SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Ruxolitinib-mediated Modulation and Dynamic Expression of P-glycoprotein in Human CD8+ T Cell Memory Subsets During Differentiation

by Kipchumba Biwott

Supervisor: Zsolt Bacso, M.D. Ph.D.

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

DEBRECEN, 2025

Ruxolitinib-mediated Modulation and Dynamic Expression of P-glycoprotein in Human CD8+ T Cell Memory Subsets During Differentiation

By Kipchumba Biwott (MSc)

Supervisor: Zsolt Bacso MD. Ph.D.

Doctoral School of Molecular Cellular and Immune Biology, University of Debrecen

Head of the **Defense Committee**:	Prof. József Tőzsér, D.Sc.
Reviewers:	Barbara Uzonyi, Ph.D. Zsolt Sarang, Ph.D.
Members of the Defense Committee:	Imre Gombos, Ph.D. Endre Kókai, Ph.D.

The PhD Defense takes place at the University of Debrecen, Faculty of Medicine, Department of Emergency Care and Oxyology, Lecture Hall, at 11:00 AM on November 6, 2025.

1. INTRODUCTION

P-glycoprotein, also known as multidrug resistance protein 1, is encoded by the ABCB1 gene and belongs to the ATP-binding cassette transporter family. It acts as an ATP-dependent efflux pump, exporting xenobiotics, toxins, and drugs from cells. Highly expressed at biological barriers such as the blood-brain barrier, gut, liver, and kidneys, it plays a crucial detoxification role.

In the immune system, P-glycoprotein is present in monocytes, dendritic cells, natural killer cells, B cells, and both CD4+ and CD8+ T cells. In cytotoxic T cells, it protects against oxidative stress, supports activation, proliferation, and persistence, and contributes to cytokine secretion and cholesterol transport functions vital for immune regulation. Overexpression of P-glycoprotein in cancer cells is a key mechanism of chemotherapy resistance, including against tyrosine kinase inhibitors like imatinib. Interestingly, memory CD8+ T cells expressing this protein show enhanced survival, making them promising candidates for immunotherapy.

The Janus kinase–signal transducer and activator of transcription pathway is another core immune regulatory mechanism. Mutations in this pathway are common in blood cancers such as polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Ruxolitinib (RUX), a selective Janus kinase 1 and 2 inhibitor, is approved for treating these disorders and graft-versus-host disease. It is primarily metabolized by cytochrome P450 enzymes, but interactions with P-glycoprotein may affect its bioavailability and efficacy. Notably, inactivation of the ABCB1 gene has been shown to enhance RUX's anti-proliferative effects in T cells, suggesting a pharmacokinetic role for the transporter.

Programmed death-1 is a co-inhibitory receptor on activated T cells that promotes immune tolerance but contributes to immune exhaustion in chronic inflammation. RUX has been reported to reduce programmed death-1 expression, potentially restoring T cell function.

In this study, we examined how RUX affects P-glycoprotein in human cytotoxic T lymphocytes under resting and activated conditions. We measured ABCB1 gene and protein expression, drug efflux using calcein-AM, and ATPase activity. Additionally, we assessed CD8 and programmed death-1 levels as markers of activation and exhaustion. Our results show that RUX inhibits efflux

activity only at high concentrations, stimulates basal ATPase activity, and suppresses verapamil-induced activation, indicating direct modulation of transporter function.

Using an in vitro T cell priming model with peripheral blood mononuclear cells and Epstein–Barr virus-transformed antigen-presenting cells, we tracked memory T cell development and differentiation. ABCB1 expression was higher in unprimed cytotoxic T cells and declined with activation; however, RUX delayed this downregulation. It also altered T cell subset distribution, increasing naive and central memory cells while reducing effector memory and terminal effector cells. Additionally, it expanded stem-like memory T cells and lowered programmed death-1 expression.

RUX modulates P-glycoprotein expression and activity, influences T cell differentiation, and suppresses immune exhaustion markers. These findings have significant implications for immunotherapy and the treatment of immune-related disorders such as graft-versus-host disease and myeloproliferative neoplasms

1.1 THE HUMAN ATP-BINDING CASSETTE TRANSPORTER SUPERFAMILY

ATP-binding cassette transporters are energy-dependent proteins that move various molecules such as sugars, amino acids, lipids, and ions across cell membranes against concentration gradients. Found across all domains of life, there are 48 identified in humans, 79 in Escherichia coli, 23 in Saccharomyces cerevisiae, and over 100 in Arabidopsis thaliana.

These transporters contain highly conserved nucleotide-binding domains characterized by Walker A and Walker B motifs. The Walker A motif (GXXXXGK(T/S)) binds the phosphate groups of ATP, while the Walker B motif (with conserved aspartate or glutamate) coordinates magnesium ions and catalyzes ATP hydrolysis. Together, these motifs power conformational changes required for substrate transport.

Additionally, ABC transporters have transmembrane domains composed of 6–12 hydrophobic helices that determine substrate specificity. Structurally, they exist as full transporters with two

nucleotide-binding and two transmembrane domains, or as half-transporters requiring dimerization. Mutations in ABC genes are linked to diseases such as cystic fibrosis, anemia, and neurological disorders.

1.1.1. Human ABC genes and associated diseases

The human ABC gene family includes the subfamilies ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG. The ABCA subfamily consists of 12 transporters, categorized into two groups based on their phylogenetic intron structures. The first subgroup includes ABCA1, ABCA2, ABCA3, ABCA4, ABCA7, ABCA12, and ABCA13, which are distributed across six chromosomes. The second group consists of five genes: ABCA5, ABCA6, ABCA8, ABCA9, and ABCA10.

Certain human diseases are closely linked to ABC genes. For example, ABCA1 is associated with cholesterol transport issues and disorders related to high-density lipoprotein (HDL) synthesis. Mutations in ABCA1 can lead to a rare genetic disorder known as Tangier Disease, characterized by low levels of HDL and Apoprotein A-I. Symptoms of this disorder include anemia, thrombocytopenia, neuropathy, and tonsillitis. The ABCA4 protein transports derivatives of vitamin A in rod photoreceptor cells. Mutations in ABCA4 can lead to Stargardt disease.

ABCB1 is a crucial transporter that exports xenobiotics from normal and tumor cells and is often linked to chemotherapy resistance. ABCB4 transports phosphatidylcholine (PC) in the liver, while ABCB11 transports bile salts. Mutations in ABCB11 are linked to pediatric hepatocellular cancer. ABCB2 (TAP1) and ABCB3 (TAP2) work together as heterodimers to transport peptides into the endoplasmic reticulum, presenting antigens via class I HLA molecules. Mutations in these genes are associated with autoimmune diseases, including ankylosing spondylitis, celiac disease, and Graves' disease. ABCB10 plays a role in protecting against reactive oxygen species.

The ABCC subfamily has varied functions such as ion transport and toxin efflux activity. Specifically, ABCC1 is a multidrug resistance protein, while the cystic fibrosis transmembrane conductance regulator (CFTR, also known as ABCC7) is implicated in the autosomal recessive disease cystic fibrosis. This disease primarily affects the mucosal lining of the lungs and digestive system. In addition, the *ABCC13* gene in other species encodes proteins responsible for transporting various molecules across cell membranes. However, in humans, *ABCC13* is likely

nonfunctional due to accumulated mutations and deletions in the gene, preventing it from producing an active protein.

The ABCD subfamily comprises ABCD1, ABCD2, and ABCD3, which are in peroxisomes. These half-transporters function as homodimers or heterodimers. ABCD1 and ABCD2 regulate the transport of long-chain fatty acids. X-linked adrenoleukodystrophy (X-ALD) is associated with variants in ABCD1. This accumulation of very long fatty acids in peroxisomes results in myelin defects in neurons, as well as malfunctions in the adrenal glands and testes. ABCD2 is linked also to the transport of very long-chain fatty acids (VLCFAs), while ABCD3 is involved in transporting branched-chain acyl-CoA into peroxisomes. Additionally, ABCD4 transports compounds in mitochondria and lysosomes, and mutations in ABCD4 can result in cobalamin deficiency.

The ABCE and ABCF subfamilies contain ATP-binding domains but lack transmembrane domains. Therefore, they do not participate in membrane transport. The ABCG1 protein is involved in cholesterol transport in macrophages, T-cell proliferation, and protection from apoptosis. ABCG2 serves as a drug-resistance protein that effluxes xenobiotics out of cells. Finally, ABCG5 and ABCG8 function as heterodimers that transport sterols in the liver and intestine. Genetic alterations in ABCG5 and ABCG8 can lead to sitosterolemia, characterized by the accumulation of cholesterol and sterols.

