UNIVERSITÄT ZU LÜBECK

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Investigation of the influence of cyclin-dependent kinase inhibitors on neutrophils activation

Inauguraldissertation

zur Erlangung der Doktorwürde
der Universität zu Lübeck
- Aus der Sektion Medizin -

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Lübeck 2025

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Tag der mündlichen Prüfung: den 08.10.2025

Zum Druck genehmigt. Lübeck, den 13.10.2025

Promotionskommission der Sektion Medizin

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1. INTRODUCTION

1.1. Overview of the immune system and breakdown of immunological tolerance

Ensuring the optimal performance of the immune system is crucial for preserving good health, as any imbalance can result in immune-related disorders[26]. Insufficient immune response can increase susceptibility to infections and cancer development, while autoimmunity plays a significant role in normal physiological processes.[102]. Additionally, it is imperative for the immune system to tolerate non-pathogenic microorganisms, such as commensals, to support the proper function of various bodily tissues, including the skin and digestive system[34].

A typical immune response is orchestrated through multiple checkpoints that identify threats, eliminate them, regulate tissue damage, facilitate memory formation, and establish tolerance. It commences with physical and chemical barriers that restrict contact with harmful substances, impeding colonization and infection by pathogens. [166]. Another relevant factor is the presence of commensal microorganisms that protect their host by competing for habitable spaces with pathogenic microorganisms[44]. Some commensal strains secrete microbial substances that regulate tissue colonization of pathogenic microorganisms and modulate the host's gene expression, inducing immune homeostasis[155]. By overcoming this first barrier, pathogens can invade cells and multiply or be phagocytized in the extracellular medium or infected cells [177]. Then, a threat of infection is recognized by innate immune system cells such as neutrophils and macrophages which process the antigen and load a small fragment into MHC2 that is displayed on the cell surface so that it can be recognized by TCD4+ via T-cell receptor. Another possibility is antigen presentation on the surface of infected cells of antigen fragments bound to MHC1 that are recognized by TCD8+ cells also via T-cell receptor[177]. Following activation, TCD8 cells release granzymes and perforins, triggering apoptosis in infected cells[166]. TCD4+ cells, when stimulated, generate

cytokines that steer the immune response towards either cell-mediated inflammation or a humoral response, known as Th1 and Th2, respectively[177]. Th1 cells secrete interleukin 2, which promotes T cell proliferation, primarily triggering a cell-mediated inflammatory response. [177]. Th1 cells also generate interferon-gamma, which establishes a positive feedback loop in the Th1 response by stimulating interleukin 12 production by macrophages. This, in turn, further enhances interferon-gamma production and inhibits Th2 cell differentiation.[177]. On the other hand, when T cells produce interleukin 4, interleukin 5, interleukin 6, and interleukin 10, it amplifies the Th2 response, resulting in B cell proliferation, maturation into plasma cells, and antibody production.[102]. Another possibility for activated B cells is to become memory B cells that often migrate to lymph nodes or spleen[166].

The B cell activation process usually occurs in the germinal centers of the lymph nodes, where somatic hypermutation occurs, resulting in an increased diversity of antibodies. Subsequently, the activated B cells exit the lymphoid tissue as plasma cells. B cells demonstrating higher avidity for antigen binding to their surface receptors will undergo greater proliferation, thereby favoring the selection of B cells that generate antibodies with superior affinity for the respective antigen.[177], making the immune response more efficient in case of reinfection by the same pathogen[182].

Before maturing and entering the circulation, developing T cells undergo positive selection in the cortex in the thymus. T cells with potential reactivity are negatively selected and eliminated in the medulla of the thymus (central tolerance). Upon exiting the thymus, mature T cells undergo a second selection (peripheral tolerance). Those with autoreactivity are eliminated or become anergic. Another important mechanism is the clonal deletion or clonal anergy, which consists of the elimination of immature B cells that recognize ubiquitous self-cell-surface antigens. However, there are always lymphocytes that escape these controls[234]. Regulatory T cells (Tregs) originate in the thymus and serve to suppress T cells that have evaded thymic selection. Although interleukin 2 induces T cell proliferation related to cell-mediated inflammatory response, it also stimulates the maintenance, expansion, and activation of regulatory Tregs

which inhibit activated T cells. Disruption of this negative feedback loop holds the potential to trigger autoimmunity.[196]

Autoantibodies, autoreactive T cells and B cells contribute to the maintenance of homeostasis, tissue repair and regeneration[177]. Cancer can be recognized and eventually eliminated by antibodies targeting the cell membrane of cancer cells, in the presence of complement, which leads to cell lysis.[15]. Furthermore, cell debris is removed from tissues by specific cross-reactive IgM to prevent chemical changes that could initiate or maintain the production of cross-reactive pathogenic IgG[146]. Nevertheless, genetic predisposition and environmental factors can generate dysregulation of innate and adaptive immunity leading to tissue destruction[177]. In this sense, neutrophils have a prominent role, being able to promote tissue damage, contributing to the development and maintenance of autoimmunity and chronic inflammatory diseases [85]. Neutrophils can modulate the function of dendritic cells, T and B lymphocytes as they synthesize many cytokines and chemokines, including IFN-1/IFN-2[200, 218]. Additionally, during inflammation, neutrophils produce neutrophil extracellular traps (NETs) composed of chromatin and antimicrobial proteins. These traps serve to immobilize pathogens and facilitate their elimination.[74]. However, NETs can be a source of modified autoantigens and alarmins that activate both immune cells and tissue-resident cells. [112]. Alarmins are capable of inducing sterile inflammation [20] and are linked to chronic inflammatory processes. [180]. The stimulation of plasmacytoid dendritic cells by autoantigens and alarmins leads to the synthesis of IFN-1 through the activation of endosomal toll-like receptors (TLRs)[125]. Potentially, NETs can exacerbate the tissue inflammatory response by activating the NLRP3 inflammasome in lupus macrophages, inducing IL-18 and IL-1β secretion[108]. In addition, NETs are able to trigger antibody production by self-reactive memory B cells through TLR9 and B-cell receptor activation and can prime T cells to respond to antigens more effectively[80, 225]

Major histocompatibility complex (MHC) is intrinsically related to antigen presentation and, therefore, to the distinction between self and non-self. Several studies have observed a correlation between autoimmunity and MHC. In addition, mutations in proteins related to the regulation of signal transduction in cells of the immune system can generate autoimmune diseases. The gene encoding

protein tyrosine phosphatase nonreceptor type 22 (PTPN22) serves as an illustration of this phenomenon. PTPN22 is expressed in hematopoietic cells and acts to inhibit T cell activation via its receptor, while also stimulating interferon-1 production by myeloid cells. [214]. Mutations in PTPN22 have been observed in conditions such as rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, Graves' disease, and Crohn's disease[26].

Moreover, additional mechanisms have been proposed to elucidate how infections may trigger autoimmunity. These include sequence homologies between pathogen-derived peptides and self-peptides, alterations from primary epitopes to other epitopes, activation of pre-existing autoreactive immune cells, and the sustained presence of viral antigens perpetuating the immune response.[234].

The addition of sugars (glycosylation) to antibody structures can modify their function in activating inflammation, their capacity to activate complement pathways, or the clearance of immune complexes (ICs) [184]. In contrast, the loss of glycosylation can also *in vivo* suppress the inflammatory response[91]. Epigenetic modifications in DNA, such as DNA methylation, are also linked to the loss of immunological tolerance [70]. Environmental factors such as tobacco smoke, ultraviolet light, pharmaceutical agents, vaccines, heavy metals, silica solvents, infectious processes and xenobiotics, and collagen/silicone implants contribute to autoimmunity [48]. In addition, several other factors can impact the function of the immune system, including senescence [51], pregnancy[3], stress[189] and nutrition[7, 11, 16, 141].

1.2. Neutrophils and inflammation

Neutrophils are the most abundant leukocytes and represent the host's first antimicrobial defense as effector cells by recognizing microorganisms through their various receptor systems. Furthermore, they can modulate the immune response by secreting several effector molecules. Neutrophils originate from the bone marrow and develop from the normal myeloid progenitor cells through the myeloblast–promyelocyte–myelocyte–metamyelocyte pathway[64]. After being released from the bone marrow, they undergo apoptosis and die within

approximately 5.4 days. [233], or they migrate back to the bone marrow, spleen, or liver to be eliminated by resident macrophages[56]. When neutrophils undergo apoptosis and are not promptly phagocytosed by macrophages or other dendritic cells, they will undergo necrosis. [237]. In spite of that, its half-life can be extended at infection sites due to the action of proinflammatory cytokines such as Granulocyte colony-stimulating factor G-CSF[233] or hypoxia with an anti-apoptotic effect[52]. The maintenance of this survival can lead to the appearance of chronic inflammatory diseases such as rheumatoid arthritis[24].

