of proteins of the IL-7 pathway. We show that TYK2 has the ability to increase p-STAT5 basal levels independently of JAK1 or JAK3 (Figure 4.14) Moreover, it also enhanced JAK1 basal and IL-7-dependent phosphorylation levels, which was also independent of JAK1 levels and JAK3 ectopic expression (Figure 4.15).

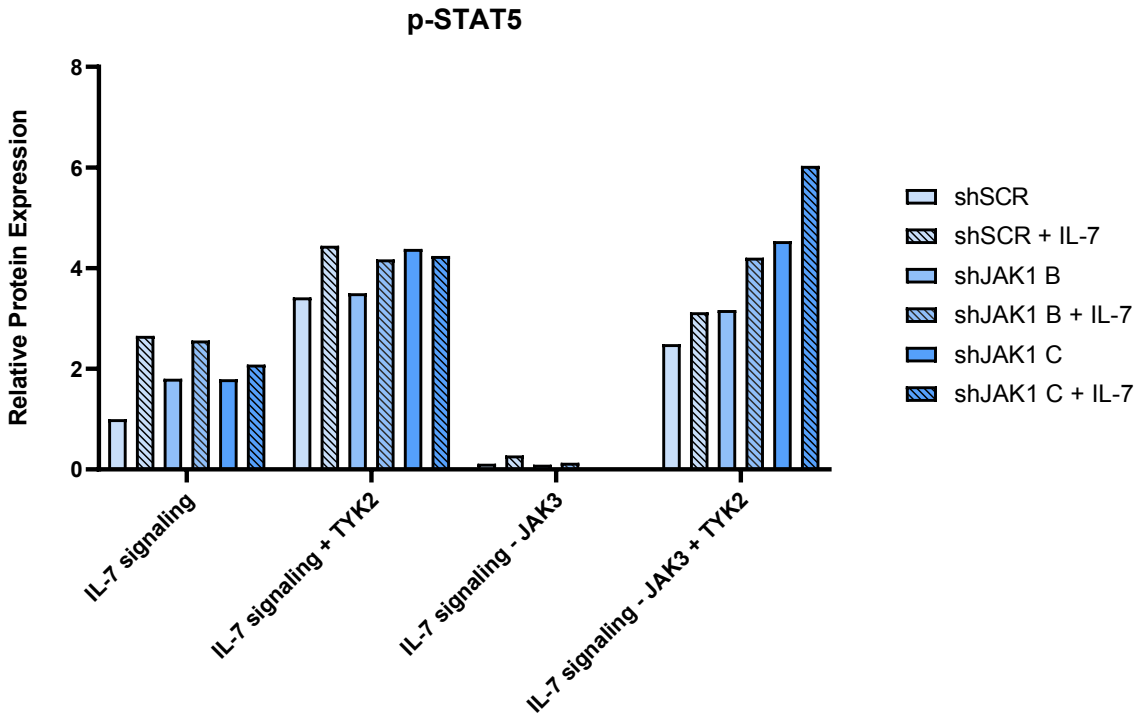

Figure 4.14. p-STAT5 quantification in all experimental conditions. Protein quantification analysis of p-STAT5 levels via ImageJ. p-STAT5 levels were normalized to total protein levels and then for β-actin. All conditions were normalized to shSCR IL-7 signaling (control).

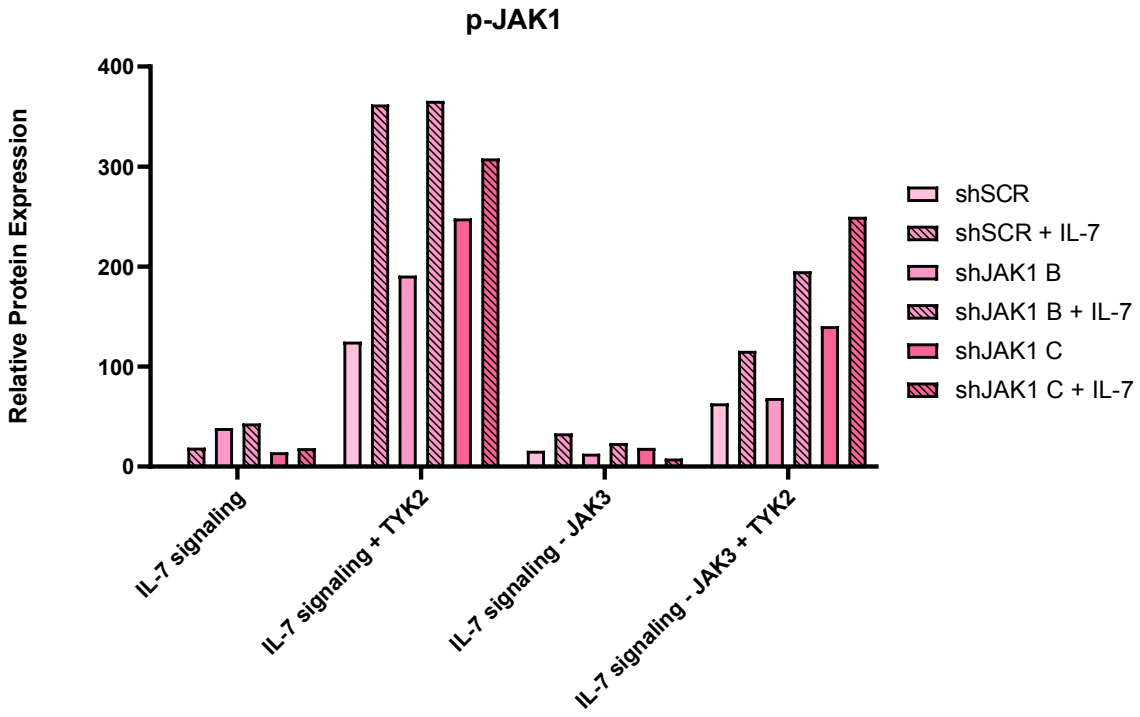

Figure 4.15. p-JAK1 quantification in all experimental conditions. Protein quantification analysis of p-JAK1 levels via ImageJ. p-JAK1 levels were normalized to β-actin. All conditions were normalized to shSCR IL-7 signaling (control).

4.3.2.1 Evaluation of endogenous TYK2 role in the activation of IL-7 signaling in the absence of JAK3

The previous results showed that even in the absence of ectopic JAK3, there was a response to IL-7, as observed in Figure 4.12. This finding contradicts the literature, which shows that JAK3 is crucial for IL-7-mediated signaling[66]. Furthermore, we noted that the HEK-293T shSCR cell line has TYK2 endogenous levels (Figure 4.12). Based on these observations, we hypothesized that the TYK2 endogenous levels may be able to activate the pathway in the absence of JAK3 in HEK-293T shSCR cells.

In order to evaluate our hypothesis, the HEK-293T shSCR cell line was transfected with the IL-7 pathway players, without and with JAK3, and TYK2 endogenous activity was inhibited with Deucravacitinib, a specific TYK2 inhibitor[103].

As shown in Figure 4.16, IL-7 induces STAT5 activation in the control conditions (DMSO), indicating that our experiment is working. When all the players in the IL-7 pathway are present, and endogenous TYK2 is inhibited, STAT5 activation still occurs, though to a lower extent.

When ectopic JAK3 isn't present, STAT5 activation after IL-7 stimulation is observed, as previously observed in Figure 4.12 in Chapter 4.3.2. Upon treatment with the TYK2 inhibitor, phosphorylation of STAT5 is no longer visualized in basal and IL-7-stimulated conditions in the absence of JAK3, as shown in Figure 4.16. These results strongly suggest that TYK2 endogenous levels are responsible for STAT5 activation when JAK3 is absent from the signaling.