1.1.2. P-glycoprotein and its structure

P-glycoprotein, or multidrug-resistance protein 1, is a member of the ATP-binding cassette transporter family and plays a key role in drug efflux. It is a single polypeptide with two homologous transmembrane domains (each with six α-helices) and two nucleotide-binding domains responsible for ATP hydrolysis. ATP binding induces conformational changes that shift Pgp from an inward- to an outward-facing state, allowing substrate export.

In humans, it is encoded by the ABCB1 gene (170 kDa), while in mice by Abcb1a and Abcb1b. Other ABC transporters involved in xenobiotic efflux include MRP1 (ABCC1) and BCRP (ABCG2). Pgp influences drug absorption, distribution, metabolism, elimination, and toxicity (ADMET), affecting bioavailability by limiting cellular drug accumulation.

Pgp substrates include a wide range of lipophilic compounds such as anticancer drugs, immunosuppressants, antibiotics, and opioids. It was first identified in colchicine-resistant CHO cells. The inward-facing conformation has a ~60 Å cavity with binding pockets formed by hydrophobic and polar residues that allow interaction with diverse molecules (250–4000 Da). The catalytic site includes Walker A, Walker B, and the conserved C motif (LSGGQ), essential for ATP hydrolysis.

1.1.3. Role of Pgp in the immune system cells and the rest of the organs

P-glycoprotein functions as an ATP-dependent efflux pump, exporting xenobiotics and drugs into compartments like bile, urine, and the intestinal lumen, contributing to detoxification and tissue protection. It is highly expressed in barrier and excretory organs such as the adrenal glands, liver, kidneys, colon, and blood-brain barrier. In the liver, it regulates cholesterol and bile secretion, while in the placenta, it protects the fetus by controlling glucocorticoid transport. P-glycoprotein also contributes to immune defense in the gut during bacterial infection and enhances renal detoxification during endotoxemia.

Beyond detoxification, P-glycoprotein plays critical roles in immune regulation. It is expressed in dendritic cells, macrophages, B cells, natural killer cells, and both CD4+ and CD8+ T cells. In T cells, it supports migration, protects against oxidative stress, and promotes survival and memory formation. It also facilitates regulatory T cell development and modulates interferon signaling. In macrophages, it encourages a shift from pro-inflammatory to anti-inflammatory states. In stem cell biology, P-glycoprotein is highly expressed in $CD34^+$ hematopoietic stem cells, supporting proliferation and regeneration. It distinguishes active, proliferative cells from quiescent ones, playing a key role in tissue renewal and immune competence.

1.2. CD8+ T CELLS AND INFECTIONS

CD8+ T cells are essential for combating intracellular pathogens and targeting and destroying cancer cells. Upon antigen recognition, naive CD8+ T cells undergo activation with significant expansion and maturation into effector and memory T cell subsets. After eliminating infected or

tumor cells, the expanded populations contract by apoptosis, and only the memory cells remain. Effector CD8+ T cells, also known as cytotoxic T lymphocytes, induce the death of target cells by engaging Fas/Fas ligand interactions and releasing the cytolytic protein perforin, which forms pores in the target cell membrane, allowing granzymes (serine proteases) to enter and initiate apoptosis, similar to caspases. Memory CD8+ T cells are crucial for providing swift and robust immune responses upon re-exposure to antigens, thereby contributing to long-term immunity.

1.2.1. Heterogeneity of CD8+ T cells

Naive CD8+ T cells become activated upon encountering antigens presented by antigen-presenting cells in lymphoid tissues. Activation triggers clonal expansion into effector T cells that combat infections or tumors. After the immune response, most effectors die, while some differentiate into long-lived memory T cells capable of rapid recall responses.

CD8+ T cells differentiate into short-lived effectors or long-lived memory subsets: central memory T cells (Tcm) and effector memory T cells (Tem). Tcm (CD62L+) reside in lymphoid organs and proliferate strongly upon reactivation, producing IL-2 and IFN-γ. Tem ($CD62L^-$) patrol peripheral tissues and respond quickly but proliferate less.

Stem cell memory T cells (Tscm), marked by CD45RA+CD62L+CCR7+CD95+, lie between naive and Tcm cells and have strong self-renewal and drug resistance. CD73+CD45RA+CD62L+ cells (young memory T cells) also show high proliferative potential. Chronic infections lead to T cell exhaustion, marked by inhibitory receptors like PD-1 and LAG-3, reduced function, and lower metabolic activity. Temra cells (CD8+CD45RA+) are terminally differentiated memory cells important in aging and chronic infection, losing co-stimulatory markers CD27 and CD28 over time.

1.2.2. CD8+ T cell exhaustion

Exhausted cytotoxic T cells represent a subset of differentiated immune cells that lose their ability to produce important signaling molecules such as interleukin-2, tumor necrosis factor alpha, and, at later stages, interferon gamma. They also show reduced cytotoxic function. Unlike memory T cells formed during acute infections, exhausted T cells fail to respond properly to survival signals like interleukin-7 and interleukin-15 due to impaired receptor signaling.

These cells are defined by the continuous expression of inhibitory immune checkpoint molecules such as programmed death-1, cytotoxic T-lymphocyte-associated protein 4, lymphocyte-activation gene 3, T cell immunoreceptor with Ig and ITIM domains, T cell immunoglobulin and mucin-domain containing-3, CD39, and 2B4. The level and combination of these molecules reflect the severity of exhaustion. Additional co-stimulatory molecules, such as CD27 and CD28, also play roles in driving exhaustion, with programmed death-1 signaling weakening the activation of the T cell receptor and CD28.

During chronic stimulation, exhausted T cells progress from early stem-like precursors to transient effector-like cells that produce interferon gamma, tumor necrosis factor, and granzyme B, and finally to terminally exhausted cells. This progression involves the gradual loss of renewal capacity and is supported by helper T cells and interleukin-21

1.2.3. Transcriptional regulation of cytotoxic T-cell differentiation

The development of cytotoxic T cells is regulated by a network of transcription factors that balance effector and memory cell differentiation. T-bet and Eomesodermin promote effector cell development and cytotoxic activity, while their absence can lead to interleukin-17-producing cells. Their expression is induced by T cell receptor activation and cytokines like interleukin-12 and interleukin-2. These pathways interact with other regulators like FOXO1 and mTOR, shaping cell fate.

Effector cells with high T-bet express markers like KLRG1 and reduced interleukin-7 receptor, indicating terminal differentiation. Cells with higher Eomesodermin are more likely to become memory cells, expressing receptors such as CD62L and CXCR4 for tissue homing. Eomesodermin is essential for memory cell survival and turnover.

Other transcription factors include BLIMP-1, which supports effector differentiation, and BCL-6, which promotes central memory T cell development. ID2 favors effector differentiation, while ID3 supports the transition to memory. These factors form opposing pairs, such as T-bet vs. Eomesodermin, BLIMP-1 vs. BCL-6, and ID2 vs. ID3, and interact with signaling pathways like STAT and ZEB proteins.

Effector differentiation is driven by signals from the T cell receptor and cytokines like interleukin-2, interleukin-12, type I and II interferons, interleukin-21, and interleukin-27. Memory formation is supported by interleukin-7, interleukin-10, interleukin-15, and transcription factors like TCF-1 and FOXO1. These complex interactions determine the balance between short-term response and long-term immunity.

1.2.4. Models of T-cell Differentiation

T cells differentiate through multiple stages. When naive CD8+ T cells encounter an antigen, they undergo specific developmental processes, giving rise to Tem and long-lasting Tcm cells. To explain the formation of memory CD8+ T cells, three main models have been suggested. These include the linear differentiation model, signal-strength model, and asymmetric-cell-fate model. In the linear differentiation model, naive T cells can differentiate into both effector and memory T cells. The signal strength model suggests that naive T cells undergo hierarchical differentiation based on the intensity and duration of signals received from APCs, where weak signals lead to cell death, strong signals drive terminal differentiation into short-lived effector T cells, and intermediate signals favor the development of central memory T cells with self-renewal capacity. In contrast, the stem cell model proposes that Tcm cells function as a reservoir of self-renewing cells capable of generating both effector and memory T cells, ensuring long-term immune protection. Together, these models explain how T cells balance immediate immune responses with the maintenance of immunological memory. The formation of heterogeneous effector cell populations is influenced by the strength of the signals received. This model posits that the signals encountered during T cell priming, such as antigen signals (Signal 1), co-stimulation (Signal 2), and pro-inflammatory cytokines (Signal 3), affect T cell heterogeneity. Weaker signals favors memory T cells formation, while stronger signals typically promote terminal effector T cells formation.

Asymmetric-cell-fate model: This model suggests that memory and effector cells can arise from a single precursor T cell during activation, resulting in the formation of self-renewing memory cells and terminally differentiated memory cells. Together, these models provide a deeper understanding of how various conditions and signals influence the differentiation and function of CD8+ T cells.