Neutrophils are rarely found in healthy skin and thus are not "skin-resident cells." Right after an injury, neutrophils are the first cells of the immune system to reach the affected region[165], and the signals that regulate neutrophil migration in acute tissue damage can be summarized in three main phases: an early neutrophil recruitment phase induced by short-term signals, an amplification phase of neutrophil infiltration in response to more persistent signals and the resolution of inflammation, which may include neutrophil reverse migration[58].

After tissue damage, damaged and necrotic cells release damage-associated molecular patterns (DAMPs), which promote early neutrophil recruitment[181] and can directly modulate neutrophil function through surface receptors[38, 63]. DAMPs activate core enzymatic and transcriptional pathways, such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), which result in the production of chemokines and lipid mediators[153]. Moreover, DAMPs also induce the production of CXC-chemokine ligand 8 (CXCL8) family chemokines and leukotrienes from surrounding tissue cells for even more sustained neutrophil recruitment[57, 197]. Exocytosis from endothelial cells can rapidly release pre-made stores of chemokines [172]. Lipid mediators such as leukotriene B4 (LTB4) and 5-oxo-eicosatetraenoic acid (5-KETE) are also potent inducers of neutrophil chemotaxis[5, 183]. LTB4, which is produced from arachidonic acid by 5-lipoxygenase and sensed by the G protein-coupled receptors (GPCR) BLT1 receptor (also known as LTB4R) to induce neutrophil polarization and migration, plays a crucial role in the amplification stage of neutrophil recruitment[5]. Probably, LTB4 is required for persistent and long-term neutrophil infiltration[124]. Infections can trigger even more pronounced neutrophil infiltration due to the virulence factors carried by pathogens. Tissue DAMPs and

pathogen-associated molecular patterns (PAMPs) create an extra level of danger signals. Furthermore, many immune cells participate and modulate neutrophil recruitment[2, 83].

During inflammation, neutrophils produce neutrophil extracellular traps (NETs) in a process called NETosis, which facilitates the immobilization of pathogens that are too large to be engulfed. NETs are complexes containing histones, granular enzymes, and peptides, including neutrophil elastase, defensins, and cathelicidins. In addition, neutrophils also secrete factor XI, which induces the formation of NETs and attracts even more neutrophils to the inflammatory site[165]. The NET formation is an active process characterized by an internal breakdown of nuclear and granular membranes, mixing these compartments' contents in the cytosol, and finally, their extracellular release via plasma membrane rupture[74]. However, when neutrophils produce excessive NETs, such as in patients with diabetes, they hinder the healing process by causing excessive tissue damage[240]. Besides, there is evidence for NETs' contributions to cancer progression, metastasis, and cancer-associated thrombosis. NETs are increased across several cancer types and predict progression and adverse outcomes [169, 212, 238], and NETs are shown to contribute to resistance to immunotherapy[220].

Neutrophils recruited to the site of infection can phagocytize pathogenic microorganisms, ROS release, and enzymes such as myeloperoxidase and lysozyme. Phagocytosis is about 100 times more efficient when the microorganism is opsonized by IC or complement. Fc and complement receptors on neutrophils bind to these molecules, increase the adhesion between particles and neutrophils, and promote cell activation[102]. Nevertheless, excess infiltration and activation of neutrophils at a site of tissue damage can cause chronic inflammation, limit injury repair or even lead to loss of organ function[30, 119]. Pathological activation of neutrophils ultimately elicits blisters and the clinical picture of EBA[118]. In this sense, it is essential to prevent tissue damage by promoting the local resolution of inflammation by removing neutrophils from the injury site [213].

How neutrophils are eliminated from tissues can influence the inflammatory status of the environment[81]. For instance, during necrosis, due to the loss of plasma membrane integrity, proteases and reactive oxygen species (ROS) are released[19, 195]. On the other hand, in apoptosis, the cessation of secretory competence occurs[198], which is a primordial element in the resolution of inflammation[222], so apoptosis is considered a non-inflammatory form of cell death[150]. Apoptotic cells emit find me and eat me signals, being later phagocytosed by phagocytic cells such as macrophages or immature dendritic cells in a process called efferocytosis, preventing secondary necrosis and release of their toxic and immunogenic contents within the tissues, thus not generating more proinflammatory cytokine production[151, 199]. It initiates the reprogramming of the inflammation toward resolution[199], and this process can be improved by cyclin-dependent kinase inhibitors (CDKIs)[8].

Unlike apoptosis, necrosis can even intensify inflammation since necrotic cells can induce the production of proinflammatory cytokines in other cells of the immune system[19]. For instance, interaction of apoptotic cells with macrophages modifies the secretory profile of macrophages, increasing the production of anti-inflammatory signals[231] and reducing proinflammatory mediators[67]. Apoptotic neutrophils secrete lactoferrin, which has an additional anti-inflammatory effect by preventing further recruitment of granulocytes, favoring the resolution of inflammation[27]. As neutrophils age, levels of the anti-apoptotic protein myeloid cell leukemia factor-1 (Mcl-1) decline and, consequently, go into apoptosis[160]. Proinflammatory cytokines such as G-CSF increase the expression of Mcl-1, prolonging the neutrophils' half-life.

Two main pathways of neutrophil apoptosis are described; extrinsic and intrinsic. The extrinsic pathway involves binding to specific surface receptors, such as Fas, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL), which in turn activate caspase 8[205]. Furthermore, the extrinsic pathway could be started without surface receptor ligands through ROS-mediated activation of sphingomyelinase in the cell membrane and subsequent generation of ceramide[203].

The intrinsic pathway is triggered by mitochondrial membrane depolarization, which results in the release of mitochondrial cytochrome-c that binds to apoptosis-promoting activating factor-1 (Apaf-1), leading to caspase 9 activation and consequent apoptosis[133]. This activation occurs when pro-apoptotic proteins from the B cell lymphoma 2 (Bcl-2) family, including Bax, Bad, Bak, and Bid, outweigh the anti-apoptotic Bcl-2 proteins, including myeloid cell leukemia factor-1 (Mcl-1) and B cell lymphoma-extra-large (Bcl-XL). Triggers for this process include DNA damage, endoplasmic reticulum stress, and exposure to pharmacological agents such as CDKIs[33]. While pro-apoptotic proteins of the Bcl-2 family have a long half-life[160], that of Mcl-1 is around 3 hours[6]. Mcl-1 levels are rapidly and dynamically regulated by the host and, in this sense, are good targets for pharmacological therapies, such as CDK9 inhibitors[138]. The maintenance of Mcl-1 levels is mediated by phosphorylation of RNA polymerase II (RNAP-II)[87, 160].

As mentioned earlier in this work, apoptosis induction can favor inflammation resolution [27]. Another way would be neutrophil reverse migration, which describes the interstitial migration of neutrophils away from inflamed sites. Neutrophils can re-enter the vasculature in a distinct process referred to as neutrophil reverse transendothelial migration[148]. However, the detailed description of this process is beyond the scope of this work.

1.3. Epidermolysis bullosa acquisita

EBA is a rare autoimmune disease, accounting for approximately 5% of autoimmune bullous diseases. An annual incidence varies between 0.08 to 0.5 cases per million individuals[121]. EBA has no predilection for gender and can occur at any age, with a median age of 50 years and peaks of onset in the first three decades and the seventh and eighth decades of life[96]. The expression of autoimmunity to type 7 collagen is HLA-DR2 associated[77]. In addition, EBA can be associated with other diseases, the most frequent being inflammatory bowel disease (IBD), especially Crohn's disease[37, 97]. Autoantibodies against type VII collagen have been detected in up to 68% of IBD patients[98]. Moreover,

medications such as vancomycin, gentamycin[59], UV-radiation[104], and contact allergy to metals can trigger EBA[13].