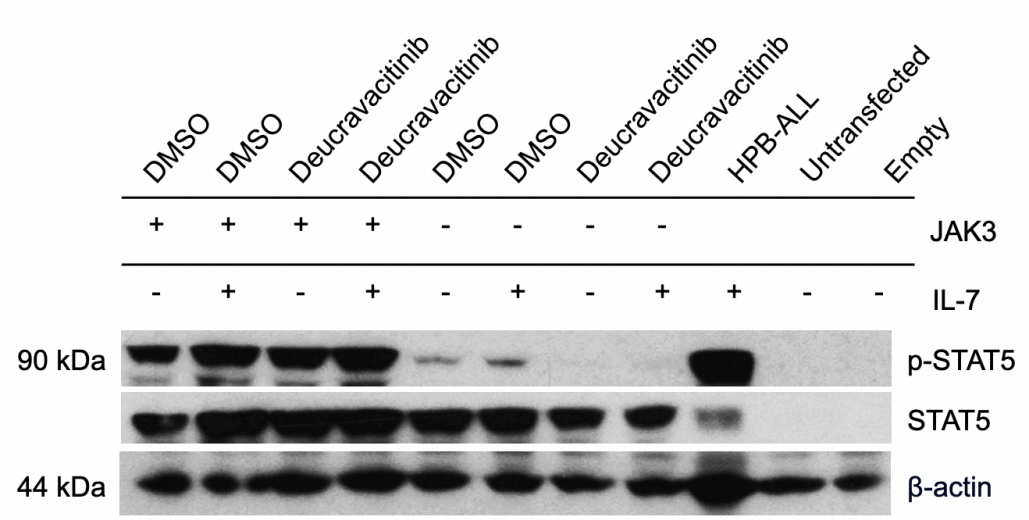

Figure 4.16. Evaluate if TYK2 endogenous levels are responsible for STAT5 activation in response to IL-7 in the absence of JAK3. HEK-293T shSCR cell line was transfected with IL-7Rα chain, common γ chain and STAT5 and, after 24 hours, treated with Deucravactinib. The cells were stimulated with IL-7 at 50 ng/mL for fifteen minutes, 48 hours after the transfection. Results suggest that TYK2 endogenous levels are responsible for STAT5 activation in response to IL-7 in the absence of JAK3. HPB-ALL cells were stimulated with IL-7 at 50 ng/mL for 15 minutes and used as a positive control; Untransfected – HEK-293T shSCR cell line untransfected; Empty – HEK-293T shSCR cell line transfected with empty constructs.

We were unable to detect p-TYK2 or p-JAK1 with the given exposure time, as demonstrated in Figure 4.17. Nevertheless, total protein levels were present.

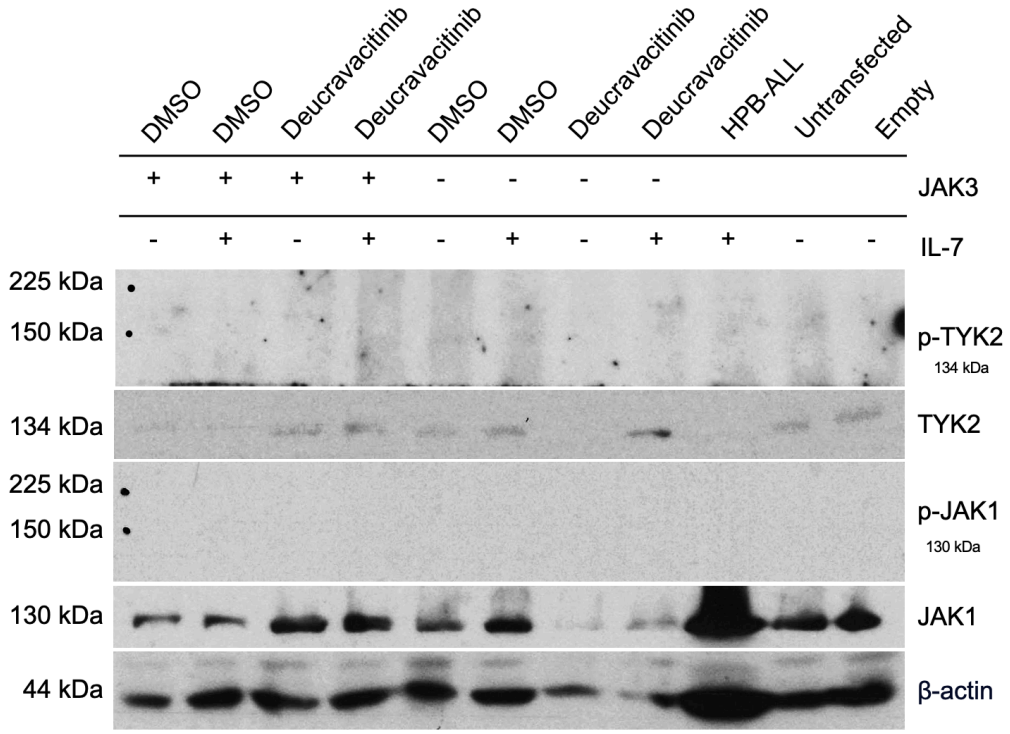

Figure 4.17. TYK2 and JAK1 expression in HEK-293T shSCR treated with Deucravacitinib. HEK-293T shSCR cell line was transfected with the IL-7Rα chain, common γ chain, and STAT5 and, after 24 hours, treated with Deucravactinib. The cells were stimulated with IL-7 at 50 ng/mL for fifteen minutes 48 hours after the transfection.
HPB-ALL cells were stimulated with IL-7 at 50 ng/mL for 15 minutes and used as a positive control; Untransfected – HEK-293T shSCR cells untransfected; Empty – HEK-293T shSCR cells transfected with empty constructs.

4.4 Evaluation of TYK2 association with the IL-7R complex in HEK-293T model

In order to ascertain the association between TYK2 and the IL-7R complex, we conducted a transfection of HEK-293T cells with the various players of the IL-7 pathway, with or without TYK2. We then proceeded to immunoprecipitate the IL-7Rα chain.

As shown in Figure 4.18, the immunoprecipitation of the IL-7Rα chain was successful, as determined by the band below 76 kDa (indicated by the black

arrow). Moreover, the IL-7R heterodimer was also precipitated, evidenced by the upper band ranging from 76 to 102 kDa (indicated by an orange arrow).

With the regular signaling pathway players (Figure 4.18, upper panel), it's possible to visualize JAK1, and JAK3 associated with the IL-7R. Moreover, it’s also possible to visualize the presence of endogenous TYK2, associated with the IL-7R complex (yellow arrow).

Upon co-transfection of TYK2 (Figure 4.18, lower panel), the association of TYK2 to the IL-7R complex is again observed, including in the IL-7 stimulation condition. Curiously, the presence of JAK3 associated with the IL-7R complex is no longer evident with TYK2 co-transfection.

Of note, all of the detected proteins associated with the IL-7R had a lower signal when the cells were stimulated with IL-7.