1.3. RUXOLITINIB JAK1/2 INHIBITOR FOR MULTIPLE DISEASES

RUX is a potent and selective oral inhibitor of Janus kinase 1 and Janus kinase 2, approved for clinical use in treating diseases such as myelofibrosis, polycythemia vera, and graft-versus-host disease. Janus kinases are enzymes that mediate the signaling of over 50 cytokines and hormones through the Janus kinase–signal transducer and activator of transcription pathway, which regulates blood cell formation, immune responses, and metabolism. The Janus kinase family includes Janus kinase 1, Janus kinase 2, Janus kinase 3, and tyrosine kinase 2.

Chemically, RUX is a cyclopentyl-propionitrile derivative that selectively binds the active site of Janus kinase enzymes, especially Janus kinase 1 and Janus kinase 2, inhibiting their function with high potency. This inhibition suppresses multiple inflammatory cytokines such as interleukin-1 beta, tumor necrosis factor alpha, interleukin-6, interleukin-12, and interferon-regulated chemokines in human immune cells.

RUX reduces inflammation triggered by bacterial components and blocks the production of monocyte chemoattractant protein-1, a key factor in immune cell recruitment. It also limits the growth of cytokine-dependent progenitor cells and malignant cells carrying Janus kinase 2 mutations.

Mutations in Janus kinase 2, particularly the V617F mutation, are strongly linked to blood cancers such as polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Other Janus kinase mutations are associated with immune deficiencies and leukemias.

RUX clinical effectiveness stems from its strong anti-inflammatory activity and ability to modulate immune responses. It reduces spleen enlargement, decreases inflammatory cytokines, limits suppressive immune cells, restores function in exhausted T cells, and enhances immune responses by upregulating antigen-presenting molecules.

1.3.1. Structure of Janus Kinases (JAKs)

Janus kinases are intracellular non-receptor tyrosine kinases with a conserved seven-domain architecture (JH1-JH7), sharing approximately 48% sequence homology among family members. The C-terminal JH1 domain functions as the catalytic kinase domain, while the adjacent JH2 pseudo-kinase domain serves a critical regulatory role despite lacking catalytic activity. Structural studies have revealed that the central JH3 and JH4 domains share homology with SH2 domains though their biological functions require further elucidation. The N-terminal FERM domain plays an essential role in receptor binding and membrane localization, facilitating interactions with cytokine receptors and subsequent signal transduction initiation. This sophisticated domain organization enables JAKs to effectively integrate regulatory functions with catalytic activity while maintaining stable receptor associations.

1.3.2. Classification of JAK Inhibitors

JAK inhibitors represent an important class of targeted therapeutics that modulate cytokine signaling through specific inhibition of JAK family tyrosine kinases. These compounds can be systematically classified based on three key characteristics: selectivity, binding mode, and binding site. First-generation inhibitors such as tofacitinib and baricitinib are non-selective, broadly targeting multiple JAK isoforms. In contrast, second-generation inhibitors, including upadacitinib and ritlecitinib, demonstrate improved selectivity for specific JAK family members, potentially reducing off-target effects. These compounds can be categorized by their binding sites as ATP-competitive inhibitors (Types I-II), allosteric modulators (Types III-V), or covalent binders (Type VI). This comprehensive classification framework provides critical insights for developing JAK inhibitors with optimized therapeutic profiles for treating autoimmune, inflammatory, and oncological diseases.

2. JUSTIFICATIONS AND OBJECTIVES

2.1. Justifications

P-glycoprotein, encoded by the ABCB1 gene, plays a pivotal role in drug resistance and immune regulation. While its function in removing xenobiotics and chemotherapeutic agents is well established, its role in immune cell function, particularly in CD8+ T cells, remains less understood. Elevated expression of Pgp in tumor cells contributes to chemotherapy resistance, limiting the effectiveness of treatment. In immune cells, however, it can enhance resilience under challenging conditions. Understanding the mechanisms underlying Pgp-mediated resistance in T cells is essential for optimizing cancer immunotherapy, managing autoimmune diseases, and improving transplantation outcomes.

RUX, a JAK1/2 inhibitor, has demonstrated immunomodulatory properties in hematologic malignancies and inflammatory conditions. However, its potential impact on Pgp activity and T cell maturation remains incompletely understood. Investigating how RUX influences Pgp expression and function in cytotoxic T lymphocytes could provide valuable insights into its therapeutic implications, particularly in graft-versus-host disease and the efficacy of chemotherapeutic agents.

By elucidating the relationship between Pgp expression, T cell maturation, and RUX treatment, this study aims to contribute to developing targeted strategies for improving immune resilience, enhancing cancer treatment outcomes, and refining immunotherapeutic approaches.

2.2. Objectives

2.2.1. General objectives

To investigate Pgp presence, activity, and expression dynamics in cytotoxic T lymphocytes during maturation, and to determine whether the JAK inhibitor RUX modulates these parameters.

2.2.2 Specific objectives

1. To quantify Pgp expression and efflux activity in CTLs and assess how RUX alters them.

2. To characterize the temporal pattern of Pgp expression throughout CTL differentiation and evaluate its modulation by RUX.

3. MATERIALS AND METHODS

3.1. Antibodies and chemicals

Unless specified, materials and chemicals were sourced from Thermo Fisher Scientific, Sigma-Aldrich, Merck Life Science Kft., or MedChem Express (Budapest, Hungary). Fluorescently-labeled mAbs included: anti-human CD16 FITC, CD19 PE, CD56 PC7, CD62L Pacific Blue (Beckman Coulter); CD3 PerCP-Cy5.5, CD4 Pacific Blue, CD45RA FITC, CD8 APC-H7 (Becton Dickinson); CD45 PO (EXBIO Praha); CD73 PE (Sony Biotechnology); CD95 PE-Cyanine7, CD127 PE-Cyanine5.5 (Thermo Fisher Scientific); CD279 (PD-1) APC-Cy7 (BioLegend). In-house anti-human CD8 (OKT8) PO and Pgp (15D3) Alexa Fluor 647 mAbs were produced from hybridoma and mouse ascites. OKT8 mAb was purified from OKT8 hybridoma supernatant using a Protein A column at 4°C.

3.2. Isolation and purification of 15D3 and OKT8 antibodies

The 15D3 anti-human Pgp mouse mAb was purified from ascites of 15D3-hybridoma cells injected intraperitoneally into ten-week-old female BALB/c and Swiss nude mice, housed at the University of Debrecen, Hungary (21-25°C, 12-hour light/dark cycle, standard pellets, and water ad libitum). Ascites were collected aseptically on day 14, stored at -80°C, and purified using a Thermo Fisher Scientific Protein G Spin Kit. Protein concentrations of 15D3 and OKT8 mAbs were measured with a NanoDropTM OneC at 280 nm. Purified antibodies were stored with 0.02% sodium azide at 4°C (short-term) or -20°C (long-term). Alexa Fluor and PO-labeled mAbs were prepared for direct immune-labeling per manufacturer instructions (Thermo Fisher Scientific).

3.3. Cell lines and generation of cytotoxic T lymphocytes

The study used:

(i) PBMCs from healthy donors (ii) NIH-3T3 MDR1 (Pgp-expressing) cells, (iii) NIH-3T3 parental cells (from Dr. Michel Gottesman, NIH) (iv) JY cells (EBV-transformed B-lymphoblastoid, high MHC class I A2/II DR expression).

Cell lines were cultured in RPMI 1640 with sodium bicarbonate, glucose, pyruvate, MEM non-essential amino acids, GlutaMAX, gentamycin/ampicillin, and 10% heat-inactivated FCS at 37°C with 5% CO_2. PBMCs were isolated from buffy coats (healthy donors, Debrecen, Hungary) via Ficoll-Paque density gradient centrifugation, with informed consent and ethics approval (OVSzK 3572-2/2015/5200). Monocytes were removed using anti-CD14 magnetic microbeads. PBLs were cultured at 2–5 × 10^6 cells/mL in RPMI with 10 mM Hepes, 50 µM 2-mercaptoethanol, and optional IL-2 (20 IU/mL). CTLs were generated by priming PBLs with heat-inactivated JY cells (4:1 ratio, heat-shocked at 45°C for 2 hours) for two 30-day cycles, with IL-2 (20 IU/mL) added every third day from day three to support memory T cell development.