The production of autoantibodies is directed to type 7 collagen (COL7) located in the basement membrane zone of the epidermis, specifically in the dense-sublamina[109]. COL7 is the main anchoring component of sublamina-dense fibrils. Therefore, its damage results in the loss of dermo-epidermal adhesion, skin fragility, and the formation of vesicles, blisters, and erosions[121]. COL7 comprises 3 domains: the N-terminal non-collagenous (NC1), collagenous, and NC2[111]. The NC1 domain is the largest antigenic portion in EBA[106], but patients with antibodies against the NC2 portion and the collagenous domains have been reported[204]. Infusing anti-COL7 antibodies in murine models clinically and histopathologically reproduces EBA[211]. In addition, immunization with recombinant NC1 fragments generated long-lasting EBA[210].

1.4. Pathophysiology of Epidermolysis bullosa acquisita

Knowledge about pathophysiology comes primarily from animal models, given the rarity of EBA in humans. In murine models, purified IgG antibodies from the sera of mCOL7-immunized rabbits are transferred, or mice are immunized with a recombinant peptide fragment from the immunogenic NC-1 domain of mCOL7. In the second model, after a few weeks, mice begin to produce antibodies against mCOL7 and then show the clinical features of EBA[21]. Accordingly, the depletion of T[209] or B cells, or dendritic cells and macrophages before the immunization phase results in no disease development[101].

The half-life of autoantibodies in the circulation is extended due to the inhibition of their catabolism by the neonatal Fc receptor (FcRn). Mice deficient in this receptor type significantly reduced clinical manifestations compared to wild-type controls in antibody-transfer models[207]. Autoantibodies against COL7 deposit on the dermal-epidermal junction (DEJ)[186]. Nonetheless, *ex vivo*, in human models only IgG1, IgG3, IgA1, and IgA3 antibodies can induce blister formation[186, 187]. A fact that has not yet been elucidated is the ability of IgA class autoantibodies to cause blisters since no complement is deposited in

DEJ[187]. After the deposition of IC in DEJ, the complement system is activated, mainly the alternative pathway[156]. Then, the recruitment of leukocytes occurs due to attraction to the lipid mediator leukotriene B4 (LTB4) and other chemoattractants[5]. Also, several cytokines are produced, modulating the inflammatory response[118].

The recruitment of neutrophils into the skin is mediated by CD18 and ICAM-1. CD18 regulates neutrophilic tissue adhesion and is essential for the clinical manifestations of EBA[41, 245]. So, the IC deposited in DEJ bind in an Fc-dependent manner to neutrophils triggering the signaling cascade, which culminates in the release of ROS, proteases, and the production of more proinflammatory cytokines amplification of the inflammatory response, and tissue destruction[118].

1.5. Signal transduction in immune complex-stimulated neutrophils

Neutrophils express a large variety of immunoreceptors and various Fc-receptors, which are primarily involved in recognition of Ig-opsonized pathogens but also participate in immune complex-mediated inflammatory processes, having the ability to inhibit or activate them[75, 188]. The most important Fc-receptors in neutrophils are the low-affinity Fcγ-receptors[29]. Crosslinking of activating Fc receptors for IgG (FcγRs) by IC induces the phosphorylation of receptor-associated γ-chains by Src kinase family members[75], triggering processes that involve the participation of Ca^{2+}, protein kinase C (PKC), RAS homologue (RHO) family and protein tyrosine kinase 2 (PYK2), PI3K-mediated TeC family and AKT signaling pathways, generating cytoskeletal rearrangement, ROS production and phagocytosis[157]. Low-affinity activating Fcγ-receptors signal through cytoplasmic ITAM motifs, which recruit the SYK tyrosine kinase and activate further signaling[75], which, in turn, activates several other signal-transduction molecules such as phosphoinositide 3-kinase (PI3K)[29]. SYK mediates the subsequent phosphorylation of the SLP-76 protein complex consisting of SLP-76, BTK, Vav, PI3K, and PLC, and this leads to the activation of downstream MAP kinases[188]. By generating phosphatidylinositol-3,4,5-tris-phosphate (PIP3), PI3K creates membrane-docking sites for Bruton's tyrosine kinase (BTK) and phospholipase Cγ (PLCγ). Activation of PLCγ leads to an

increased intracellular calcium level and triggers other downstream signaling events[171]. PI3K and p38 mitogen-activated protein kinase (MAPK) are critical to achieving this phosphorylation of protein kinase B (PKB) that, in turn, stimulates the chemotaxis[53]. Additionally, PI3K converts Phosphatidylinositol 4,5-bisphosphate (PIP2) into PIP3, and PLC hydrolyses PIP2 into IP3 and DAG. The accumulation of PIP3 in the plasma membrane initiates the recruitment of proteins containing the pleckstrin homology (PH) domain with the appropriate specificity and, as such, is directly involved in, e.g., F-actin assembly through the SCAR/WAVE complex, which consequently generates cytoskeletal rearrangements essential for neutrophil motility and phagocytosis[89]. IP3 triggers calcium mobilization, further favoring appropriate cytoskeletal changes and allowing chemotaxis or release of intracellular granules. DAG activates PKC isoforms, a family of serine and threonine kinases involved in the regulation of the NADPH oxidase system and activation of MAP kinases and NF-κB, which may trigger transcriptional regulation of genes[217]. The NADPH oxidase catalyzes the generation of oxygen radicals. It becomes active after coupling a cytosolic multienzyme system composed of 4 components ($p47^{phox}$, $p67^{phox}$, p40phox, and Rac2) with transmembrane proteins ($p22^{phox}$ and $gp91^{phox}$, which form cytochrome b558). Activated PKC isoforms and MAPKinases phosphorylate $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [17]. BTK is phosphorylated by Src-family protein tyrosine kinases or by SYK at Tyr_{551} in the SH1 domain, followed by autophosphorylation at Tyr_{223} in the SH3 domain[174]. BTK is probably involved in activating and inhibitory FcR signaling pathways, leading to reduced BTK activation by cross-linking inhibitory FcR (FcγRIIB)[168]. BTK is required for neutrophil migration. The expression of the lineage-determining transcription factors and granule proteins are BTK dependent. In addition, BTK is a critical gatekeeper of neutrophil responses because the production of ROS is increased after the engagement of Toll-like receptors (TLRs) or tumor necrosis factor (TNF) receptors in BTK-deficient neutrophils, which is reversed by the transduction of recombinant BTK[242].

Signaling through activating immunoreceptors is counterbalanced by signals emerging from ITIM-containing inhibitory immunoreceptors[188]. After ligand binding, ITIMs are phosphorylated by Src-family kinases, recruiting the tyrosine

kinases SHP-1, SHP-2 [219]and inositol phosphatases SHIP-1 and SHIP-2[192]. SHP-1 can dephosphorylate SYK and various components of the SLP-76 complex. SHIP-1 and -2 convert PIP3 to PIP2[188].The activation pathway also inhibits autoregulation through PKC-activating SHP-1[107]. The net outcome of ITAM and ITIM signaling, regulated strictly spatiotemporal at the neutrophil plasma membrane, determines the strength and nature of the downstream process affected[188]. PKC has also been shown to recruit and activate SHP-1 upon FcR signaling, indicating a potential PKC-SHP-1-mediated negative feedback loop of FcR[107].

Small GTPases of the more prominent Rho family expressed by human neutrophils are Ras-related C3 botulinum toxin substrate (Rac) proteins (Rac1, Rac2, and RhoG), RhoA, and Cell division control protein 42 homolog (Cdc42), with several other RhoGTPases expressed at lower levels[90]. Migration of neutrophils to inflammatory or infectious sites[103], phagocytosis, the NADPH oxidase, degranulation, and dynamic actin rearrangements are also regulated by Rho GTPases[90]. Besides, Rac is known to play a role in forming ROS and degranulation [123], and Cdc42 activation is essential for cell migration upon chemoattractant stimulation[151]. P-Rex1 activates RhoG, which regulates the DOCK2-ELMO complex to activate Rac signaling following GPCR activation of neutrophils[55]. PI3Ks phosphorylate the membrane lipid PIP2, producing PIP3[73], which in turn instead, activates Rac[31]. PIP3 also regulates DOCK GEFs, which are proteins that regulate chemotactic speed, neutrophil polarization, and persistent directionality[122].

The signaling pathways in IC stimulated neutrophils have not yet been fully understood. Nevertheless, some key enzymes and receptors have already been identified (Fig. 1). Together, these signaling pathways lead to neutrophil activation that in turn promotes tissue destruction and the formation of subepidermal blistering[23, 41].