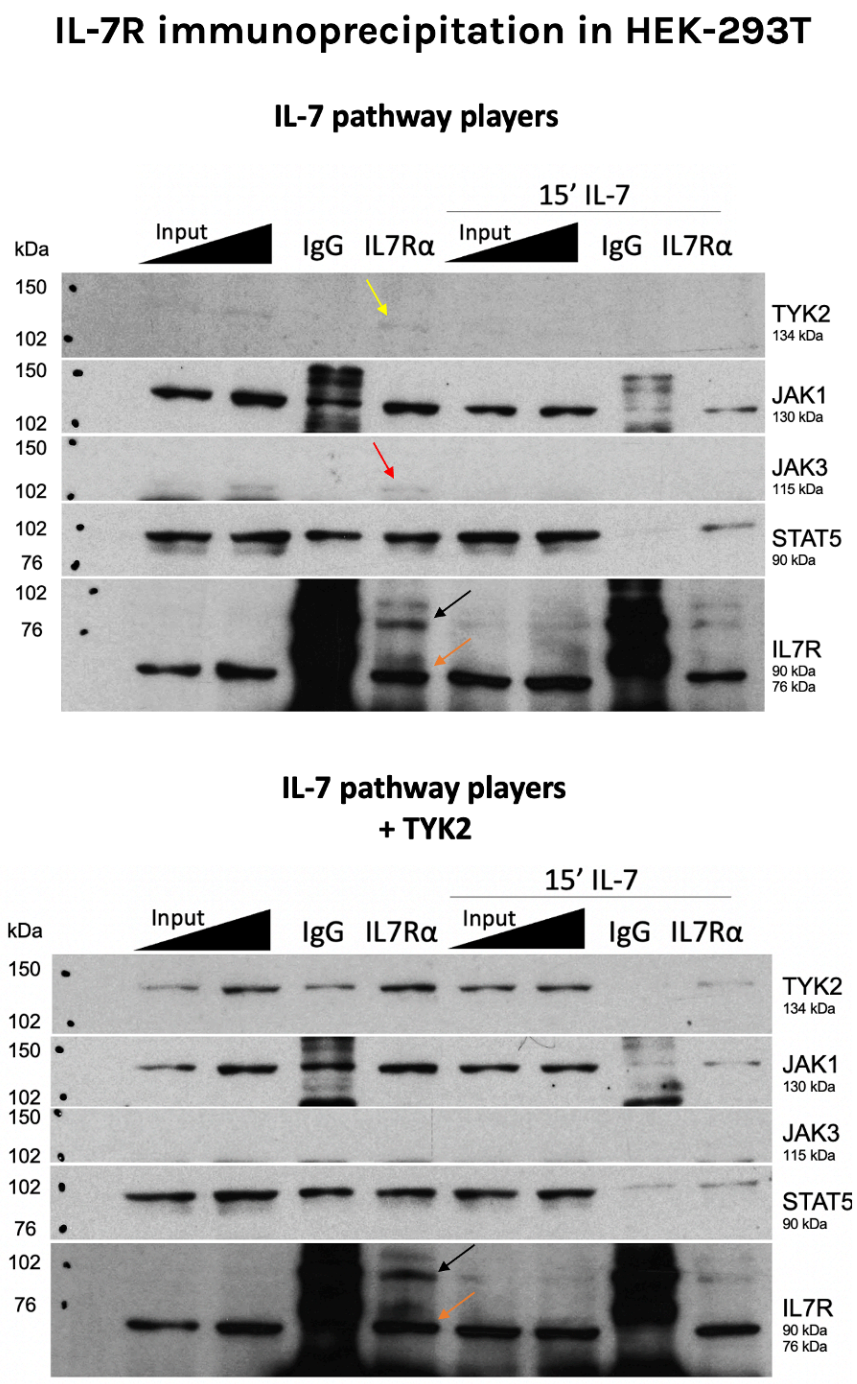

Figure 4.18. IL-7Rα chain immunoprecipitation in HEK-293T IL-7/IL-7R signaling pathway ectopic model. Immunoprecipitation of the IL-7Rα chain was performed in HEK-293T cells transfected with the different players of the IL-7 pathway (upper panel) and with TYK2 (bottom panel). Both conditions were unstimulated and stimulated with IL-7 at 50 ng/mL for fifteen minutes. The yellow arrow indicates TYK2 and the red arrow JAK3. The black arrow indicates the IL-7R heterodimer and the orange arrow the IL-7Rα chain. Input is characterized by 10 μg and 30 μg total protein extracts, respectively. TYK2 is found in association with the IL-7R complex.

To validate these results, TYK2 immunoprecipitation was performed next, and the association with IL-7R was evaluated. TYK2 immunoprecipitation was successful in both experimental conditions, as observed in Figure 4.19.

With the regular signaling pathway players (Figure 4.19, upper panel), it's possible to observe the IL-7Rα chain in the total protein extracts in the input, represented by the band below 76 kDa. The IL-7R heterodimer is also present in the input, represented by the band between 76 kDa and 102 kDa. Curiously, in the TYK2 immunoprecipitated condition, the molecular weight of the IL-7Rα chain seems to be higher than the band observed in the input, closer to the 76 kDa marker, suggesting a possible conformational change (orange arrow). In what regards JAK1 and JAK3, we observed that JAK1 is associated with TYK2 after IL-7 stimulation, and JAK3 is only present in the input before stimulation.

By co-transfecting TYK2 (Figure 4.19, lower panel), the same changes in the molecular weight of the IL-7Rα, are found in the immunoprecipitation condition. JAK1 is observed in association with TYK2 in both IL-7 stimulated and unstimulated conditions, and JAK3 remains unassociated.

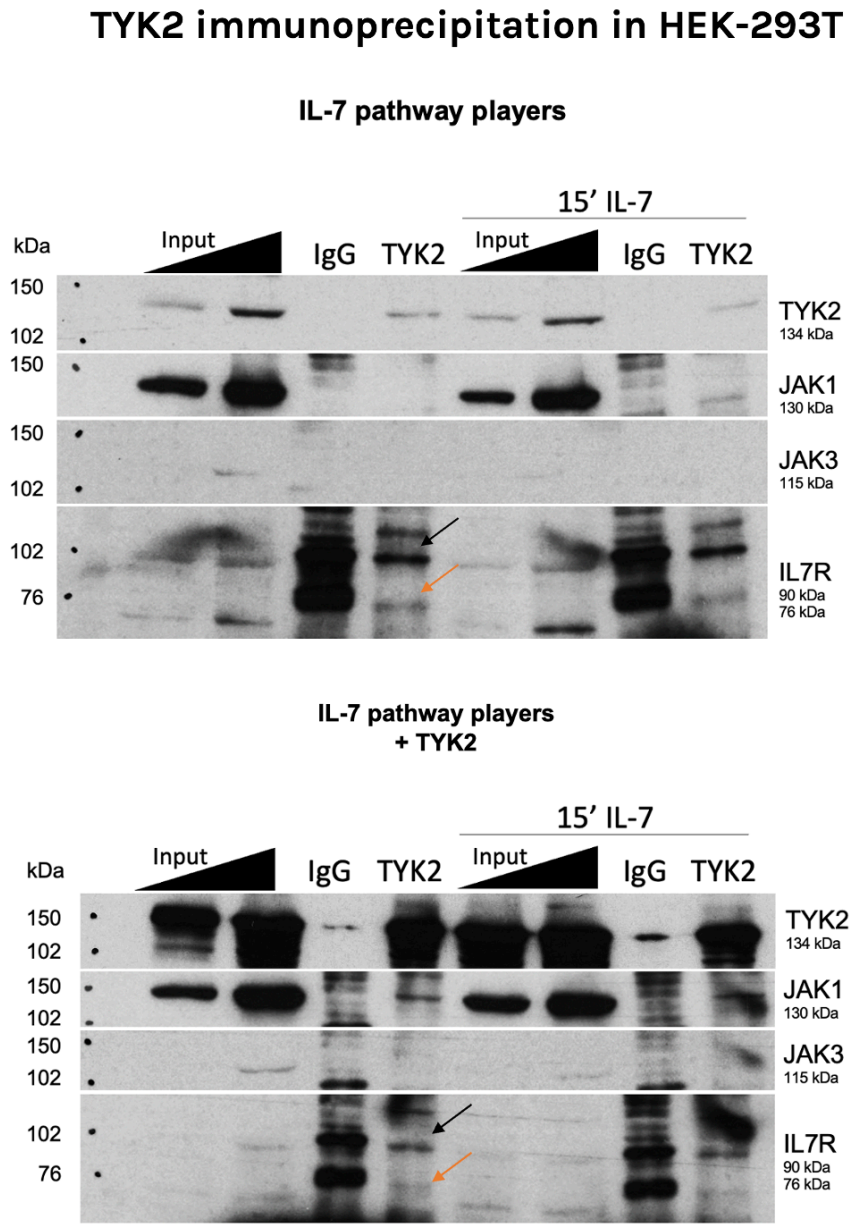

Figure 4.19. TYK2 immunoprecipitation in HEK-293T IL-7/IL-7R signaling pathway ectopic model. Immunoprecipitation of TYK2 was performed in HEK-293T cells transfected with the different players of the IL-7 pathway (upper panel) and with TYK2 (lower panel). Both conditions were unstimulated and stimulated with IL-7 at 50 ng/mL for fifteen minutes. The black arrow indicates the IL-7R heterodimer and the orange the IL-7Rα. Input is characterized by 10 μg and 30 μg total protein extracts, respectively. IL-7R complex is found in association with TYK2.

Our results show that, in this HEK-293T IL-7/IL-7R signaling pathway ectopic model, TYK2 is associated with the IL-7R complex.

4.5 Evaluation of TYK2 association with the IL-7R complex in HEK-293T model, in the absence of JAK1 and JAK3

Since TYK2 showed an association with the IL-7R complex, we sought to understand if altering the presence of JAK1 or JAK3 would impact TYK2's interaction with the complex. For this, the experimental setting used in Chapter 4.3.2 was performed, but only using the HEK-293T shSCR and HEK-293T shJAK1 C cell lines.

First, the shJAK1 C cell line was transfected with all the IL-7 pathway players and then co-transfected with TYK2, to evaluate if there were differences in TYK2's association with the IL-7R complex in the presence of low levels of JAK1 protein. Subsequently, IL-7Rα immunoprecipitation was conducted in unstimulated and stimulated cells.

In the condition with the IL-7 pathway players (Figure 4.20, upper panel), the IL-7Rα chain was effectively precipitated. However, the heterodimer band (represented by the black arrow) is much weaker than in the previous precipitations (e.g. Figure 4.18, upper panel). Moreover, in this condition, TYK2 doesn't show an association with the IL-7R, and it is only found in the input.

With the co-transfection of TYK2 and by comparing the upper panel with the lower panel in Figure 4.20, it is possible to observe that the heterodimer band of the receptor is more prominent than before. Moreover, JAK3's association with the receptor increases when TYK2 is co-transfected. This observation was only made with JAK1 lower levels and was not found in the immunoprecipitations with JAK1 normal endogenous levels (Figure 4.18). Regarding TYK2's association with the IL-7R with lower JAK1 levels, there is a slight signal.