3.4. Measurement of Pgp and CD8 protein on cell surfaces

In this procedure, T cells were initially washed with PBS by centrifugation at 1200 rpm for 5 minutes. After the washing step, the cells were counted using a hemocytometer and adjusted to a concentration of 5 million cells per mL. Subsequently, 250,000 cells were plated in a 96-well format. To facilitate the detection of Pgp and CD8, we added 10 mg/mL of Alexa647-15D3 anti-Pgp antibody and 10 µg/mL of Alexa488-OKT8 anti-CD8 antibody to the cells. The mixture was gently vortexed and incubated on ice for 45 minutes. After incubation, the cells were washed twice with ice-cold glucose PBS to remove unbound antibodies. The cells were then resuspended in ice-cold glucose PBS containing 2 µM Hoechst dye. Pgp intensity measurements were subsequently performed using a Novocyte 3000 RYB flow cytometer.

3.5. Isolation of total RNA from CD8+ and CD8- cells

CD8+ and CD8- human primary T cells were sorted using a FACS ARIA III. Total RNA was isolated from MDR1, NIH-3T3, PBL-derived, and JY-preactivated CD8+ and CD8- cells using TRI Reagent® (TR118, Molecular Research Center). Approximately 1 × 10^6 cells were lysed in 1 mL Trizol™, homogenized, and mixed with 200 µL chloroform per 1 mL Trizol. After vortexing and incubating at room temperature for 3-5 min, samples were centrifuged at 15,000 rpm for 15 min at 4°C. The aqueous phase was transferred, mixed with 500 µL isopropanol, and incubated for 15-20 min. After centrifugation at 15,000 rpm for 10 min at 4°C, the RNA pellet was washed with

800 µL 70% ethanol, vortexed, and centrifuged again. The pellet was air-dried for 5-10 min, resuspended in 20-50 µL RNase-free water (AccuGENE® LONZA), and incubated at 65°C for 10 min. RNA yield, purity, and integrity were assessed via Nanodrop (A260/A280, A260/A230 ratios) and stored at -20°C.

3.6. Real-time quantitative PCR

Real-time quantitative PCR TRIzolate Reagent (UD GenoMed) was used to isolate total RNA, and a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe the RNA to cDNA in accordance with the manufacturer's instructions. The TaqMan probes Hs00184500_m1 ABCB1, Hs00184500_m1 ACTB, Mm02619580_g1 Actb, Hs02786624_g1 GAPDH, and Mm99999915_g1 Gapdh were used in real-time q-PCR to measure the quantity of mRNA. A Roche LightCycler 480 Instrument II was used for real-time monitoring. Human and mouse β-actin and GAPDH reference genes, chosen through housekeeping gene stability analysis with the RefFinder program, were used for gene expression normalization. The delta-delta cycle threshold (ΔΔCT) approach was used to measure gene expression.

3.7. Inhibitory effects of zosuquidar and RUX in NIH-3T3 Cells and CTLs

The ABCB1 transporter's functional activity was assessed using a calcein accumulation assay with inhibitors Zosuquidar (ZQ) and RUX, serially diluted across 12 U-bottom 96-well plates. NIH-3T3 cells with human ABCB1 (MDR1) served as positive controls, and untransfected NIH-3T3 cells as negative controls, maintained at 80-90% confluence. Calcein-AM was added (5 nM for NIH-MDR, 1 nM for CTLs) to 0.5 × 10^6 cells/well, followed by anti-CD8 Alexa647-OKT8 antibodies. Cells were incubated at 37°C with 5% CO2 for 30 min, washed twice with ice-cold glucose-PBS (1% FBS) at 1200 RPM for 5 min, and resuspended in 150 µL Hoechst 33342 (20 µg/mL) on ice in the dark. Calcein intensity was measured using a Novocyte 3000 RYB flow cytometer. The transport activity factor (TAF) was calculated as TAF = (MFI(inh) - MFI(0)) / MFI(inh), where MFI(inh) is mean fluorescence intensity with inhibitor and MFI(0) without, ranging from 0 to 1.

3.8. Experiment setup of T cell activation and treatment in human PBLs

T cells were activated by coating 24-well plates with 2 µg/mL anti-CD3/CD28 Dynabeads, incubated overnight at 4°C, and washed with PBS. One million PBLs were added to wells for TCR activation. RUX (100 µM) was added to CD3/CD28-activated wells (activated + RUX) or non-activated wells (RUX-only), with untreated/unstimulated cells as controls. Treatments lasted 72 hours (Table 2). Post-incubation, 250,000 cells per group were stained in a 96-well U-bottom plate with anti-PD1 (APC), anti-Pgp (Alexa488-UIC2 with ZQ inhibitor), and anti-CD8 (Pacific Orange-OKT8) antibodies on ice for 45 minutes in the dark. Cells were washed with PBS, resuspended in glucose-PBS with Hoechst dye, and analyzed by flow cytometry.

3.9. Preparation of membranes from NIH 3T3 mouse fibroblast cells

NIH 3T3 cells expressing wild-type Pgp, mutant variants, or lacking Pgp were used for membrane preparation via the Sarkadi method. Cells were washed thrice with 10 ml buffer containing 0.05 mg/ml PMSF, centrifuged at 300 × g for 5 min at 4°C, and resuspended in 10 ml ice-cold TMEP buffer (50 mM Tris-HCl, 50 mM mannitol, 2 mM EGTA, 2 mM DTT, 1X protease inhibitor, 0.5 mM PMSF). Cells were homogenized for 25 min, centrifuged at 500 × g for 10 min at 4°C to remove nuclear debris, and the supernatant was centrifuged at 28,000 × g for 1 hr at 4°C to isolate membrane proteins. The pellet was resuspended in 1-2 ml TMEP buffer, stored at -80°C, and protein concentration was measured using the Lowry method

3.10. ATPase activity measurement

ABCB1-specific ATPase activity was measured by quantifying inorganic phosphate release from ATP hydrolysis, isolating ABCB1 activity using inhibitors: Na-azide (F0F1 complex), Ouabain (Na+/K+ ATPase), EGTA (Ca^{2+} ATPases), and vanadate (ABCB1 nucleotide-binding domains). Membrane samples (5 µg protein in 60 µL) in a 96-well plate were treated with an ATPase premix (51 mM MOPS, 64 mM KCl, 6.4 mM Na-azide, 0.65 mM EGTA Tris, 2.6 mM DTT, 1.28 mM Ouabain) ± 100 µM Na-orthovanadate. Inhibitors (RUX at 1, 10, 100 µM; ZQ at 10 µM; CSA at 20 µM) ± 40 µM verapamil were applied for 10 min at 37°C. ATP-Mg^{2+} (3.5 mM) initiated the reaction (25 min, 37°C), stopped with 5% SDS. Inorganic phosphate was quantified

colorimetrically with a phosphomolybdate complex (1% ammonium molybdate, 0.014% potassium antimony tartrate, 2.5 M sulfuric acid, 20% acetic acid, 1% ascorbic acid), measured at 700 nm after 30 min at 22°C. ABCB1-specific activity was calculated by subtracting vanadate-positive from vanadate-free ATPase activity.

3.11. Experimental setup to measure the change in phenotypes and Pgp expression

The phenotypes of peripheral blood lymphocytes and Pgp expression were measured in PBLs that were unprimed (JY-non-exposed) and after JY-exposure (JY-primed). Multicolor flow cytometric measurements were carried out to determine the change in the population proportion and PgP expression in different populations (T, B, NK, and NKT, $CD4^+$ and CD8+ T cells). Four independent repeated experiments were carried out.

3.12. Experimental setup of the T cell activation and treatment in unprimed and JY-primed CTLs

CTL maturation experiments used JY-primed PBLs after one-month differentiation, compared to unprimed PBLs from the same two donors. Unprimed experiments (3 repeats) yielded 12 FCS files across 4 activation/treatment groups, while JY-primed experiments (4 repeats) yielded 16 FCS files. TCR activation involved coating 24-well plates with 2 µg/ml CD3/CD28 Dynabeads, incubated overnight at 4°C, washed with PBS, and seeded with 1 million JY-primed or unprimed cells. RUX was added to separate wells with CD3/CD28 (activated + RUX), cells only (RUX-only), or untreated/unstimulated cells (control). Treatments lasted 72 hours.

3.13. Flow cytometry

Calcein intensity, Pgp, and CD8 expression were measured in NIH-3T3 MDR, NIH-3T3, and CTLs using Novocyte 3000 RYB and BD Biosciences FACS Aria III flow cytometers, with data processed in FCS Express v6.0. Lymphocytes were stained with mAbs, incubated for 15 minutes, washed with PBS, and analyzed on FACS Canto II (BD FACSDiva v6.1.3), collecting 100,000 events per sample, with analysis in FlowJo v10.9.0.