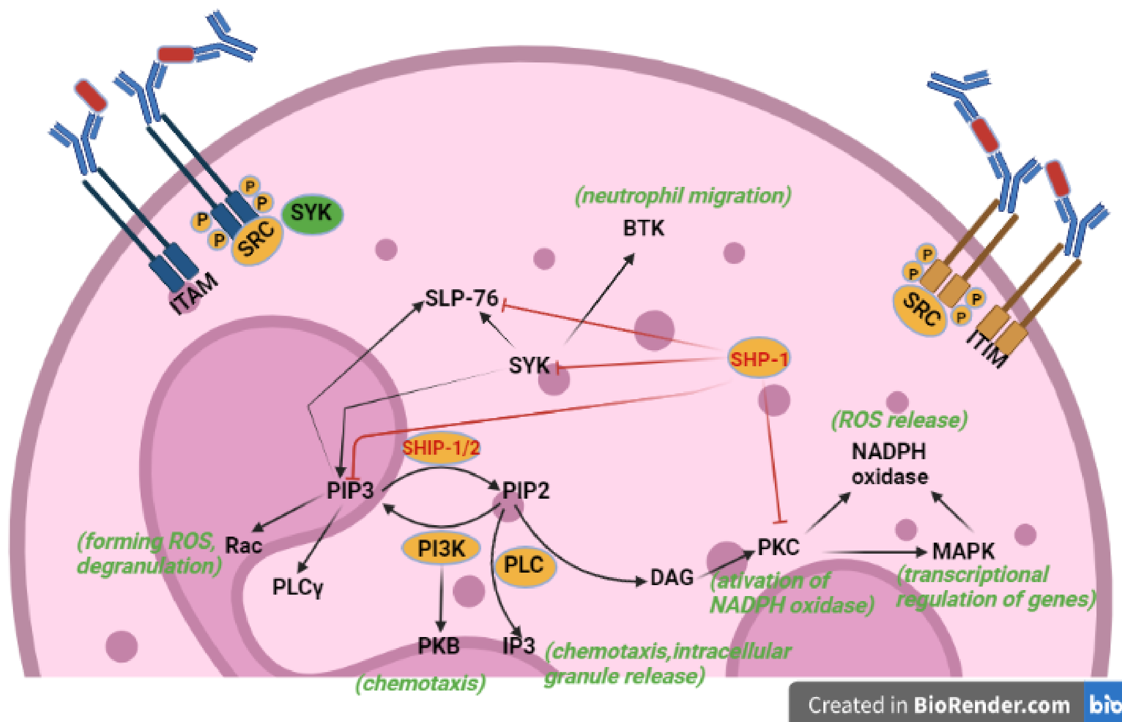


Fig. 1 Schematic depiction of signaling via immunoreceptor ITAM and ITIM motifs in neutrophils. *The interaction of immune complexes (IC) with ITAM and ITIM type receptors triggers a series of reactions leading to neutrophil activation and inhibition of such activation, respectively. Src family kinases phosphorylate both receptors.[188]. ITIM phosphorylation may recruit two major classes of phosphatases, the tyrosine phosphatases SHP-1 and SHP-2[219]and the inositol phosphatases SHIP-1 and SHIP-2[192]. SHP-1 can dephosphorylate SYK and the various components of the SLP-76 complex. SHIP-1 and -2 convert PIP3 into PIP2[188]. On the other hand, ITAM phosphorylation results in the recruitment and activation of the pivotal tyrosine kinase SYK and, consequently, in the activation of neutrophils[157]. The black and red arrows signify activating and inhibitory pathways, respectively. Image created with BioRender.com*

1.6. Kinome analysis of immune complex-stimulated neutrophils

Previously unpublished work by the research group Lübecker Institut für Experimentelle Dermatologie (LIED) was the starting point to support this research. After stimulation of neutrophils with IgG1 IC, 141 selective inhibitors were analyzed at a concentration of 1μM, of which 31 showed inhibitory substances effecting ROS release. So, 27 were revalidated in neutrophil activation

assays *in vitro*, and 8 had good results in murine models of EBA (unpublished data by Dr. Bieber). In parallel, the kinase activity in IC-activated human neutrophils using the novel PamGene technology was analyzed. This recent technique makes it possible to measure more than 100 tyrosine and serine/threonine kinases in real-time activity profiles [9]. In this way, it was possible to demonstrate that 41 kinases had their activity modulated by the stimulation ICs, including CDKs.

Interestingly, one major group of kinases, namely cyclin-dependent kinases (CDK), is activated by ICs. Based on the results (Fig. 2), a selection of CDKs was made, and suitable inhibitors were selected for this thesis. A favorable IC_{50} value and a good specificity for the corresponding kinase were significant for selecting the substances for *in vitro* analysis and a possible later application in animal models.

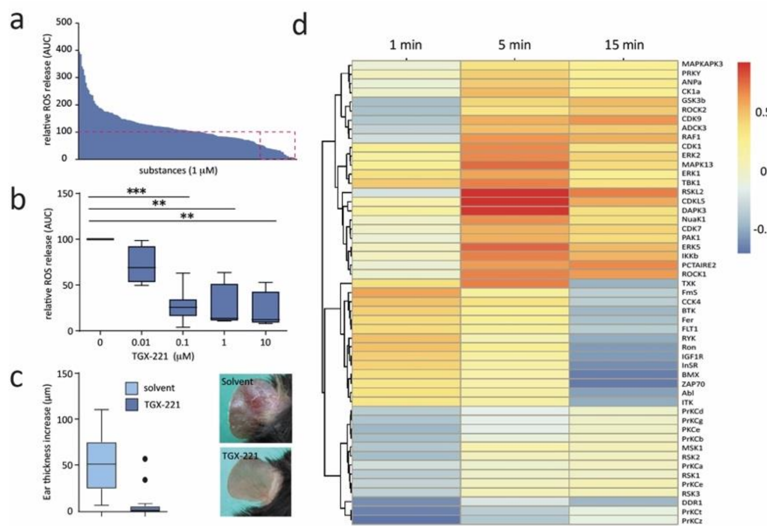


Figure 2. Screening and kinome analysis in neutrophils after activation by IC. *(a) Summary of the screening: Human neutrophils were stimulated with IC composed of human collagen type VII (hCOL7) and anti-hCOL7. ROS release was determined to measure the activation of neutrophils. Thirty-one Inhibitors reduced IC-induced ROS release by at least 50% or more. (b) The in vitro validation confirmed, among other things, that the PI3Kß-selective inhibitor TGX-221 as an inhibitor of IC-induced ROS release from neutrophils and (C) was also effective in vivo. (D) Results of the longitudinal kinome analysis of IC-activated neutrophils identify 41 kinases with modulated activity, 12 of which overlap with the screen shown in (A).*

1.7. Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) are protein kinases that need a separate subunit - a cyclin - that provides domains essential for enzymatic activity[142]. CDKs are responsible for regulating several cellular processes, including life cycle,

transcription, metabolism, communication, and apoptosis. During cell division, CDKs ensure that each cell correctly replicates its DNA and divides it evenly among the resulting cells. If the cell division is defective, the cells will go into apoptosis. Dysregulation of this process can cause cancers and neurodegenerative diseases (e.g., Alzheimer's and Parkinson's)[178]. In human cells, 20 CDKs and 29 cyclins have already been described[32].

The cell cycle is divided into 4 phases: G0/G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis). In the S phase, several checkpoints ensure that the daughter cells have the same chromosomal content[224]. The cell cycle is blocked when DNA damage is detected, allowing DNA repair before it progresses to mitosis[248]. If DNA repair fails or cells cannot program themselves to respond to cellular stress induced by the pause in their cycle, cells enter p53-induced apoptosis[120]. Three main checkpoints during the cell cycle are primarily regulated by cyclins, their partner CDKs and by CDKIs[158]. Just before entering phase S, the first checkpoint occurs in phase G1. It is controlled by cyclin-dependent kinase p16 inhibitor, an inhibitor of CDK4/6 activity, ensuring the arrest of cell cycle progression. The second checkpoint is located at the transition of the G2/M phase when misduplicated DNA copies and cells with DNA damage are eliminated. This moment is regulated by p53 and activated in response to DNA damage. Active p53 causes inhibition of proteins, such as p21, with CDK1/cyclin B to arrest the passage of cells from G2 into mitosis. The third checkpoint occurs during chromosomal alignment and daughter chromatid retraction[230].

In 2006, it was discovered that neutrophils and eosinophils could go into apoptosis due to CDKIs[193]. Leitch and colleagues showed that neutrophils express CDK1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 to various levels. However, the most strongly expressed CDKs were CDKs 7 and 9[132].