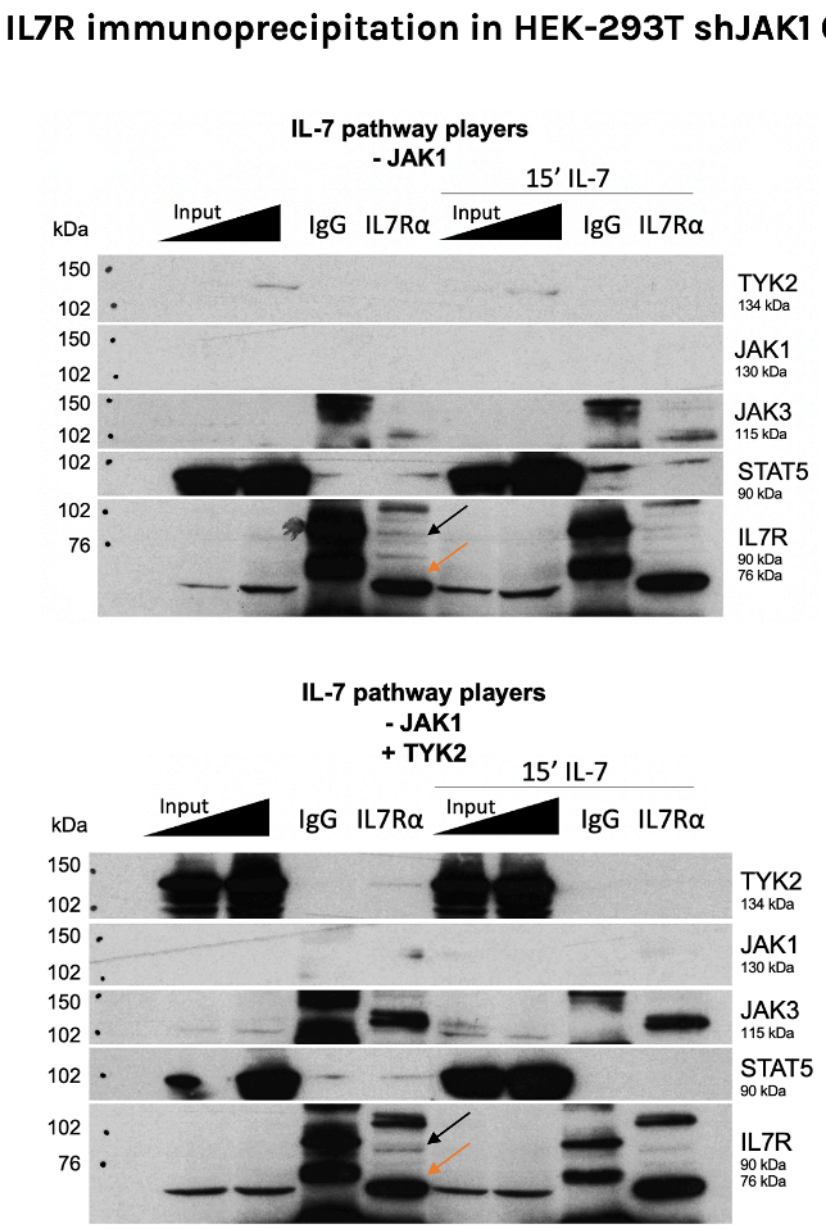

Figure 4.20. IL-7Rα chain immunoprecipitation in HEK-293T JAK1 knockdown cells. Immunoprecipitation of the IL-7Rα chain in the HEK-293T shJAK1 C cell line transfected with the IL-7 pathway players (upper panel), and with TYK2 (lower panel). Both conditions were unstimulated and stimulated with IL-7 at 50 ng/mL for fifteen minutes. The black arrow indicates the IL-7R heterodimer, and the orange arrow indicates the IL-7Rα chain. Input is characterized by 10 μg and 30 μg total protein extracts, respectively.

Regarding the removal of JAK3 from the IL-7 pathway, in the condition with the normal players (Figure 4.21 upper panel), the IL-7Rα chain immunoprecipitation was successful, as determined by the presence of the band below 76 kDa and the second band between 76 kDa and 102 kDa, indicating the heterodimer. However, TYK2 wasn't found associated with the IL-7R.

In the condition with ectopic TYK2, the IL-7Rα chain and the IL-7R heterodimer immunoprecipitation were also successful, as shown in Figure 4.21 lower panel. Nonetheless, TYK2 was once again not found associated with the precipitated proteins, and we were not able to observe JAK1 or STAT5 associated with the IL-7R complex. This suggests that JAK3 may be necessary for assembling the IL-7R complex, as neither JAK1 nor STAT5 were present in the precipitate condition and could explain why TYK2 is not associated to the complex in these conditions.

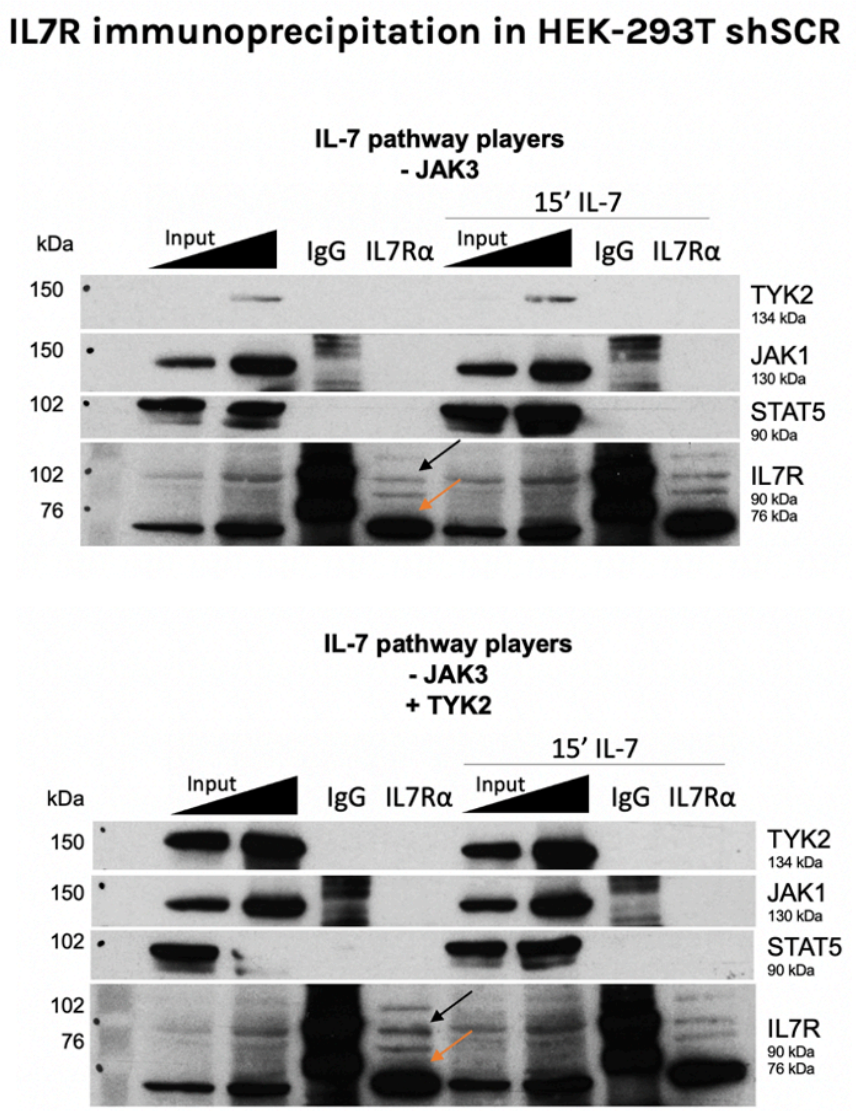

Figure 4.21. IL-7Rα chain immunoprecipitation in HEK-293T model in the absence of JAK3. Immunoprecipitation of the IL-7Rα chain in the HEK-293T shSCR cell line transfected with the IL-7 pathway players, except for JAK3 (upper panel) and with TYK2 (lower panel). Both conditions were unstimulated and stimulated with IL-7 at 50 ng/mL for fifteen minutes. The black arrow indicates the IL-7R heterodimer and the orange arrow the IL-7Rα chain. Input is characterized by 10 μg and 30 μg total protein extracts, respectively.

Our results showed that in the absence of JAK3, TYK2 is not associated with the IL-7R. In what regards JAK1 lower levels, TYK2 has a weak association. However, we cannot discard the possibility that for the IL-7R complex to assemble, it requires JAK1 and JAK3, and this could be the reason why TYK2 is not found in the absence of these two proteins.

4.6 Evaluation of TYK2 association with the IL-7R complex in TAIL7 cells

We also attempted to evaluate the association between TYK2 and the IL-7R complex in the T-ALL cell line TAIL7, which expresses IL-7R endogenously, by immunoprecipitating the IL-7Rα.