Data preprocessing involved PeacoQC quality control, doublet removal (FSC area vs. height/width), and gating WBCs (FSC vs. SSC) and CD45+ lymphocytes (CD45 vs. SSC). Lymphocyte subsets were gated by markers. For Pgp and CTL phenotyping, sequential gating isolated CD8+ T cells, with 3200 cells down-sampled from 28 files (N=89,600) for multidimensional analysis. UMAP visualized high-dimensional data, and FlowSOM identified T cell meta-clusters, optimized to avoid over-/under-clustering. Clusters were overlaid on samples, and their percentages among CD8+ lymphocytes were compared across conditions.

3.14. Statistical analysis

Data normality was assessed using Kolmogorov–Smirnov and Shapiro–Wilk tests. Nonparametric data were analyzed with the Mann-Whitney test, and parametric data with Student's t-test. One-way ANOVA with Tukey's post hoc test was used for multiple comparisons ($p<0.05$) in GraphPad Prism v9.0. A chi-square test in Excel evaluated CTL subset variations across activations/treatments, with Bonferroni-adjusted post hoc comparisons. Effect size was measured using Cramer's V, categorized as large, medium, small, or negligible.

4. RESULTS

4.1. PART 1: The Presence and Functional Role of The Pgp in Cytotoxic T Lymphocytes

We conducted several cellular, biochemical, and molecular tests to systematically examine how RUX affects the phenotype and function of cytotoxic T lymphocytes. Following a description of baseline and antigen-induced alterations in Pgp and CD8 expression, we evaluated the impact of RUX on Pgp mRNA levels, transporter activity, ATPase activity, and PD1 alterations.

4.1.1. Dynamic expression of Pgp and CD8 in quiescent and antigen-primed human lymphocytes

Pgp expression was compared in quiescent CD8+ lymphocytes (CTL13, CTL14) and JY-primed CD8+ lymphocytes (CTL1, CTL4) from separate human donors. JY-priming, involving biweekly cultivation with JY cells for 28 days, promoted mature cytotoxic and memory T cell development.

Pgp expression was slightly but significantly higher in unprimed (CTL13, CTL14) than JY-primed (CTL1, CTL4) lymphocytes, likely due to T cell transition from naive to effector phenotypes and memory cell production. JY-primed CTLs showed significantly higher CD8 expression, indicating enhanced activation, differentiation, and cytotoxic capacity from repeated antigen exposure. CD8 expression varied significantly across donors, unlike Pgp expression, which showed no significant donor variability.

4.1.2. Ruxolitinib modulates Pgp mRNA expression in TCR-activated human T cells

ABCB1, encoding Pgp, is key in chemoresistance and immune cell activity by expelling xenobiotics. We studied RUX's effect on ABCB1 mRNA in JY-primed CD8+ T lymphocyte cultures compared to NIH-3T3 MDR1 cells (high ABCB1, ~500x CTL levels) and untransfected NIH-3T3 cells (undetectable ABCB1). Expression was normalized to ACTB due to lower variability than GAPDH. In CD8+ T cells from five donors, JY-priming reduced ABCB1 mRNA by ~95% compared to naive PBMC CD8+ cells, reflecting maturation to effector phenotypes. Acute 72-hour anti-CD3/CD28 TCR activation also lowered ABCB1 expression, but RUX co-treatment preserved it in both CD8+ and CD8- T cells, indicating RUX modulates Pgp via TCR signaling pathways, consistent across short-term and long-term cultures

4.1.3. Dose-dependent inhibition of P-glycoprotein activity by ruxolitinib and ZQ in cytotoxic T lymphocytes.

Pgp transporter activity in human CTLs was assessed using a calcein efflux assay, where calcein-AM fluorescence inversely correlates with Pgp efflux, quantified by the transport activity factor (TAF). Dose-response curves with ZQ, a Pgp-specific inhibitor, showed effective Pgp inhibition in NIH-3T3 MDR1 cells (IC_{50}: 1.779–2.847 × 10^{-7} M) and CTLs (IC_{50}: 1.809–3.498 × 10^{-6} M), with CTLs requiring higher ZQ concentrations due to lower Pgp expression. Calcein-AM concentration was reduced in CTLs to enhance assay sensitivity. RUX also inhibited calcein efflux dose-dependently, with IC_{50} values of 1.002–1.572 × 10^{-5} M in NIH-3T3 MDR1 cells and 3.003–8.543 × 10^{-5} M in CTLs, indicating weaker Pgp interaction compared to ZQ. RUX's profile suggests it acts as a low-affinity Pgp substrate or modulator in CTLs.

4.1.4. Ruxolitinib directly stimulates Pgp ATPase activity and interferes with verapamil-induced activation

Using NIH-3T3 MDR1 cell membrane extracts, ATPase assays assessed RUX's interaction with Pgp. RUX increased basal Pgp ATPase activity dose-dependently (1, 10, 100 μM), with 100 μM nearly doubling activity ($p < 0.05$), similar to verapamil (40 μM, $p < 0.01$). Pgp inhibitors ZQ (10 μM) and CSA (20 μM) reduced basal ATPase activity, though not significantly. RUX slightly reduced verapamil-induced ATPase activation, but the effect was concentration-independent and insignificant. ZQ significantly inhibited verapamil-induced activation ($p < 0.001$), while CSA's inhibition was evident but not significant. These results suggest RUX directly interacts with Pgp as a substrate or modulator, influencing drug interactions.

4.1.5. Ruxolitinib impedes PD-1 expression and modulates CD8 and Pgp levels in human CD8+ T cells following acute activation

Due to its ability to postpone lymphocyte maturation, RUX is used therapeutically in myeloproliferative diseases and graft-versus-host disease, where aberrant T cell proliferation is caused by altered JAK-STAT signaling. To ascertain whether the maturation-delaying effect of RUX could be replicated *in vitro*, we monitored the surface expression of PD-1, CD8, and Pgp after activating PBLs with CD3/CD28 beads for 72 hours.

Since we were interested in CD8+ cytotoxic T cells, we used flow cytometry to examine the CD8+ and CD8- subsets independently. Fluorophore-conjugated antibodies against CD8, PD-1, and Pgp were used to stain cells, and the mean fluorescence intensity (MFI) was measured.

Acute CD3/CD28 activation confirmed effective T cell activation by dramatically upregulating PD-1 expression in CD8+ cells ($p < 0.0001$) compared to the control. With much lower PD-1 levels than activated, untreated controls ($p < 0.0001$), RUX administration dramatically slowed this induction, indicating that RUX attenuates or delays activation-associated phenotypic maturation.

An inhibitory effect on activation-induced alterations was further supported by the fact that CD8 expression levels were dramatically upregulated upon CD3/CD28 stimulation ($p < 0.0001$), but

that this upregulation was partially blocked by RUX ($p < 0.0001$). CD3/CD28 stimulation also markedly increased surface expression for Pgp ($p < 0.0001$), while RUX administration markedly inhibited this induction, albeit not as much as for PD-1.

The effects of RUX appear to be more noticeable in CD8+ T cells, as the CD8- population showed little changes in PD-1, CD8, or Pgp expression across circumstances, and the differences were not statistically significant when compared to control cells. Overall, these results corroborate RUX's function as an inhibitor of activation-associated maturation events in T cells by showing that it inhibits PD-1 induction and modifies CD8 and Pgp expression in activated CD8+ T lymphocytes.

4.2. PART 2: P-glycoprotein expression in maturing CD8+ T cells and the effect of ruxolitinib

To investigate the expression of Pgp during T-cell maturation, we examined its expression in earlier and later memory states of human CD8+ T cells, as well as the maturation modulatory effect of RUX. Previous studies have shown that Pgp is expressed in young memory CD8+ T cells.

4.2.1. JY-priming predominantly generates specific CD8+ T cell phenotypes and increases Pgp expression in these populations

Peripheral blood lymphocytes from humans were cultured with proliferation-inhibited JY cells. The JY cell line, derived from B-lymphoblasts, exhibits a high surface expression of MHC class I A*02:01 proteins, making it an effective antigen-presenting cell. This interaction triggers a robust CD8+ T-cell allotype immune response in mixed lymphocyte cultures. Previous research has shown that the resulting cytotoxic T lymphocyte clones exhibit strong and specific cytotoxic activity against JY target cells. In the present study, 8-color flow cytometry was primarily used to analyze live cells.

The overall frequency of T cells did not differ significantly between JY-primed lymphocytes and unprimed/non-exposed cells. However, there was a marked upregulation of CD8+ T cell and a significant reduction in CD4+ T cells, highlighting the effectiveness of MHC I restriction. Furthermore, the frequencies of NK and NKT cells increased significantly, whereas the proportion of B cells in JY-primed cells decreased sharply compared to their non-exposed counterparts.