Cyclin-dependent kinase 1 (CDK1), also known as Cdc2, facilitates the transition from the G2 phase into mitosis by interacting with cyclin B1[142]. Checkpoint kinases, such as Wee1-like protein kinase (WEE1) and checkpoint kinase 1 (CHK1), control their activity and prevent daughter cells from replicating errors in the DNA[167]. One work demonstrated that CDK1 catalyzes the

phosphorylation of Mcl-1 on Ser_{64} *in vitro* in human cholangiocarcinoma cell line KMCH-1 and contributes to the antiapoptotic activity of Mcl-1 by enhancing its binding to proapoptotic Bcl-2 family[117].

CDK2 controls the G1/S and S/G2 transitions in dividing cells[45], in addition to regulating the phosphorylation of several transcription factors[45]. CDK2 protects against apoptosis through phosphorylation of C-terminal domain (CTD) of RNAP-II at Ser_2[163], and Mcl-1[42, 43]. CDK2–cyclin-E binds Mcl-1 to phosphorylate it on Thr_{92} and Thr_{163} to increase its stability, and CDK2 also facilitates the phosphorylation of Mcl-1 on Ser_{64} to sequester the proapoptotic factor Bcl-2-interacting mediator of cell death (Bim) [42, 43]. CDK2I (siRNA or CVT-313) in human diffuse large B cell lymphoma induced apoptosis associated with low levels of Mcl-1[65].

The retinoblastoma tumor suppressor protein (Rb) is a transcriptional regulator[236] that undergoes inactivation through phosphorylation by CDK4/6 in both normal and cancer cell cycles[194]. Furthermore, CDK3, in conjunction with cyclin C, phosphorylates Rb at S807/811 during the G0/G1 transition, facilitating efficient cell exit from G0[179].

In early G1, retinoblastoma gene product (Rb) is phosphorylated by CDK4 and CDK6 in association with D-type cyclins and this hypophosphorylated Rb binds to E2F family members, which prevents the transcription of E2F-responsive genes by physically blocking E2F's transactivating domain and by recruiting chromatin-modifying enzymes that actively repress transcription[226], driving a gradual increase in CDK2 activity that is required to initiate CDK2-Rb positive feedback and CDK4/6-independent cell-cycle progression[115].

During late G1, activated CDK2-cyclin-E together with CDK4/6- cyclin-D phosphorylate Rb at S807/811[46] and produce a hyperphosphorylated isoform of Rb that no longer has affinity for E2F and this initiates the transcription of genes required for cell cycle progression[246]. Moreover, CDK2 coordinates the cell cycle to centrosome duplication[149] by phosphorylating target centrosome proteins such as nucleophosmin[4].

CDKI 4/6 is already the subject of several types of research in the treatment of advanced-stage breast cancer and hematological malignancies[113]. In addition, animal models showed that Palbociclib (CDK4/6 inhibitor) reduced the inflammatory lesions in the kidney and skin in lupus-prone mice compared to controls[247]. These drugs prevent cell cycle progression from the G1 to the S phase of the cell cycle[143].

CDK5 involves several biological processes: neuron migration, neuroplasticity, neurogenesis, cell differentiation, endocrinological processes, and angiogenesis. Nevertheless, little is known about its role in the cell cycle[12, 100, 142, 235]. CDK5 may control the neutrophil secretory process via vimentin phosphorylation[130].

CDK7 controls the cell cycle by phosphorylating CDKs 1, 2, 4, and 6 in their T-loop to promote their activities[201]. CDK4 and CDK6 activity is rapidly lost following CDK7 inhibition[202]. CDK7 phosphorylates the CDK2/cyclin E complex allowing the cell to exit the G1 state and enter to S phase[208]. It also helps at the end of the S phase to activate the CDK1/cyclin B complex so the cell can enter mitosis[68]. In addition to, CDK7 phosphorylates RNAP-II CTD at Ser_5 to facilitate transcription initiation[239]. CDK7 also phosphorylates CDK9, which phosphorylates the RNAP-II CTD at Ser_2 to drive transcription elongation[128]. CDK7 phosphorylates and therefore regulates several transcription factors[68], including p53[116], retinoic acid receptor[191], oestrogen receptor[36] and androgen receptor[129].

CDK9 is a component of the positive transcription elongation factor (P-TEFb) complex involved in transcriptional regulation[147]. It regulates the transcription of primary inflammatory response genes via RNAP-II, including genes encoding inflammatory cytokines and the neutrophil pro-survival protein Mcl-1[216]. CDK9 activity and Mcl-1 drastically decrease before neutrophils go into apoptosis[233]. On the other hand, proinflammatory cytokines such as G-CSF increase the expression of Mcl-1, consequently prolonging the half-life of neutrophils[28]. Inhibition of CDK9 increases Mcl-1 degradation, inducing apoptosis, but does not affect the levels of Bcl-2A, which is a protein that changes its levels during aging and neutrophil apoptosis. Neutrophils lose cyclin T-1

expression as they age, which could cause a decrease in CDK9 levels. Mcl-1 probably has relevant anti-apoptotic activity since its expression drops drastically after 9h of cell culture, and only 20% of activity remains after 20h[233]. In addition to inducing apoptosis, CDK9 inhibitor compounds can selectively reduce neutrophil infiltration and promote the resolution of inflammation *in vitro* and *in vivo*[94, 137, 138, 193].

In summary, CDKs 1, 2, 4 and 6 regulate the cell cycle[135] while CDK7, 8, 9, 12 and 13 phosphorylate the C-terminal tail of RNA polymerase II (CTD), regulating gene transcription (Fig. 3)[105]. CDK7 also activates CDKs1, 2, 4 and 6, thus indirectly influencing the cell cycle[49, 69]. CDK3 promotes the transition from quiescence (G0) into G1 by phosphorylating Rb[190]. CDK5, 10, 11, 14–18 and 20, on the other hand, have diverse functions that are tissue specific. For instance, CDK10 and CD11 control the transcription of multiple transcription factors, hormone receptors, associated regulators, and splicing factors[135].