Our results showed that only the IL-7Rα chain was precipitated instead of the usual IL-7Rα chain and heterodimer, as previously demonstrated in other immunoprecipitation experiments (Figure 4.22). In concordance with the heterodimer not being precipitated, JAK3, which binds to the common γ chain, was not observed in the immunoprecipitated condition. Of note, it was also absent in total protein extracts, represented in the "input" condition in Figure 4.22. Additionally, the presence of TYK2 was only observable in the input of the stimulated condition at very low levels.

These results demonstrate that the IL-7R complex was not precipitated, as JAK3 and the heterodimer aren't detected. Moreover, TYK2 was not detected either, which may suggest that, in the IL-7R complex, TYK2 does not associate with the IL-7Rα chain.

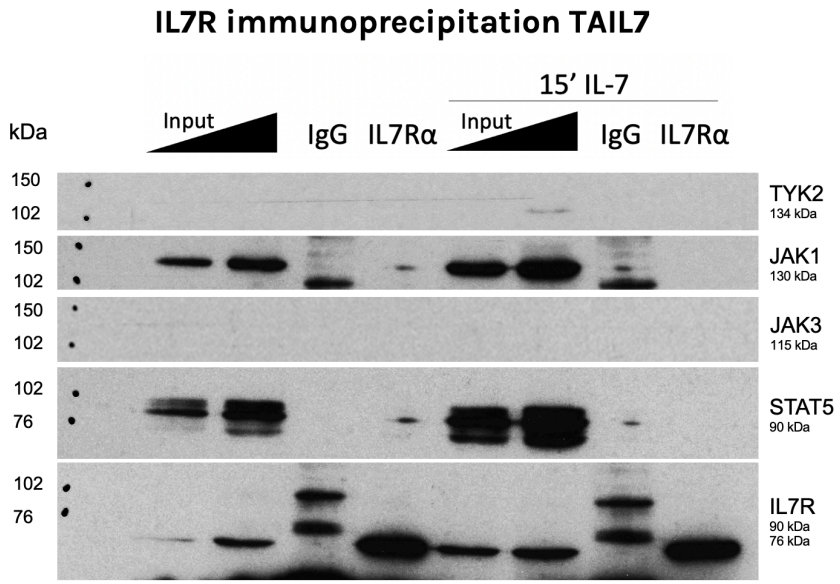

Figure 4.22. IL-7R immunoprecipitation in TAIL7 cell line. TAIL7 cells were deprived of IL-7 for 24h and afterwards stimulated with IL-7 at 50 ng/mL for fifteen minutes. Immunoprecipitation of the IL-7Rα chain was attempted. Input is characterized by 10 μg and 30 μg total protein extracts, respectively.

4.7 Evaluation of TYK2 inhibition biological impact in TAIL7 cells

In order to evaluate the biological role of TYK2 in IL-7-dependent T-ALL cells, TAIL7 cells were treated with increasing concentrations of Deucravacitinib, and cell viability, activation, and IL-7R surface expression were assessed.

TAIL7 cells require IL-7 for their survival. For instance, when these cells are cultured without the cytokine for 24 hours, 50% of the population dies. However, upon stimulation with IL-7, their viability increases[65]. Thus, to understand if TYK2 has a role in IL-7-dependent viability in T-ALL cells, we inhibit TYK2 and evaluated its impact in IL-7 induced cell viability.

However, the control condition (DMSO) in Figure 4.23 shows no major increase after IL-7 stimulation. This indicates that the time-point (72h) at which the cells were collected, and their viability was assessed, was not the appropriate one, and a longer time-point should be considered to observe alterations in the viability.

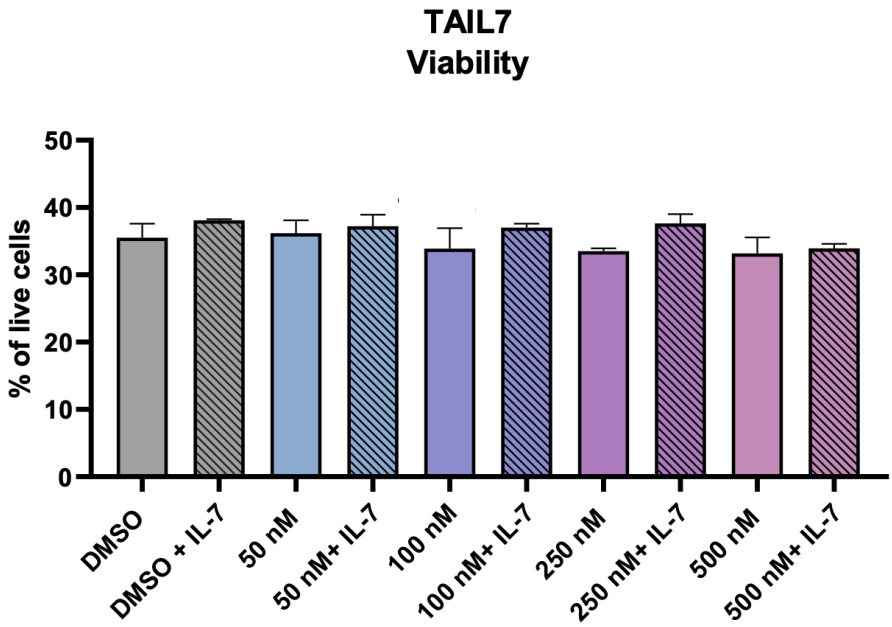

Figure 4.23. Viability assay in TAIL7 cells treated with Deucravacitinib. This experiment was performed once. The cells were deprived of IL-7 for 24 hours, then treated with increasing concentrations of Deucravacitinib (50 nM, 100 nM, 250 nM and 500 nM), and stimulated with IL-7 at 20 ng/mL. For the vehicle condition, the volume applied was the same as the highest concentration from the inhibitor (500 nM). Cells were microscopically evaluated 24 hours and 48 hours upon treatment with TYK2 inhibitor and IL-7 stimulation, and then collected at 72 hours for flow cytometry analysis. Graphics were made in GraphPad.

Another objective was to understand if inhibiting TYK2 would affect TAIL7 activation. TAIL7 activation occurs in the presence of IL-7, and it leads to an increase in cell size, which can be analyzed by flow cytometry using SSC and FSC parameters[65]. As shown in Figure 4.24, activated TAIL7 cells correspond to the subpopulation gated within Live cells.

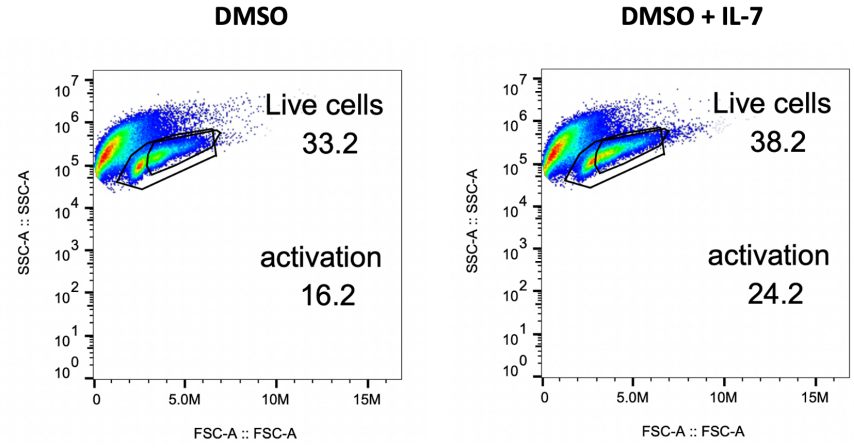

Figure 4.24. Flow cytometry analysis of TAIL7 activation.
Plot on the left are TAIL7 cells in the DMSO condition. Plot on the right are TAIL7 cells in DMSO condition stimulated with IL-7.

As we can appreciate in Figure 4.25, IL-7 induces TAIL7 activation in the DMSO condition. We couldn't perform statistical tests because the experiment was performed only once, corresponding the error bars to the three technical replicates done for each condition. Nonetheless, we can appreciate that the activation fold induced by IL-7 is lower at the highest concentration of the inhibitor (1.225) when compared to the DMSO control condition (1.368). The results suggest a tendency for lower IL-7 activation of TAIL7 cells when TYK2 is inhibited. However, this needs to be confirmed with new experiments and assessed at later time points and possibly higher concentrations of the inhibitor.