This study also examined the proportion of Pgp-expressing cells across different T-cell subsets in PBL-derived lymphocyte cultures. Within the T-cell population, there was a significant overall increase in the percentage of Pgp-positive cells. Notably, the fraction of Pgp-expressing cells increased in both CD4+ and CD8+ T cells, while it markedly decreased in NK and NKT cells. Although there was a significant increase in Pgp-expressing B cells, this finding is less relevant due to the substantial reduction in the total number of B cells.

4.2.2. Memory subpopulations identified during JY-primed CD8+ T cell maturation

Naive T cells are conventionally characterized as CD45RA+CD62L+, central memory T cells as CD45RA-CD62L+, effector T cells (Te) as CD45RA-CD62L-, and terminally differentiated effector T cells (Temra) as CD45RA+CD62L-. Additional subpopulations within these groups were identified based on previously published research.

We utilized multi-parametric flow cytometry to analyze PBLs, and cultures stimulated with JY cells, labeling them with specific surface markers. Immune clusters were determined based on the expression levels of these markers, visualized in a heat map, and integrated into a comprehensive dataset. FlowSOM clustering was employed to identify immune subsets, which were then mapped onto individual samples, allowing for comparison of their proportions among CD8+ T cells. Dimensionality reduction using UMAP facilitated the visualization of the high-dimensional data. Across both JY-exposed and non-exposed cytotoxic T lymphocytes, we identified 13 distinct CD8+ subsets: naive T cells, TeCD73+CD95+, TeCD95+, TemraCD127+, TemraCD127-, TymCD127+, TymCD127-, Tscm, TemCD95+, TcmCD95+CD127+, TcmCD73+CD95+, TcmCD95+, and TcmPD1+.

4.2.3. P-glycoprotein-expressing CTL subsets were identified in both JY-primed and unprimed cultures.

Among the 13 identified subsets, five exhibited significantly elevated levels of the multidrug resistance protein Pgp: TymCD127+, TymCD127-, Tscm, TcmCD95+, and TcmPD1+ . Notably, the TcmPD1+ subset, comprising central memory T cells expressing PD1, displayed the highest Pgp levels, representing a novel finding. This was followed by the young memory CD127+ subset (TymCD127+), consistent with previous observations. The remaining three subsets TymCD127-, Tscm, and TcmCD95+ showed progressively lower Pgp expression.

We further assessed the average Pgp expression across these memory subpopulations under different conditions of maturation, activation, and treatment. The analysis confirmed that TymCD127+ and TcmPD1+ subsets exhibited the highest Pgp levels. Notably, priming, activation,

and RUX treatment consistently sustained significant Pgp expression, with RUX showing a tendency to further enhance Pgp levels.

It is important to note that elevated Pgp levels are not necessarily required for their physiological function. Even resting naive T cells exhibited detectable surface Pgp expression. The increased Pgp levels observed in cancer cells, however, represent a specific adaptation that supports multidrug resistance (MDR) during chemotherapy.

4.2.4. Phenotypic polarization reflects typical adaptive immune responses in the JY-primed CTL maturation model following TCR activation.

Our goal was to distinguish between T-cell effector and memory phenotypes by exposing PBLs to antigen-presenting B cells, using the JY cell line to induce priming during the culture period. We analysed CD8+ T cell polarization under various activation conditions, resulting in a comprehensive dataset comprising 89,600 CD8+ cells. A contingency table was constructed to categorize each cytotoxic T lymphocyte phenotypic subset across different activation and treatment conditions. The changes observed in all subsets at various treatment and maturation stages. We also calculated the effect sizes for these changes, emphasizing the biological significance of the statistically significant variations.

4.2.5. Differentiation of CD8+ T cell subsets derived from human peripheral blood lymphocytes in response to TCR activation.

In an in vitro system with CD3/CD28 TCR activation, the absence of thymic naive T cell production leads to a reduced naive T cell fraction and increased TeCD95+ and TeCD73+CD95+ effector subsets. Most transformations are driven by the TCR signal, with robust findings supported by healthy donors and strong CD3/CD28 bead activation. Memory subsets, particularly small intermediate ones with high Pgp (TymCD127+, Tscm, TemCD95+, TcmCD95+CD127+, TcmPD1+), decrease when precursor supply is limited, while early-stage-derived subsets, including short-lived effectors, increase before undergoing apoptosis. Notably, Pgp-expressing TcmCD95+ rises from 9.5% to 36.5%, and young memory TymCD127- (high CD95) slightly increases from 9.9% to 12.7%.

4.2.6. Activation-dependent changes in the CD8+ subsets in JY-primed and unprimed CTLs

TCR activation of unprimed subsets led to a significant downregulation of TymCD127+ and Tscm subsets, while TymCD127- and TcmCD95+ subsets were upregulated. TcmPD1+ levels showed no significant change. In unprimed versus JY-primed (JY-exposed) subsets of TCR-unactivated cells, TymCD127+ was upregulated, whereas Tscm, TymCD127-, TcmCD95+, and TcmPD1+ were downregulated. In unprimed versus JY-primed subsets of TCR-activated cells, there was a significant increase in TcmPD1+ and significant downregulation of TymCD127+, Tscm, TymCD127-, and TcmCD95+. When comparing unactivated and TCR-activated subsets in JY-primed cells, we observed a significant increase in TcmPD1+ cells and a non-significant increase in TymCD127-, alongside significant downregulation of TcmCD95+. TymCD127+ and Tscm subsets exhibited non-significant downregulation. The blue arrows denote the direction of population changes in memory subsets expressing Pgp due to the treatment. Subsets listed in bold reflect large effect sizes, indicating significant immunological impacts, while non-bolded subsets represent medium or small effect sizes.

4.2.7. Differentiation of long-lived CD8+ T cell subsets in JY-primed human peripheral blood lymphocyte cultures.

The next stage of differentiation was evaluated in one-month-old IL-2-expanded T cells, which were initially cultured through two cycles of JY-cell-primed mixed lymphocyte cultures. Within these cultures, CD8+ T cell subsets differentiated by recognizing allogeneic self-antigens presented by JY cells, leading to a robust CD8+ T cell response. The addition of IL-2 to the one-month culture facilitated the differentiation and accumulation of long-lived memory subpopulations.

Marked reduction in naive cells and subsets such as TymCD127-, TemCD95+, TemraCD127+, and TcmCD95+CD127+. Conversely, the most notable increases were observed in the TeCD95+ and TeCD73+CD95+ subsets.

When comparing JY-primed T cells after one month of maturation to unprimed PBLs, there was a sustained loss of naive cells and a more pronounced rise in effector cells, exceeding the changes seen after 72 hours of acute TCR activation. The previously expanded TeCD95+ and TeCD73+CD95+ subsets continued to grow, while the smaller TemraCD127+ subset showed significant expansion. Additionally, other memory subsets changed, with long-lived memory cells emerging during the retraction phase. Early TymCD127+ and TcmCD73+CD95+ subsets increased, while the central memory population of TcmCD95+ cells and the larger TymCD127- subset, which primarily differentiates into effector cells, showed a decline. The smaller TymCD127+ subset, characterized by high Pgp expression, transitioned into TcmCD73+CD95+ memory subsets.

4.2.8. Differentiation of late-stage CD8+ T cell subsets in TCR-reactivated, JY-primed cultures

In JY-primed one-month-old cultures with TCR activation via CD3/CD28 beads, the TeCD95+ effector population significantly increases to ~80% (from 40% unprimed), replenished from smaller intermediate memory subsets as naive precursors are nearly absent. UMAP maps show reduced intermediate populations. The T-cell memory recall response is robust, with consistent trends in memory subsets during initial and recall TCR activation. Long-lived memory subsets with high Pgp expression persist. Late TCR activation sees a decline in most subpopulations, including young memory TymCD127+ and central memory TcmCD73+CD95+ cells, except for the small TcmPD1+ subset, which expands significantly despite its few percent size, mirroring earlier high-Pgp subset behavior.

4.2.9. Ruxolitinib delays CD8+ T cell maturation

RUX inhibits the Jak-1 and Jak-3 signaling pathways, critical for TCR activation and T-cell maturation. We examined RUX's impact on 13 identified CD8+ subsets maturation in unprimed

and JY-primed systems. Multiple trials (control, activated, treated, and treated-activated for 72 hours) assessed RUX's effects on CD8+ T-cell maturation. RUX was administered during TCR activation in both unprimed and JY-primed cells. UMAP analysis was used to evaluate outcomes, enabling visualization of RUX's effects. The UMAP plot for unprimed, activated, and treated cells revealed that RUX preserved naive cells (red cluster) post-activation. Additionally, TCR activation increased TcmCD95+ cells in unprimed groups (light green clusters in UMAP plots), while JY-priming reduced naive cells and elevated TeCD95+ cells.