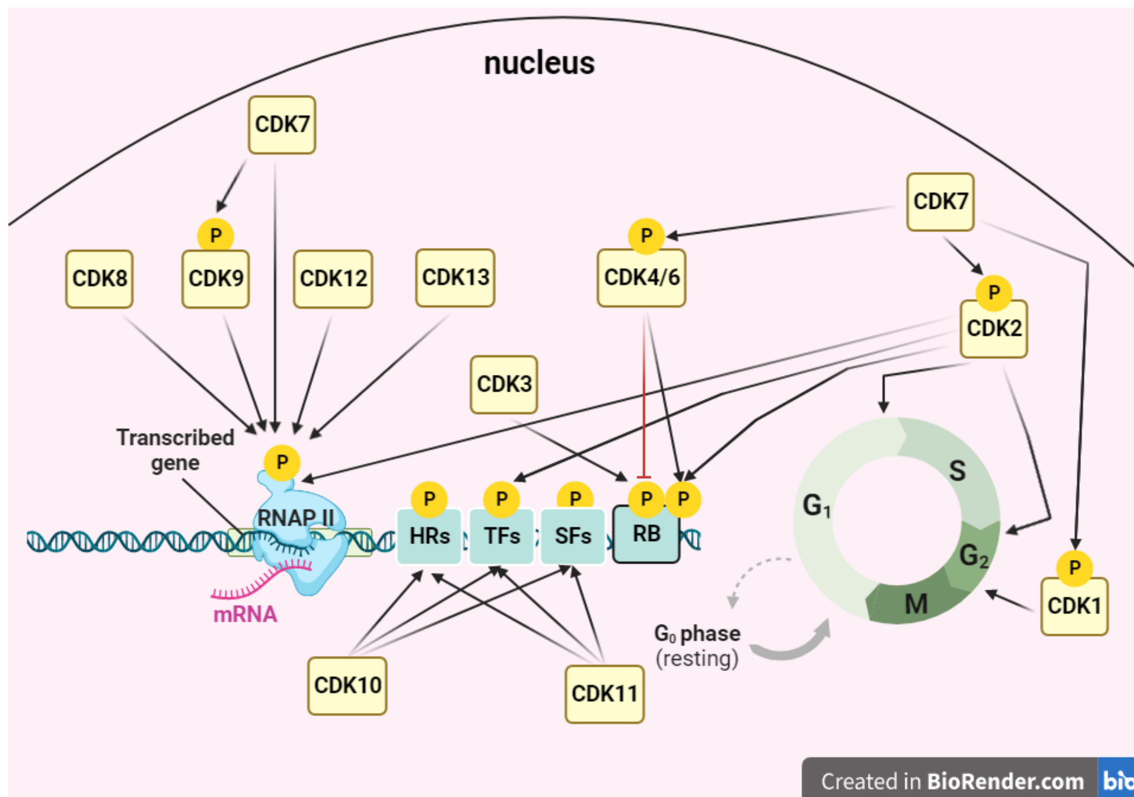


Fig. 3 Schematic representation of CDK signaling during the cell cycle and gene transcription. *In the cell nucleus, CDKs phosphorylate RNAP-II, transcription factors (TFs), hormone receptors (HRs), splicing factors (SFs), retinoblastoma gene product (Rb) and other CDKs[129]. In this way, gene transcription and the cell cycle progression are modulated. CDKs 1, 2, 4, and 6 act individually at different stages of the cell cycle, ensuring that each cell correctly replicates its DNA and divides it evenly among the resulting cells[129]. CDK3 phosphorylates Rb to promote the transition from quiescence (G0) into G1[190]. CDK7, 8, 9, 12 and 13 phosphorylate RNAP-II, regulating gene transcription[105]. In addition, CDK7 activates CDKs1, 2, 4 and 6[49, 69]. Rb exhibits 2 phosphorylation states: hypophosphorylated that prevents transcription of E2F-responsive genes, preventing entry into G1[226]. and a hyperphosphorylated isoform that activates transcription of E2F responsive genes and stimulates S phase entry[46]. CDK4/6 activation induces Rb inactivation and thereby E2F activation, driving a gradual increase in CDK2 activity that is required to initiate CDK2-Rb positive feedback and CDK4/6-independent cell-cycle progression[115]. CDK10 and CD11 control the transcription of multiple transcription factors, hormone receptors and associated regulators, or splicing factors[135]. The black and red arrows signify activating and inhibitory pathways, respectively. Image created with BioRender.com.*

1.8. Aim of this thesis

Treatment of EBA is based on local care that includes the prevention of physical trauma and infection, the use of non-adherent dressings, topical corticosteroids, and, if necessary, the use of systemic medications such as corticosteroids, dapsone, cyclosporine, colchicine, intravenous immunoglobulin, and sometimes additionally plasmapheresis[139].

Unfortunately, EBA patients respond poorly to therapy: on average, it takes 9 months to induce remission[114]. Better documented for patients with bullous pemphigoid, iatrogenic immunosuppression contributes significantly to increased mortality [127] which could be more than doubled compared to a control of the same age and sex [66].

Due to the inadequate treatment options and the increasing incidence of pemphigoid diseases, effective and safe therapeutic strategies for patients with pemphigoid diseases are urgently needed[140].

Based on these data, the effect of CDKs inhibition on IC-induced neutrophil activation *in vitro* was analyzed to validate new therapeutic options for treating EBA or other IC-induced autoimmune diseases and enhance our knowledge of the disease development. For this purpose, neutrophils from healthy blood donors were isolated, activated with IC *in vitro*, and treated with selected CDK inhibitors. ROS and FACS (Fluorescence-activated cell sorting) assays were performed, comparing neutrophil activation in the presence of CDK inhibitors with positive and negative controls.

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Equipment

Tab. 1

Equipment	Manufacturer
Centrifuge 5804 R	Eppendorf, Hamburg
Centrifuge Heraeus Megafuse 1.0R	Kendro Laboratory Products GmbH, Hanau
Centrifuge D60-20	NeoLab, Heidelberg
Centrifuge Mikro 20	Hettich, Tuttlingen
Flow Cytometer, MACSQuant® Analyzer 10	Miltenyi Biotec GmbH, Bergisch Gladbach
GloMax® Discover	Promega, Fitchburg, USA
Microliter single channel pipet, research and researchplus:10µL,100µL,200µL,1000µL	Eppendorf, Hamburg
Microliter multichannel pipet research plus:10µL, 200 µL	Eppendorf, Hamburg
Microscope, Kern OCM-1	Kern & Sohn, Balingen
Neubauer counting chamber, double subnet	Hecht Assistent, Sondheim vor der Rhön
Safety cabinet Safe 2020 class II	Thermo Electron LED GmbH, Langenselbold
Laboratory shaker microplate, TPM 4	LLG Labware, Meckenheim
pH meter pH 526	Labor-pH Meter inoLab® pH 7110
Pipet assistant pipetus®	Herschmann Laborgeräte GmbH & Co, Eberstadt
HERAcell 240i CO2 Incubator	Kendro Laboratory Products GmbH, Hanau
Vortex shaker, Vortexgenie 2	Scientific Industries Inc., Bohemia, USA
uniVACUUSYS Vacuum aspiration System	LLG Labware, Meckenheim
Captair chem	Erlab, France

2.1.2. Consumables

Tab. 2

Consumable	Manufacturer
Micro plate, 96 well, PS, F-bottom, white	Greiner Bio-One GmbH, Frickenhausen
Microplate,96well, PS, V-bottom, transparent	Greiner Bio-One GmbH, Frickenhausen
AcroPrep™ Advance 96-well filter plates	Pall Corporation, Port Washington, USA
Falcon® 5mL round bottom polystyrene tube with cell strainer snap cap	Corning, Corning, USA
Adhesive foil seal, transparent	Sarstedt, Nümbrecht
PCR single cap 8er-softstrips 0.2 mL, colorless	Biozym, Hessisch Oldendorf
Pipet tip: 10 µL	Labsolute®/Th.Geyer, Renningen
Pipet tip: 200 µL, 1000 µL	Sarstedt AG & Co. KG, Nümbrecht
S-Monovette® K3 EDTA, 9 mL	Sarstedt AG & Co. KG, Nümbrecht
Tube 15 mL	Sarstedt AG & Co. KG, Nümbrecht
Tube 50 mL	Sarstedt AG & Co. KG, Nümbrecht
Serological plastic pipet 5mL,10mL,25mL	Sarstedt AG & Co. KG, Nümbrecht
Vacuum pump	LLG Labware, Meckenheim

2.1.3. Chemicals and Biochemicals

Tab. 3

Chemicals and Biochemicals	Manufacturer
Anti-human Collagen VII IgG1	Synthesized in the Lübeck Institute of Experimental Dermatology (Recke et al., 2010)
Bovine serum albumin fraction V (BSA)	Roche, Basel, Schweiz
$CaCl_2$, powder, water-free	Carl Roth GmbH + Co. KG, Karlsruhe
DMSO	Sigma-Aldrich, St. Louis, USA
Ethanol, 96 % denatured	Carl Roth GmbH + Co. KG, Karlsruhe
HCl	Th. Geyer, Renningen
Human Collagen VII E-F	Recombinant synthesized in the Lübeck Institute of Experimental Dermatology (Recke et al.,2010)
Ion-free water (Ampuwa Plastipur)	Fresenius Kabi AG,Bad Homburg vor der Höhe
Luminol ≥97 %	Sigma-Aldrich, St. Louis, USA
Na_2CO_3	Merck KGaA, Darmstadt
$NaHCO_3$	Fluka Chemie AG, Buchs
Na_2HPO_4	Carl Roth GmbH + Co. KG, Karlsruhe
$NaHCO_3$	Fluka Chemie AG, Buchs
Na H_2PO_4	Merck KGaA, Darmstadt
NaOH	Merck KGaA, Darmstadt
Tween® 20	Sigma-Aldrich, St. Louis, USA

2.1.4 Buffer, Solutions, and Media

Tab. 4

Buffer, Solution, or Media	Composition*
Annexin V binding-buffer	BioLegend, San Diego, USA
Blocking buffer (BB)	1x PBS: 1 % BSA, 0.05 % (w/v) Tween 20
Carbonate-bicarbonate buffer	1Ld H_2O : Na_2CO_3 5.3g/L, $NaHCO_3$:5.04g/L; pH 9.6
CL medium	RPMI-1640,1% FCS, w/Glutamin, 25mM HEPES, 2 g/L Glucose
D (+) glucose solution, 100 g/L	Sigma-Aldrich, St. Louis, USA
FACS buffer	1x PBS: 0.5 % BSA
Fixation buffer	BioLegend, San Diego, USA
Fetal calf serum (FCS)	Bio & Sell, Feucht
HEPES buffer, 1 M	PAN™ Biotech, Aidenbach
MACSQuant storage solution	Miltenyi Biotech GmbH, Bergisch Gladbach
MACS Quant® running buffer	Miltenyi Biotec GmbH, Bergisch Gladbach
MACS Quant washing solution	Miltenyi Biotec GmbH, Bergisch Gladbach
PBS 10x	1L H_2O :18g/L Na_2HPO_4 x H_2O ,80g/L NaCl; pH 7.2
Polymorphprep™	Alere Technologies AS, Norway
RPMI 1640 w/ Phenol red	Lanza, Belgium
RPMI 1640 w/ stable. Glutamine, w/o Phenol red, w/o glucose	Genaxxon bioscience, Ulm
Washing buffer (WB)	1x PBS: 0.05 % (w/v) Tween 20