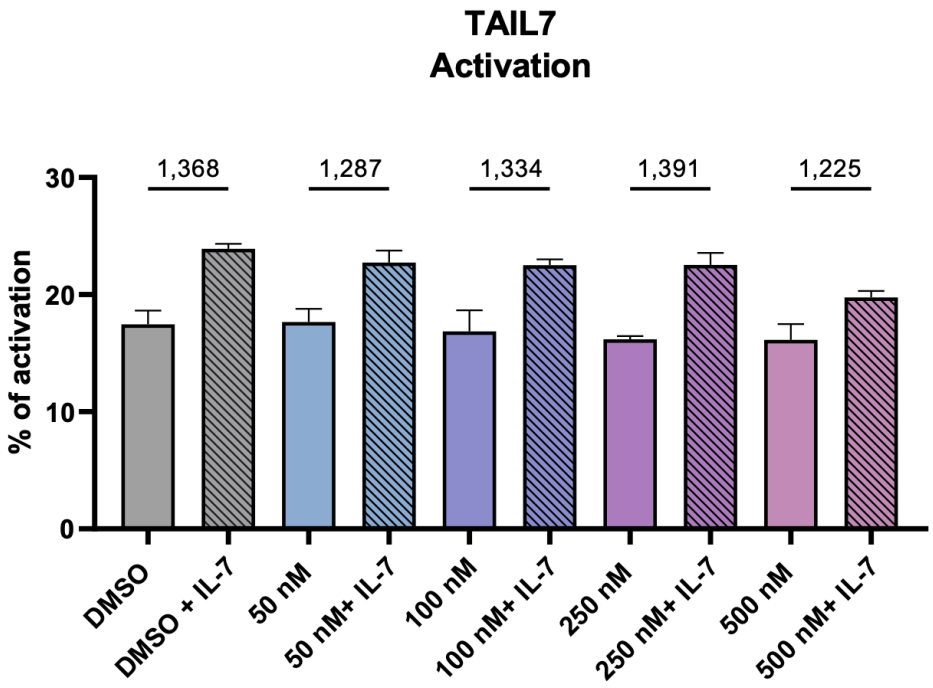

Figure 4.25. Activation assay in TAIL7 cells treated with Deucravacitinib. This experiment was performed once. The cells were deprived of IL-7 for 24 hours, then treated with increasing concentrations of Deucravacitinib (50 nM, 100 nM, 250 nM, and 500 nM), and stimulated with IL-7 at 20 ng/mL. For the vehicle condition, the volume applied was the same as the highest concentration from the inhibitor (500 nM). Cells were microscopically evaluated 24 hours and 48 hours upon treatment with TYK2 inhibitor and IL-7 stimulation, and then collected at 72 hours for flow cytometry analysis. For each condition, three technical replicates were performed.
The values above the columns represent the fold change between conditions. Graphics were made in GraphPad.

TAIL7 cells suffer receptor internalization after IL-7 stimulation, which is necessary for IL-7-mediated signaling and cell activation[66]. As such, IL-7R surface expression was also evaluated in order to understand if TYK2 inhibition could be functionally associated with the receptor membrane levels.

As shown in Figure 4.26 in the DMSO condition, stimulating the cells with IL-7 led to a decrease in IL-7R surface expression, indicating that our experiment worked.

Figure 4.26 shows a clear tendency for the receptor membrane levels to decrease as the concentration of the inhibitor becomes higher in the presence of IL-7. In fact, the difference in fold change between the DMSO condition (0,509) and the 500 nM condition (0,364) was 0,145.

Different hypotheses can be formulated regarding TYK2's role in the membrane receptor levels. One of them could be that TYK2 is stabilizing the receptor in the membrane, and its inhibition results in impaired receptor membrane levels in the presence of IL-7. The second could be that TYK2 participates in the recycling route of the receptor, as signaling activation induces receptor internalization and either results in receptor recycling or degradation. TYK2 inhibition could impair the recycling pathway and decrease the membrane levels, moreover, this would explain why this phenomenon only occurs in the presence of IL-7. The third hypothesis could be that TYK2 inhibition could even induce a faster internalization.

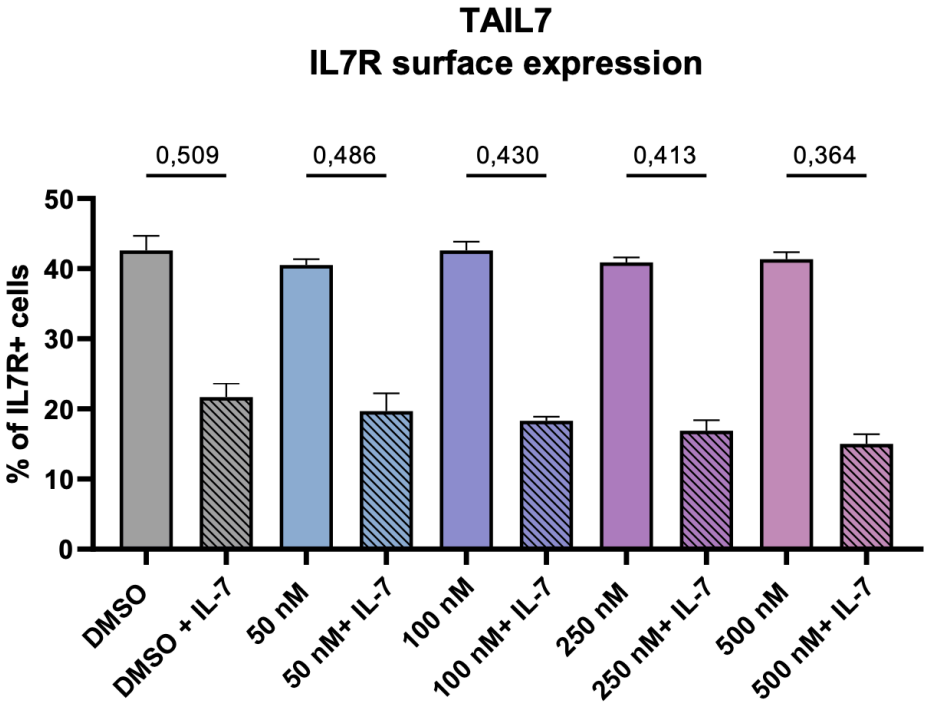

Figure 4.26. IL-7R surface expression evaluation in TAIL7 cells treated with Deucravacitinib. This experiment was performed once. The cells were deprived of IL-7 for 24 hours, then treated with increasing concentrations of Deucravacitinib (50 nM, 100 nM, 250 nM and 500 nM), and stimulated with IL-7 at 20 ng/mL. For the vehicle condition, the volume applied was the same as the highest concentration from the inhibitor (500 nM). Cells were microscopically evaluated 24 hours and 48 hours upon treatment with TYK2 inhibitor and IL-7 stimulation, and then collected at 72 hours for flow cytometry analysis. For each condition, three technical replicates were performed.
The values above the columns represent the fold change between conditions. Graphics were made in GraphPad.

This experiment needs to be repeated as it was only performed once. Nonetheless, it allowed us to evaluate where optimization needs to be performed and also make certain observations. Despite our viability assay didn't show an increase in cell viability in the presence of IL-7, we were able to observe changes regarding cell activation and IL-7R surface expression.

Our findings demonstrate a slight decrease in TAIL7 activation at the highest inhibitor concentration. Similarly, when evaluating IL-7R surface expression, there was a tendency for the IL-7R membrane levels to reduce as the inhibitor concentration increased, but only in the presence of IL-7.

From this experiment, we were also able to detect parameters that require optimization. The first is to use in future experiments a higher time point, such as 96 hours or 120 hours, even though at the 72-hour time point, alterations in the viability should start to be observable, according to the literature[65]. Moreover, as

the activation results only suggest alterations with the highest inhibitor concentration, it would be helpful to increase the concentrations of Deucravacitinib.

5 Discussion

T-cell acute lymphoblastic leukemia is a subgroup of acute lymphoblastic leukemia, the most common childhood cancer, accounting for 15% of pediatric and 25% of adult cases[34]. T-ALL results from the malignant transformation and expansion of T-cell progenitors. Nowadays, the event-free survival exceeds the 85% mark in children, however, in adults, it is significantly lower, with only 20-40% of cases achieving event-free survival[38,48]. Patients are exposed to intensive multiagent chemotherapeutic agents and often relapse having poor outcomes[38]. T-ALL is associated with genetic alterations and one of the most common altered pathways is the IL-7 signaling pathway, which displays major roles in T-cell development[24,64,65].

Our laboratory is dedicated to gain a deeper understanding of the mechanisms involved in IL-7 signaling, to uncover its significance in the context of T-ALL and ultimately identify new therapeutic targets and develop novel therapeutic strategies. To characterize the signaling landscape in IL-7-dependent T-ALL, we employed Reverse Phase Protein Arrays to evaluate the changes in the phosphorylation levels of several proteins in response to IL-7. Our experiment yielded significant results, indicating that phosphorylation of TYK2, a Janus kinase protein never before associated with the IL-7 signaling, occurs upon IL-7 stimulation. Thus, this project aimed at understanding the role of TYK2 in the IL-7/IL-7R signaling pathway and also in the context of IL-7-dependent T-ALL.