4.2.10. Effects of TCR-activation and ruxolitinib in the unprimed immune subsets

To investigate the impact of TCR activation on unprimed CTLs, we stimulated these cells with CD3/CD28 beads, both in the presence and absence of RUX in JY-primed samples. TCR activation resulted in an increase in Pgp-expressing subsets, notably TcmPD1+, as well as non-Pgp-expressing subsets such as TeCD95+ and TcmCD95+. Conversely, TCR activation caused a decrease in the frequencies of subsets such asTemraCD127+, TemCD95+, TcmCD95+CD127+, and TemCD95+ subsets.

4.2.11. Effects of TCR-activation and ruxolitinib in the JY-primed immune subsets

To assess the impact of TCR activation on JY-primed CTLs. TCR activation resulted in an increase in the Pgp-expressing TcmPD1+ subset, as well as in the non-Pgp-expressing TeCD95+ subsets. Interestingly, TCR activation led to a striking upregulation of the TeCD95+ subset, while RUX treatment inhibited this activation-induced increase compared to the activated group.

Non-Pgp-expressing subsets, including TemraCD127+, TemraCD127-, TcmCD95+, TcmCD95+CD127+, TcmCD73+CD95+, and naive CD8+ T cells, were downregulated during TCR activation. Surprisingly, the TeCD73+CD95+ subset exhibited activation-induced downregulation, whereas RUX treatment led to its upregulation.

Overall, the observed patterns suggest a potential maturation delay due to RUX; however, these changes were not statistically significant.

4.2.12. Effects of ruxolitinib on the maturation of CD8+ T cells

Contingency table analysis confirmed the maturation-delaying effect of RUX, revealing significant differences in cell numbers. Notably, naive cell counts were consistently higher in RUX-treated samples compared to those treated with pure TCR-activation samples, across both unprimed and JY-primed conditions.

The effects of RUX treatment during TCR activation were further quantified by assessing the normalized cumulative change across all subsets. This metric reflects the total differences observed at each of the four phases of the maturation process in JY-priming TCR activation experiments.

Notably, naive cells, as well as the TemraCD127+, TemraCD127-, and Pgp-expressing TymCD127+, Tscm, and TcmPD1+ subpopulations, showed significant positive cumulative differences (indicated in bold). Additionally, TeCD73+CD95+ cells also demonstrated a positive trend, although it was less significant. In contrast, the subpopulation most negatively affected was the TeCD95+ effector cells, which experienced a modest reduction, along with a decrease in TymCD127- cells.

These findings indicate that RUX treatment diminishes the main short-lived effector subpopulation while maintaining small, long-lived Pgp-expressing memory populations, consequently delaying CD8+ T cell maturation.

4.2.13. Changes in the naive population across the maturation phases

The four major stages of maturation utilized in this study, and in conjunction with RUX treatments that alter maturation, significant changes in major subsets characterizing the adaptive CD8 immune response were reliably identified. Specifically, during the acute T cell receptor (TCR) activation of PBLs, the initial population of naive cells experienced a reduction from 19.7% to 0.4%. After the one-month mark, the percentage of naive cells was measured at 0.9%, which became negligible during the repeated “recall” TCR activation at 0.03%. Consequently, by the conclusion of the four maturation stages, the naive cell population had completely transitioned into more mature cell

states, as there was no available cell supply from the thymus. The samples treated with RUX exhibited a notable upward trend relative to their previous untreated stages, thereby indicating that RUX treatment effectively delays the maturation of naive cells.

During the one-month culture period, which included two cycles of JY cell allogeneic antigen presentation, a substantial number of allogeneic T cell clones were generated due to the abundant allogeneic antigens. This phenomenon results in an increase in the effector population during the retraction phase, a phase typically marked by a decrease during the contraction phase of a standard immune response. The fourth maturation phase, responsible for generating a recall signal, involves reactivating all T cells using beads, similar to the activation process in the second phase of the maturation system. A limitation of this system is the continuous increase in effector cells, which deviates from the expected contraction phase and could obscure certain aspects of memory population behavior. However, a notable advantage is the robust and synchronous TCR activation facilitated by the abundance of allogeneic antigens via beads or JY cells, which allows for reliable monitoring of small memory populations.

4.2.14. Changes in the effector populations across the maturation phases

The effector cell populations TeCD95+ and TeCD73+CD95+ increase across four maturation stages. TeCD95+ starts at 15.2% (14.9% in PBLs), rising to 43.2% (42.4% in PBLs) after acute TCR activation with anti-CD3/CD28, 67.4% (56.9% in PBLs) after one month of JY-cell TCR activation, and 86.1% (78% in PBLs) after recall activation with anti-CD3/CD28 beads. TeCD73+CD95+ begins at 0.3%, grows to 8-11% across stages, but remains ~10% of TeCD95+.

RUX treatment suppresses effector cell production, especially TeCD95+, while naive cells and most memory subsets increase. TeCD73+CD95+ deviates, declining initially but expanding after one month and increasing with RUX, similar to naive cells.

4.2.15. Changes in the memory subsets across the maturation phases

As PBLs mature over four phases, the naive subset decreases, while effector populations expand. Memory subsets decline from 65.2% to 13.9% across phases. Initially, six major memory subsets (TemraCD127-, TemCD95+, TymCD127-, TcmCD95+, TcmCD95+CD127+, Tscm) comprise

62.4%, with four minor subsets (TcmPD1+, TymCD127+, TemraCD127+, TcmCD73+CD95+) below 3%.

In the expansion phase, TcmCD95+ surges from 9.5% to 36.5%, TymCD127- slightly increases from 10% to 12.7%, while others decline. Both later decrease over a month, with TymCD127- nearly vanishing. In the contraction phase, memory subsets drop to 31.7%, with major subsets (TemraCD127-, TemCD95+, TcmCD95+, TcmCD95+CD127+) falling to 24.9% total. Smaller subsets (TymCD127+, TcmCD73+CD95+, TemraCD127+) rise, while high-Pgp subsets (TymCD127-, Tscm, TcmPD1+) decrease.

In the final reactivation phase, TcmPD1+ and TymCD127- (high-Pgp) increase to 1.5% each, while TemraCD127- and TcmCD95+ drop to 4.8% and 4.1%. These four subsets dominate (86.3% of memory cells), with TemraCD127+ nearly disappearing (0.06%). Acute TCR activation drives expansion, dampened by RUX, resembling transit-amplifying cell behavior. High-CD127 subsets (TemraCD127+, TymCD127+, TcmCD95+CD127+, TemCD95+) decrease post-TCR activation, possibly transforming into effectors. TcmPD1+ expands with repeated TCR signals, while smaller subsets generally increase and larger ones decrease.

5. DISCUSSION

PART 1

Our research offers a new mechanistic insight into how RUX modulates Pgp expression/activity, T cell activation, and differentiation, thereby impacting CTL function. RUX inhibits PD-1 and CD8 surface expression post-TCR stimulation and influences Pgp at transcriptional and functional levels, underlining its role in immune surveillance and memory development.

We hypothesized that dynamically regulated Pgp expression supports stem-like memory T cell persistence. JY-primed CTLs showed slightly reduced Pgp protein levels versus unprimed cells. Naive CD8+ T cells had ~20-fold higher ABCB1 mRNA levels than long-term activated effectors, suggesting Pgp downregulation with differentiation.

To assess the RUX-Pgp relationship, we performed calcein-AM efflux assays. RUX inhibited Pgp with IC_{50} values of ~13 µM in NIH-3T3 MDR1 cells and ~58 µM in CD8+ T cells, exceeding normal plasma concentrations. Clinically, high Pgp levels reduce RUX efficacy, but local accumulation may still lead to inhibition. ATPase assays showed RUX significantly increased Pgp ATPase activity dose-dependently, peaking at 100 µM, and modulated verapamil-induced activation, confirming RUX's modulatory role. As per Seelig's classification, RUX acts as a substrate and modulator stimulating ABCB1 transcription and ATPase activity unlike traditional inhibitors.

RUX also reduced PD-1 upregulation and suppressed CD8 and surface Pgp elevation during CD3/CD28 stimulation. This supports studies linking RUX to PD-1 signaling interference and persistent TCR signaling. While ABCB1 mRNA showed sharp activation-induced changes, Pgp protein varied modestly likely reflecting T cell subset heterogeneity. Overall, RUX appears to delay T cell maturation and preserve a more naive-like phenotype.

Therapeutically, RUX's ability to inhibit activation, downregulate PD-1, and modulate Pgp suggests benefits for autoimmune diseases, transplant tolerance, or cancer immunotherapy. However, its interaction with Pgp raises concerns about pharmacokinetics and drug interactions.

A key limitation is the in vitro model, which may not fully capture in vivo immune complexity. Future work should assess long-term effects on memory development and validate findings in

clinical samples or animal models. Understanding subset-specific Pgp regulation will be crucial for refining therapeutic strategies.