***If delivered ready to use, the manufacturer is indicated**

2.1.5. Antibodies and fluorescent Dyes

Tab. 5

Fluorescent Dye	Clone	Manufacturer
PE/Cy7 conjugated mouse anti-human CD14 antibody	63D3	BioLegend GmbH, San Diego, USA
Pacific Blue conjugated mouse anti-human CD16 antibody	3G8	BioLegend GmbH, San Diego, USA
APC conjugated mouse anti-human CD18 antibody	1B4/CD18	BioLegend GmbH, San Diego, USA
Brilliant Violet 510 conjugated mouse anti-human CD45 antibody	2D1	BioLegend GmbH, San Diego, USA
PerCP/Cyanine5.5 conjugated mouse anti-human CD62L antibody,	DREG-56	BioLegend GmbH, San Diego, USA
PE-conjugated mouse anti-human CD193 antibody,	5E8	BioLegend GmbH, San Diego, USA
FITC conjugated Annexin V		BioLegend GmbH, San Diego, USA
Zombie NIR		BioLegend, San Diego, USA
Propidium iodide		Miltenyi Biotech GmbH, Bergisch Gladbach

2.1.6. Dyes

Tab. 6

Dye	Manufacturer
Tryptan blue	Sigma Aldrich, St. Louis, USA

2.1.7. CDKIs

Tab. 7

Inhibitor	Catalog Nr.	Target Kinase	IC_{50}	Deliverer
Palbociclib (PD-0332991) HCl	S1116	CDK4/6	11 nM/16 nM	Selleckchem, Houston, USA
BMS-265246	S2014	CDK1/2	6 nM/9 nM	Selleckchem, Houston,USA
AS2863619	S8903	CDK8/19	0.6099 nM/ 4.277 nM	Selleckchem, Houston, USA
THZ2	S7969	CDK7	13.9 nM	Selleckchem, Houston, USA
SR-4835	S8894	CDK12/CDK13	99 nM/4.9 nM	Selleckchem, Houston, USA
Purvalanol B	S0500	cdc2-cyclin B/CDK2-cyclin A/ CDK2-cyclin E/CDK5-p35	6 nM/ 6 nM/ 9 nM/ 6 nM	Selleckchem, Houston, USA
BAY 1251152	S8730	PTEFb/CDK9	3 nM	Selleckchem, Houston, USA
OTS964	S7648	TOPK / CDK11 with Kd of 40 nM	28 nM	Selleckchem, Houston, USA

K03861	S8100	CDK2(WT), CDK2(C118L), CDK2(A144C), and CDK2(C118L/A144)	Kd 50Nm/ 18.6nM/ 15.4 nM/ 9.7 nM	Selleckchem, Houston, USA
BSJ-4-116	S9859	CDK12	unknown	Selleckchem, Houston, USA
LY3143921 hydrate	S9650	CDC 7	unknown	Selleckchem, Houston, USA

2.1.8. Softwares

Tab. 8

Software	Manufacturer
GloMax® Discover System Software	Promega, Fitchburg, USA
GraphPad Prism 7	Graphpad Software Inc., San Diego, USA
MACSQuantify software	Miltenyi Biotech GmbH, Bergisch Gladbach
Microsoft Office 365	Microsoft Corporation, Seattle, USA

2.1.9. Declaration on the acquisition and use of human biomaterials for this work

All experiments were approved by the Ethics Committee of the University of Lübeck: Subproject "Untersuchung des Einflusses verschiedener Signaltransduktionsinhibitoren auf die Aktivierung von neutrophilen Granulozyten" as part of the application "Pathogenese und Therapie Autoantikörper-induzierter Gewebsschädigung Blutentnahmen bei gesunden Proband*innen - (AZ 20-463)" and carried out in accordance with the Helsinki Declaration. Healthy volunteers donated blood with written informed consent.

2.1.10. Preparation of buffers and solutions

Tab. 9

Buffers/ solutions	Composition
Phosphate buffered saline (PBS) 10x for 1 L	NaCl: 80 g NaH_2PO_4: 18g H_2O dist.: fill up to 1 L pH 7.2
CL medium for 500 mL	RPMI-1640 leukocyte medium without phenol red: 472.5 mL FCS: 5 mL Glucose solution (100 g/L): 10 mL HEPES (1M): 12.5mL
Washing buffer (ROS Assay) for 1 L	H_2O dest.: 900 mL 10x PBS: 100 mL Tween® 20: 0,5 mL pH 7,2

Blocking Buffer (FACS Assay) for 1 L	H_2O dest.: 900 mL 10x PBS: 100 mL BSA: 10 g 2 hours at 4°C in the refrigerator to dissolve BSA
FACS Buffer for 1 L	H_2O dest.: 900 mL 10x PBS: 100 mL BSA: 5 g 2 hours at 4°C in the refrigerator to dissolve BSA
Carbonate-bicarbonate Buffer for 1 L	Na_2CO_3: 5.3 g $NaHCO_3$: 5.04 g H_2O dest.: fill up to 1 L pH 9.6
Annexin V Binding Buffer 1x	$CaCl_2$: 2.8 mg NaCl: 82 mg HEPES: 10 µL DPBS: fill up to 10 mL pH 7.4

2.2. Methods

2.2.1. Isolation of human neutrophils for use in ROS assay from peripheral blood using Polymorphprep™

Peripheral blood was drawn from healthy volunteer donors into 4 Monovettes containing EDTA as anticoagulant totaling 36 ml, layered over an equal volume of polymorphprep (PMP) solution, and centrifuged at 500xg for 35 minutes at 21°C. The acceleration and deceleration of the centrifuge for this step were set to the minimum value (minimum value = 0). In the subsequent centrifugations, these values were assigned to the maximum (maximum value = 9). PMP contains 13.8% (w/v) sodium diatrizoate and 8.0% (w/v) polysaccharides and creates a density gradient in which several fractions are formed where possible separate mononuclear cells (MN) from polymorphonuclear leukocytes (PMN). Erythrocytes sink through the PMP due to their hypoosmolarity to PMP, and settle at the bottom of the tube. After the centrifugation, two white bands, which correspond to leukocytes, can be visualized (Fig. 4). The lower band where PMNs are located is carefully aspirated using a 1000µl micropipette and transferred to a 50ml tube, where cold (4 °C) RPMI medium(Genaxxon, Ulm) is added to make 50ml and centrifuged at 300xg and 4 °C for 10 minutes.

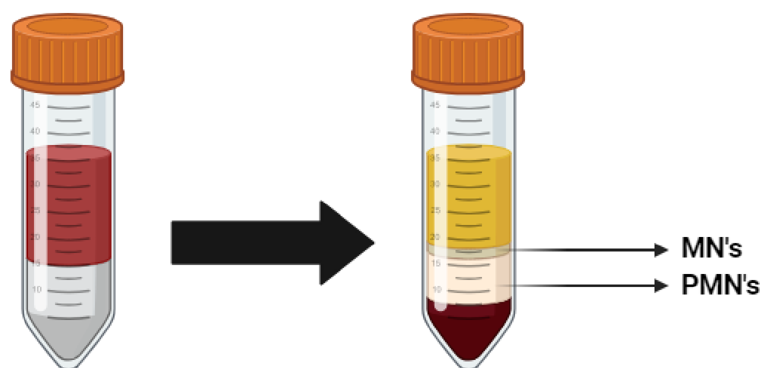


Fig. 4 Schematic depiction of the different fractions in the purification of PMNs using Polymorphprep^{TM}. *Left tube shows whole blood above the PolymorphprepTM. After centrifugation, the right tube shows different bands in which two white bands correspond to MNs: mononuclear cells and PMNs: polymorphonuclear leukocytes. Image created with BioRender.com*

After the centrifugation, the supernatant was discarded, and the pellet was resuspended in 5ml distilled water for 45 seconds. Lysis was stopped by adding 5ml 2x PBS solution, adding 15ml of cold DPBS, and centrifuged at 300xg and 4 °C for 10 minutes. The supernatant was discarded, and the lysis process can be repeated one more time if the pellets are still reddish.