5.1 Validate IL-7-mediated TYK2 phosphorylation

In the first part of this project, we were able to validate the RPPA results and demonstrate that D1 hWT-IL-7R, TAIL7, and BA/F3 hWT-IL-7R cell lines suffer TYK2 phosphorylation after IL-7 stimulation (Figure 4.2).

D1 cells are thymocytes, and TAIL7 cells are T-cell blasts arrested at an immature thymocyte stage,[65,113] and BA/F3 cells are murine pro-B cells[114]. The later TYK2 activation in the BA/F3 hWT-IL-7R cell line could be due to differences regarding cell lineage. As such, TYK2 activation in the IL-7 signaling could be

more prominent in the T-cell lineage than in the B-cell lineage. Nonetheless, this later response to IL-7 could also be due to the biological state of these cells. As for example older cells tend to respond slower to stimuli.

5.2 Understand the role of TYK2 in the IL-7 signaling pathway

Following our confirmation of TYK2 phosphorylation in response to IL-7 signaling pathway activation, we sought to understand how mechanistically this process unwinds. Is TYK2 phosphorylation a byproduct of IL-7 signaling activation or does TYK2 take part in IL-7 signaling cascade? To address this question, we took advantage of an ectopic model of IL-7/IL-7R signaling pathway already established in the lab[115]. This system uses the HEK-293T cell line which, apart from JAK1, lacks the expression of several key players in the IL-7 pathway, including IL-7Rα, common γ chain, JAK3, and STAT5.

Using this ectopic approach, we demonstrated that co-transfection of the IL-7 signaling players and TYK2, leads to TYK2 phosphorylation upon IL-7 stimulation, (Figure 4.4). On note, phosphorylation of TYK2 could be observed in the absence of IL-7, an effect that can be attributed either to TYK2's autophosphorylation self-ability, a capacity that has been previously reported in the literature [116,117]; or to the operation of another pathway within these cells.

Interestingly, overexpression of TYK2 also resulted in STAT5 activation prior to IL-7 stimulation. TYK2 has been shown to have the ability to interact with STAT5 before cell stimulation with IFNα[118]. More specifically, in the absence of IFNα, TYK2 was associated with STAT5, and both proteins were in their phosphorylated state[118]. As such, it could be possible that TYK2 overexpression could lead to an enhancement of basal STAT5 phosphorylation in the absence of IL-7 stimulation[118]. Moreover, in our experiment, cell stimulation with IL-7 also led to a considerable increase in the levels of phosphorylated STAT5.

We also aimed to understand the impact of TYK2 overexpression on JAK1 and JAK3 activation using the same experimental approach. However, even though several antibody optimizations were attempted, we were not able to visualize JAK1 or JAK3 phosphorylation. We then hypothesized that the time

point at which these proteins activation was being analyzed was not the best one. To address if this was the case, we conducted a kinetic experiment (Figures 4.5 and 4.6) and analyzed p-JAK1 and p-JAK3 levels at different time points after IL-7 stimulation. Although we still failed to detect JAK1 and JAK3 phosphorylation in all the time points tested (Figure 4.6), we were able to observe STAT5 activation as early as 5 minutes after cell stimulation with IL-7, with clearer results at the 15 minutes time point (Figure 4.5). Since IL-7-induced STAT5 activation occurs downstream of JAK1 and JAK3 activation[63], this experiment allowed us to validate the time point of 15 minutes post IL-7 stimulation chosen for analysis of IL-7 signaling pathway players activation.

Interestingly, with this experiment, we also observed that not only does TYK2 overexpression led to basal STAT5 phosphorylation, but it also results in a faster response to IL-7 in what regards activation of STAT5 (Figure 4.5).

Since TYK2 is a Janus kinase, just like JAK1 and JAK3, we sought to understand if TYK2 could have a redundant function with either JAK1, JAK3 or both in the IL-7 signaling cascade. Although the HEK-293T ectopic model allowed us to easily manipulate the IL-7 signaling players being expressed at a specific condition, since these cells express endogenously JAK1, a sub-line knockdown for JAK1, had to be generated.

In what regards a potential redundant role between TYK2 and JAK1, we observed that TYK2 overexpression leads to increased levels of basal STAT5 phosphorylation independently of JAK1 expression levels since this effect was observed both in control condition (shSCR cells) and in JAK1 KD conditions (shJAK1 B, and shJAK1 C cell lines) (Figure 4.11 A and B). Moreover, we further observed that in the shSCR and in the shJAK1 B cell lines, p-STAT5 levels increase after IL-7 stimulation. Although IL-7-induced STAT5 activation was less robust than the observed in the control condition (shSCR in Figure 4.10 A and Figure 4.14), it could be suggestive of TYK2 overtaking the function of JAK1. However, not only we did not observe the same effect in shJAK1 C cell line, which had no response to IL-7 in what regards STAT5 activation; as TYK2 overexpression led to increased levels of basal and IL-7-induced levels of p-JAK1 (Figure 4.11 A and C, and Figure 4.15) which can be responsible for the pathway activation. TYK2's ability to activate JAK1 is not surprising since it is widely

accepted that these two JAKs participate in several JAK/STAT pathways,[79,89,92,119] and are able to interact constitutively[117].

It is essential to acknowledge that this part of our study might need to be further optimized or re-drawn. Instead of doing a knockdown of the JAK1 gene, a knockout might be more beneficial, for instance, using the CRISPR-Cas9 technique, as the knockdown cells used might have different expression levels of JAK1, which could result in different results, as we saw signaling activation in the shJAK1 B cell line with TYK2 overexpression but not in the shJAK1 C cell line. Another point is that the remaining JAK1 levels in these two cell lines may be sufficient to induce signaling (Figure 4.10), which can compromise our results and cover the effect of TYK2 in the pathway. Another possible approach is to complement our approach with a JAK1 inhibitor, for example, Ruxolitinib, which is a JAK1 and JAK2 inhibitor, and it wouldn't affect TYK2 or JAK3 activity.

Aiming at understanding if TYK2 could replace JAK3's function in the IL-7 signaling cascade, we used the same IL-7/IL-7R ectopic model but this time removing JAK3 from the co-transfections. Our results demonstrated that, as previously observed for control and JAK1 knockdown conditions, TYK2 overexpression induces basal STAT5 activation in all conditions (Figure 4.13 A and B). This effect thus seems to be independent of JAK3, which is, to some extent, expected since TYK2 is known to collaborate with JAK1 and JAK2 in various signaling pathways but has not been reported as interacting with JAK3[77,78]. Moreover, we showed, once again, that JAK1 activation is exacerbated in the presence of TYK2 ectopic expression, and it is independent of JAK3 (Figure 4.15). We were also able to observe that co-transfection of TYK2 in the absence of JAK3 increased STAT5 activation in the shSCR cell line in the presence of IL-7. This could be suggestive that TYK2 is able to display JAK3's functional role. However, in the control condition of this experiment (Figure 4.12), STAT5 activation was already present in the absence of JAK3, even if at low levels. This means we cannot confirm that the co-transfection with TYK2 is responsible for signaling activation in the absence of JAK3. Nonetheless, we showed that TYK2 endogenous levels were responsible for that specific STAT5 activation when JAK3 was not present (Figure 4.16). Thus, and although pending further

confirmation, we can hypothesize that TYK2 can exert JAK3's role in the IL-7 signaling cascade.

5.3 Analyze the association of TYK2 to the IL-7R signaling complex

We wondered if TYK2 could be physically associated with the IL-7R complex. Through immunoprecipitation of the IL-7Rα, we found that TYK2 was associated with the IL-7R complex (Figure 4.18) in both conditions with endogenous and overexpressing levels of TYK2 and in the presence and absence of IL-7. Interestingly, and although this observation needs to be confirmed, when TYK2 was overexpressed, JAK3 was no longer present in the IL-7R complex.

TYK2 association to the IL-7R complex was further investigated by performing TYK2 immunoprecipitation and analyzing IL-7Rα pull-down (Figure 4.19). Intriguingly, although we identified a band pattern that seems to be consistent with that of IL-7Rα chain and IL-7R heterodimer, it had a higher molecular weight than that observed in total protein extracts (Figure 4.19). This observation requires further investigation, but changes in the molecular weight of a protein may be indicative of a conformational change. Studies have identified such conformational changes in receptors through WB analysis[120]. It is known that the IL-7R can suffer different post-translational alterations, such as N-glycosylation and disulfide bond formation[120–122]. Curiously, N-glycosylation increases the interaction of IL-7 to the IL-7R, [121,122] and if TYK2 were to be associated with this form of the receptor, it could give insight into how this protein exacerbates the IL-7 signaling.