PART 2

Pgp, an ABC transporter known for multidrug resistance in cancer, also plays key roles in immunity. Recent murine studies link Pgp to immune memory. This study explores Pgp expression in human CD8+ memory T cell subsets at the single-cell level to understand its memory-related functions.

CD8+ T cells differentiate into naive, effector, and memory cells. Effector cells clear antigens, then contract, leaving memory cells. Using an in vitro long-term IL-2-supplemented mixed lymphocyte culture with JY APCs, we modeled adaptive responses. Flow cytometry showed CD8+, NK, and NK-T cell upregulation and B/CD4+ T cell downregulation. JY co-culture increased Pgp expression on CD8+, CD4+, and B cells, aligning with prior findings.

Peripheral blood typically contains 40% naive, 20–25% effector/Temra, and the rest as Tcm. Our model produced four CTL stages: unprimed/unactivated, unprimed/TCR-activated, JY-primed/unactivated, and JY-primed/TCR-activated. Stages 1–2 represent acute responses; stages 3–4 model memory development and recall. RUX effects were studied across all.

We classified subsets using CD45RA and CD62L markers and refined them with CD73, CD95, CD127, and PD-1 to define 13 CTL populations. During the acute phase, TcmCD95^{+} dominated, followed by TymCD127^{-}. In memory phase, TymCD127^{+} prevailed, while late-reactivated TcmPD1^{+} expanded. Tscm remained stable at 2–4%.

CD73 marked young memory cells with high Pgp and ALDH1—traits of stemness and drug resistance. TCR activation reduced CD127 in chemo-resistant TymCD127^{-} cells. CD95 identified Tscm cells with self-renewal and survival features. Tscm and Tym share CD95, Pgp, and other markers, supporting their role as long-lived memory precursors.

CD127 signaled IL-7 receptor expression critical for survival and homeostasis in memory/naive cells. PD-1 (CD279) identified late-stage/exhausted CTLs, including Tex and Tex-stem cells. Our TcmPD1^{+} subset likely reflects Tex-precursors with high Pgp and asymmetric division potential.

Tym, Tscm, and Tcm share features with Tex-precursors, expressing markers like CD27, CD28, and CXCR3.

Our data show highest Pgp expression in TcmPD1+, Tscm, TymCD127^{-}, and TymCD127+ subsets, indicating roles in drug efflux, stress protection, and quiescence. These properties support their stem-like memory function.

JAK/STAT pathways modulate CTL maturation. Using RUX, a JAK1/2 inhibitor, we found that inhibition reduces effector production while favoring memory differentiation. IL-12 (JAK1/2) promotes effectors; IL-7/15 (JAK1/3) sustain memory. RUX thus shifts balance toward memory cells. Future studies should test JAK2-specific inhibitors like fedratinib in this context.

RUX delayed differentiation, increasing naive and reducing effector cells. Memory subsets responded heterogeneously: small ones expanded, large ones contracted post-TCR stimulation. This suggests a stable, slowly dividing memory core sustained by asymmetric division and cytokine signals.

RUX also improves outcomes in inflammatory diseases like COVID-19 by dampening effector CTL maturation. our findings align, showing enhanced memory subsets post-activation and improved persistence.

Memory subsets are continuously generated and maintained. Small, Pgp+ memory populations (TymCD127+, TcmPD1+) form a self-renewing quiescent pool in low-redox niches like bone marrow. PD-1 doesn't fully block proliferation, enabling these cells to surpass replicative limits and support long-term immunity important for cancer vaccines.

In summary, we map CD8+ T cell maturation post-TCR activation. Naive T cells develop into effectors and central memory subsets, with TeCD95^{+} and TcmCD95^{+} expanding over time. Advanced memory cells like Temra emerge later. These findings are relevant for optimizing immunity in chemotherapy, vaccination, and immunotherapy.

6. SUMMARY

This study investigated the interaction between ruxolitinib (RUX), an FDA-approved JAK1/2 inhibitor, and P-glycoprotein (Pgp/ABCB1/MDR1), an ATP-binding cassette transporter involved in drug resistance and immune regulation. Pgp exports xenobiotics and is expressed in immune cells, including T lymphocytes. While high Pgp expression is linked to poor responses in autoimmune diseases, it enhances the resilience of memory T cells, supporting immune recovery after chemotherapy.

To evaluate this interaction, human primary T cells and NIH-3T3 MDR1 cells were used. RUX inhibited Pgp activity in a dose-dependent manner (at non-therapeutic levels), downregulated PD-1 and Pgp in activated T cells, activated basal Pgp ATPase activity, and interfered with verapamil-induced activation, suggesting functional modulation.

Molecular analyses revealed that ABCB1 mRNA was high in unprimed T cells but declined with activation. RUX treatment upregulated ABCB1 in activated T cells, indicating its role as a transcriptional regulator. These findings may improve chemotherapy outcomes and aid in GVHD management.

The effect of RUX on T-cell maturation showed a shift toward naive and central memory (Tcm) CD8+ T cells, with reduced effector and effector memory (Tem) subsets. This reprogramming may enhance therapeutic efficacy in myelofibrosis and immune disorders. Using an in vitro model, T cells were primed with JY antigen-presenting cells, and maturation was tracked over a month. RUX exposure promoted Pgp+ long-lived memory T cell maintenance and delayed effector differentiation. It also expanded $CD127^{+}$ memory T cells, supporting long-term immune memory.

In conclusion, this study demonstrates that RUX modulates Pgp function and T cell differentiation, with implications for autoimmune disease, transplantation, and cancer therapy. Targeting Pgp in memory T cells could improve the effectiveness of immunotherapies by enhancing memory cell survival and immune resilience.

7. FUNDING

Stipendium Hungaricum Scholarship grant for Kipchumba Biwott ID 409037 funded this project

8. LIST OF PUBLICATION RELATED TO THE DISSERTATION

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/439/2025.PL
Subject: PhD Publication List

Candidate: Kipchumba Biwott
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10090211

List of publications related to the dissertation

1. **Biwott, K.**, Singh, P., Baráth, S., Nyariki, J. N., Hevessy, Z., Bacsó, Z.: Dynamic P-glycoprotein expression in early and late memory states of human CD8+T cells and the protective role of ruxolitinib.
Biomed. Pharmacother. 182, 1-14, 2025.
DOI: http://dx.doi.org/10.1016/j.biopha.2024.117780
IF: 7.5 (2024)

2. **Biwott, K.**, Lkhamkhuu, A., Ghaffar, N., Papp, A. B., Tarban, N., Goda, K., Bacsó, Z.: Ruxolitinib Modulates P-Glycoprotein Function, Delays T Cell Activation, and Impairs CCL19 Chemokine-Directed Migration in Human Cytotoxic T Lymphocytes.
Int. J. Mol. Sci. 26 (13), 1-21, 2025.
DOI: http://dx.doi.org/10.3390/ijms26136123
IF: 4.9 (2024)

List of other publications

3. **Biwott, K.**, Gitonga, F., Jepchirchir, C., Gitau, G. W., Wafula, O. P., Amwayi, P. W., Isaac, A. O., Nyariki, J. N.: Alcohol spiked with zolpidem and midazolam potentiates inflammation, oxidative stress and organ damage in a mouse model.
Forensic Toxicol. 42, 55-61, 2024.
DOI: http://dx.doi.org/10.1007/s11419-023-00674-w
IF: 3

4. Gellén, G., Klement, É., **Biwott, K.**, Schlosser, G., Kalló, G., Csősz, É., Medzihradszky-Fölkl, K., Bacsó, Z.: Cross-Linking Mass Spectrometry on P-Glycoprotein.
Int. J. Mol. Sci. 24 (13), 1-24, 2023.
DOI: http://dx.doi.org/10.3390/ijms241310627
IF: 4.9

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

5. **Biwott, K.**, Isaac, A. O., Mwaeni, V. K., Omwenga, G., Ngugi, M., Nyariki, J. N.: Vitamin B12 and coenzyme Q10 ameliorated alcohol-driven impairment of hematological parameters, inflammation, and organ damage in a mouse model.
Nutrire. 48 (1), 1-19, 2023.
DOI: http://dx.doi.org/10.1186/s41110-023-00197-9

6. Gitonga, F., **Biwott, K.**, Gitau, G. W., Wafula, O. P., Amwayi, P. W., Isaac, A. O., Nyariki, J. N.: Coenzyme Q10 Ameliorates potassium cyanide-induced toxicosis in a mouse model.
Sci. Afr. 12, 1-10, 2021.
DOI: http://dx.doi.org/10.1016/j.sciaf.2021.e00815

Total IF of journals (all publications): 20,3
Total IF of journals (publications related to the dissertation): 12,4

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

04 July, 2025