Further investigation needs to be performed in order to understand if TYK2 binds preferentially to a glycosylated form of the receptor. For instance, the same experiment could be performed but pre-treating the cells with an endoglycosidase protein. If TYK2 preferentially binds to a glycosylated form of the receptor, treating the cells with an endoglycosidase should decrease its binding to the IL-7R, which could be observed in immunoblot[83].

We also tried to evaluate if changes in the IL-7R complex, particularly the absence of JAK1 and JAK3, impact TYK2's ability to bind to the IL-7R complex. Our results demonstrate that either in the absence of JAK1 (Figure 4.20) or JAK3 (Figure 4.21), TYK2 is not associated with the IL-7R complex. Different studies have highlighted that JAK kinases, besides having a role in phosphorylating substrates, can also stabilize proteins, including receptors[83–86,96,116]. This could explain why TYK2 is not associated with the IL-7R complex, as it could need JAK1 and JAK3 for stabilization. Our immunoprecipitations, including in the absence of JAK1 and JAK3, also showed that the WB band of the IL-7R heterodimer was present, although not as prominent when the JAKs were present. This could mean that there are remaining levels of the receptor dimer, but at lower levels. As such, the probability of seeing TYK2 associated with the heterodimer could be reduced and explain why we don't observe TYK2 associated with the IL-7R complex in the absence of JAK1 and JAK3.

We have further tried to investigate if TYK2 associates with the IL-7R complex in TAIL7, a T-ALL cell line that expresses the IL-7R and that is IL-7 dependent. Unfortunately, we have reason to believe that only the IL-7Rα chain of the receptor was precipitated (Figure 4.22). This hypothesis is supported by the absence of JAK3 and the heterodimer detection, as JAK3 couples directly with the common γ chain. Thus, since we didn't observe TYK2 in the IL-7R pulldown extract, we could speculate that in the IL-7R complex TYK2 does not associates with the IL-7Rα. Nonetheless, we cannot disregard the possibility that, in T-ALL cells TYK2 does not associate with the IL-7R complex.

5.4 Characterize the functional role of TYK2 in IL-7-dependent T-ALL cells

To investigate the biological function of TYK2 in the context of IL-7 signaling-dependent T-ALL cells, we treated TAIL7 cells with increasing concentrations of Deucravacitinib, a TYK2-specific inhibitor, and evaluated its impact on leukemia cell viability, activation, and IL-7R surface expression.

The TAIL7 cell line is an IL-7-dependent T-ALL cell line and, as such, it requires IL-7 for its survival and viability. In fact, in the absence of IL-7 in culture

conditions, 50% of the population dies in 24 hours, but the viability can be increased after the introduction of the cytokine. According to the studies that have characterized this cell line, 72 hours after stimulation, it should be possible to start to observe an increase in viability[65]. Based on our results, the 72-hour time point wasn't the most suitable for evaluating cell viability since our control condition didn't respond to IL-7 properly (Figure 4.23), as it showed almost no increase in cell viability. Even though at this time point we should observe an increase, in future experiments, it would be beneficial to use a longer time point, such as 96 hours or even 120 hours. Therefore, we couldn't draw any conclusions regarding TYK2's impact on TAIL7 cell viability. Furthermore, enhancing Deucravacitinib's concentration might also be promising. A study using the human T-ALL cell line HPB-ALL, demonstrated that using a TYK2 inhibitor (NDI-031301) for 72 hours, decreases cell viability with an IC50% of 2,143 nM, a much higher concentration than the ones used in our experiments[123].

TAIL7 activation occurs after IL-7 stimulation, and it is characterized by an enhancement in cell size[65]. We were able to observe an increase in cell size in the control condition (DMSO) upon IL-7 stimulation, indicating that the cells were activated after stimulation (Figure 4.25). At the highest concentration of the inhibitor (500 nM), the differences between the unstimulated cells and the stimulated ones had a lower fold change than the DMSO condition, which could mean that TYK2 inhibition is impairing the activation process. However, we were not able to perform statistical tests, so this hypothesis needs further confirmation. Besides, further testing with higher concentrations is also necessary to evaluate the extent of this possible correlation.

When the IL-7 signaling is activated, it leads to receptor internalization, reducing IL-7R surface expression,[66] which can be observed in Figure 4.26 (DMSO versus DMSO + IL-7 condition). In this regard, our results show that, as the inhibitor's concentrations increased, there was a reduction in the surface levels of the IL-7R in the presence of IL-7.

TYK2 has been associated with the capacity of stabilizing the IFNAR1 receptor, more precisely by preventing its internalization in endosomes[83]. If having a similar function in the IL-7 signaling pathway, this could explain why IL-7R surface levels decrease when TYK2 is inhibited. However, this only happens

when the signaling is activated by IL-7. As such, if this were to be the mechanism through which TYK2 exerts its role in the IL-7R pathway, a decrease in the IL-7R surface levels should also occur in absence of IL-7.

Another study investigated has investigated TYK2 chaperone capacity in the thrombopoietin receptor (TpoR) pathway, which uses JAK2 and TYK2 as signal transducers[97]. This receptor, like the IL-7R, suffers internalization and can be either degraded or recycled back to the surface after stimulation with its corresponding cytokine, thrombopoietin (Tpo)[97]. In this study, the authors demonstrated that JAK2 and TYK2 protect the receptor from proteasomal degradation by enhancing the recycling route[97].

If TYK2 were to have a role in the recycling route of the IL-7R, it would make sense that its inhibition would result in decreased membrane levels upon IL-7 stimulation, as the receptor would not be able to return to the membrane. Nonetheless, new studies are required to test this hypothesis.

6 Conclusion

Through our research, we have shown that IL-7 has the ability to phosphorylate TYK2 in four distinct cell lines, utilizing both ectopic and endogenous approaches.

We demonstrated that overexpression of TYK2 using an ectopic approach enhances STAT5 basal phosphorylation and increases JAK1 activation, independently of JAK3 and JAK1 expression.

Our research also showed compelling evidence that TYK2 is associated with the IL-7R complex and, most likely, not with the IL-7Rα chain.

The results have also suggested that inhibiting TYK2 in the context of IL-7-mediated T-ALL might reduce IL-7-induced cell activation and IL-7R surface levels. However, further testing is required.

7 Future perspectives

Further research is required to fully comprehend the interaction of TYK2 with the IL-7R complex and its putative function. Employing the same ectopic approach but with the integration of supplementary measures may yield more advantageous outcomes.

One area that warrants further investigation is the question if TYK2 is able to replace JAK1 function. Our experiments using a JAK1 knockdown revealed that a gene knockout or protein inhibition, is required. Additionally, we found a potential link between TYK2's ability to perform JAK3 activity, as we showed TYK2 is able to restore signaling in the absence of JAK3, and TYK2 endogenous levels are responsible for signaling activation in the absence of JAK3. However, further investigation is necessary to confirm this hypothesis. So, using the same experimental model in future experiments, it would be beneficial to use a TYK2 inhibitor.

Our ectopic approach showed a possible association between TYK2 and the IL-7R complex. However, it is necessary to validate this in a physiological approach, for instance through immunoprecipitation of the IL-7R in TAIL7 cells, which we also must repeat. Nonetheless, the immunoprecipitation performed in the TAIL7 cell line showed compelling evidence that TYK2 might not be associated to the IL-7Rα chain. TYK2 could be associated to the common γ chain, and to evaluate this observation, it would be beneficial to do the immunoprecipitation of the common γ chain and check for TYK2 pull down. Another possible technique to validate the association between TYK2 and the common γ chain could be the implementation of a Proximation Ligation Assay.

Additionally, determining whether TYK2 has a stronger association with a modified receptor could aid in analyzing its function in the IL-7 signaling. We also hypothesized that TYK2 could have a role in IL-7R recycling. Thus, it would be beneficial to do an immunofluorescence assay and check for TYK2 and the IL-7R in endocytic vesicles, specifically the ones associated with receptor recycling.

In order to gain a better understanding of TYK2's functional role in IL-7-dependent T-ALL, we must optimize our experiments by increasing the time point

at which we collect cells and utilizing a higher concentration of the TYK2 inhibitor. Additionally, repeating the experiments is necessary to verify if our observations have statistical significance. Moreover, incorporating IL-7-dependent PDXs or IL-7-dependent T-ALL patient samples and subjecting them to the same viability, activation, and receptor surface expression assays would also provide a valuable complementary approach to our current methodology. This could help deepen our understanding of the role of TYK2 in IL-7-mediated T-ALL and potentially offer new insights for therapeutic intervention.

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