If this is not the case, the washing step follows. The supernatant was discarded, resuspended in 20ml CL medium for washing, centrifuged at 300xg and 4 °C for 10 minutes, and finally resuspended in 3ml CL medium. Cell concentration was calculated using a Neubauer Counting Chamber. For this purpose, a 1:10 dilution of 10 µL of the cell suspension, 10 µL of trypan blue, and 80 µL of CL medium were prepared. Vital staining with trypan blue was carried out to determine the cell count. The polyanionic azoar dye is excluded from living cells. These appear light under the microscope, while dead cells are stained dark blue. The number of living cells was calculated using the appropriate dilution factor ($1*10^5$), and the cell suspension was diluted to the desired level ($2*10^6$ cells /mL).

2.2.2. Preparation of kinase inhibitors

The inhibitors were delivered in powder form and stored in this state at -20° C. until the beginning of the dilution. The inhibitors listed in table 7 were diluted initially in DMSO at a concentration of 10 mM, and further dilution steps produced later concentrations of 1 mM, 0.1 mM, and 0.01 mM again in DMSO (Tab. 10). The exception was Palbociclib whose initial dilution was 5 mM, its saturation point. Nevertheless, it followed later the same dilution scheme. Each solution was aliquoted into in 2 µL and stored in a freezer at -20°C. The 10 mM stock solution was frozen at -80°C for future experiments.

Tab. 10 Concentration of the kinase inhibitors in corresponding steps of the experiment

Inhibitor concentrations in the stock solution	Inhibitor concentrations after adding 10 µL CL medium	Final inhibitor concentration in the wells of the 96-Well plate [V = 200 µL]
10 mM	2 mM	10 µM
1 mM	200 µM	1 µM
0.1 mM	20 µM	0.1 µM
0.01 mM	2 µM	0.01 µM

2.2.3. ROS release assay

2.2.3.1. Measuring of ROS release

The effect of CDKs inhibitors on neutrophil activation can be measured using a ROS release assay. After IC binds to FcR, an intracellular signal transduction cascade triggers the release of ROS. For this purpose, a white 96-well microtiter plate was coated with the antigen, followed by an antibody to form IC. Then PMNs were added to bind with IC, stimulated, and neutrophils can release ROS, which can be measured on the GloMax® Discover after adding luminol. Four different concentrations of CDKI with IC were investigated in duplicates for each CDKI. In parallel, four controls, also in duplicate, were carried out: a positive control (IC without inhibitor) and three negative controls (1st coating with no coating, 2nd coating only with antigen, 3rd coating only with antibodies) (Fig.5).

The day before this measurement, a white 96-well microtiter plate was coated with 50 µL of recombinantly synthesized human collagen VII E-F (2.5 µg/mL in carbonate-bicarbonate buffer), except for the 1st and 2nd negative controls that were added only with 50 µL carbonate- bicarbonate buffer[186]. Then the plate was wrapped in foil and incubated at 4 °C overnight.

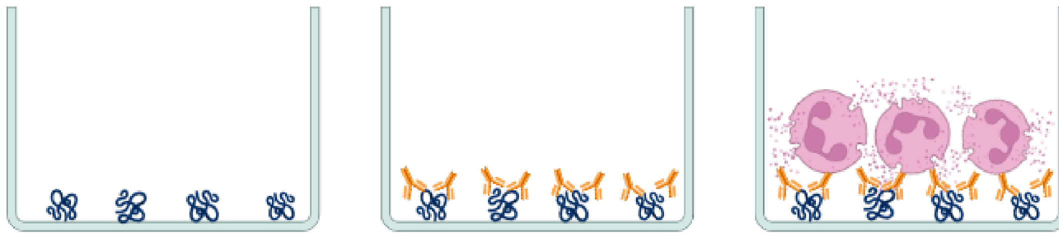


Fig. 5 Schematic coating steps of the ROS release assay. *In the first step, the wells of the white 96-well plate are coated with the antigen, a recombinantly synthesized human COL7. The corresponding antibodies (IgG1) are added in the second step and form ICs with the COL7. In the last step, freshly isolated human PMNs are added to the wells and are activated by the IC. Then, the effect of the CDKIs on neutrophils' ROS release can be measured and compared to positive controls without inhibitors. Image created with BioRender.com*

The next day the wells were washed three times with 250 µL washing buffer (PBS-T) to remove the antigen solution from the plate. Then 100 µL blocking buffer (BB) was added to each well and incubated for at least one hour at room temperature on an orbital shaker to prevent antibodies' non-specific binding. After the incubation, the plate was rewashed three times with 250 µL PBS-T per well, then 100 µL antibody solution (1.8 µg/ml anti-hCOL7-IgG1 in BB[186]) was added to the wells. Alternatively, 100 µL BB was added to the 3rd negative control well and all wells were incubated for at least 1.5 hours. The wells were rewashed with 250 µL PBS-T and once with 100 µL CL medium per well. Then 99 µL of CL medium was added to each well. In parallel, the aliquoted kinase inhibitors and a pure DMSO at -20 °C were thawed at room temperature, briefly centrifuged for a few seconds, and brought to the four desired dilutions. For this purpose, 2 µL of each set of four of the kinase inhibitors (10 mM, 1 mM, 0.1 mM, and 0.01 mM) and the pure mixture DMSO were dissolved separately in 8 µL of CL medium. Each solution was mixed briefly using a microliter multichannel pipette, and then 1 µL of inhibitor solution was added to the plate by dilution per well. Moreover, 1 µL of the diluted DMSO mixture (0.005 % final concentration) was added to each control well. The corresponding protocol was previously selected in GloMax® Discover, the device was preheated to 37 °C, and the injector was primed with the 600 µL of luminol solution. In the last step, the PMNs were quickly added to the wells using a microliter multichannel pipette.

100 μL per well with a concentration of 2*10^6 cells / mL. The luminescence was measured by a luminescence microplate reader after the injection of 10 μL luminol per well at regular intervals (62 measuring points per well within 2 hours). Around 5 mg of luminol was weighed on an analytical scale for the chemiluminescence reaction to measure ROS release. It was dissolved in NaOH 2 M and dH_2O (for 2 mg luminol: 8 μL NaOH and 1.1 mL d H_2O) shortly before the start of the measurement (ca. 62 cycles) which lasted 2 hours.

ROS, such as superoxide anions and H_2O_2, upon IC-induced stimulation, were released by neutrophilic granulocytes and reacted with luminol which is converted into the excited 3-aminophthalic acid dianion by H_2O_2, changing the 3-aminophthalic acid dianion to less excited state results in the emission of light, which the GloMax® Discover can measure. The light emitted is inversely proportional to the level of ROS production by the PMNs[71]. From this connection, a conclusion can be drawn about the activity of the cells via the measured luminescence.

2.2.3.2. Determining the purity of neutrophil granulocytes via flow cytometry

The experiments were always performed under the same conditions: cell suspension with a concentration of approximately 2*10^6 cells/mL and at least 85% neutrophils to avoid contamination with other cell groups, which could falsify the results. For this purpose, a control sample with 0.2 to 1.0 x10^6 cells was transferred to a 1.5 ml Eppis and 500 μL FACS buffer added. Then, it was centrifuged at 300g for 5 minutes at 4°C. The supernatant was removed carefully, and the pellets were resuspended with 100 μL antibody-mix composed of anti-CD14 and anti-CD16 antibodies (Tab. 11) and incubated for 20 minutes at 4°C in the dark. After this step, 400 μL FACS buffer was added to the sample and then filtered to eliminate large particles that could otherwise clog the flow cytometer. Finally, propidium iodide staining 1:100 was added directly to the sample, and the solution was analyzed via flow cytometry (Fig. 6). Propidium iodide binds to nucleic acids of permeable cells, staining nuclei of necrotic cells. Neutrophils strongly express CD16 on their surface, while monocytes and macrophages, CD14[126, 185].

For this reason, CD14^{pos} cells and necrotic cells were excluded, and CD16^{pos} cells were included into analysis.

Tab. 11 Composition of the antibody-mix

Dye	Fluorophore conjugate	Volume per well [µL]
FACS buffer	-	98
Anti-human CD14-antibody solution	APC conjugated mouse anti-human CD14 antibody	1
Anti-human CD16- antibody solution	Pacific Blue conjugated mouse anti-human CD16 antibody	1

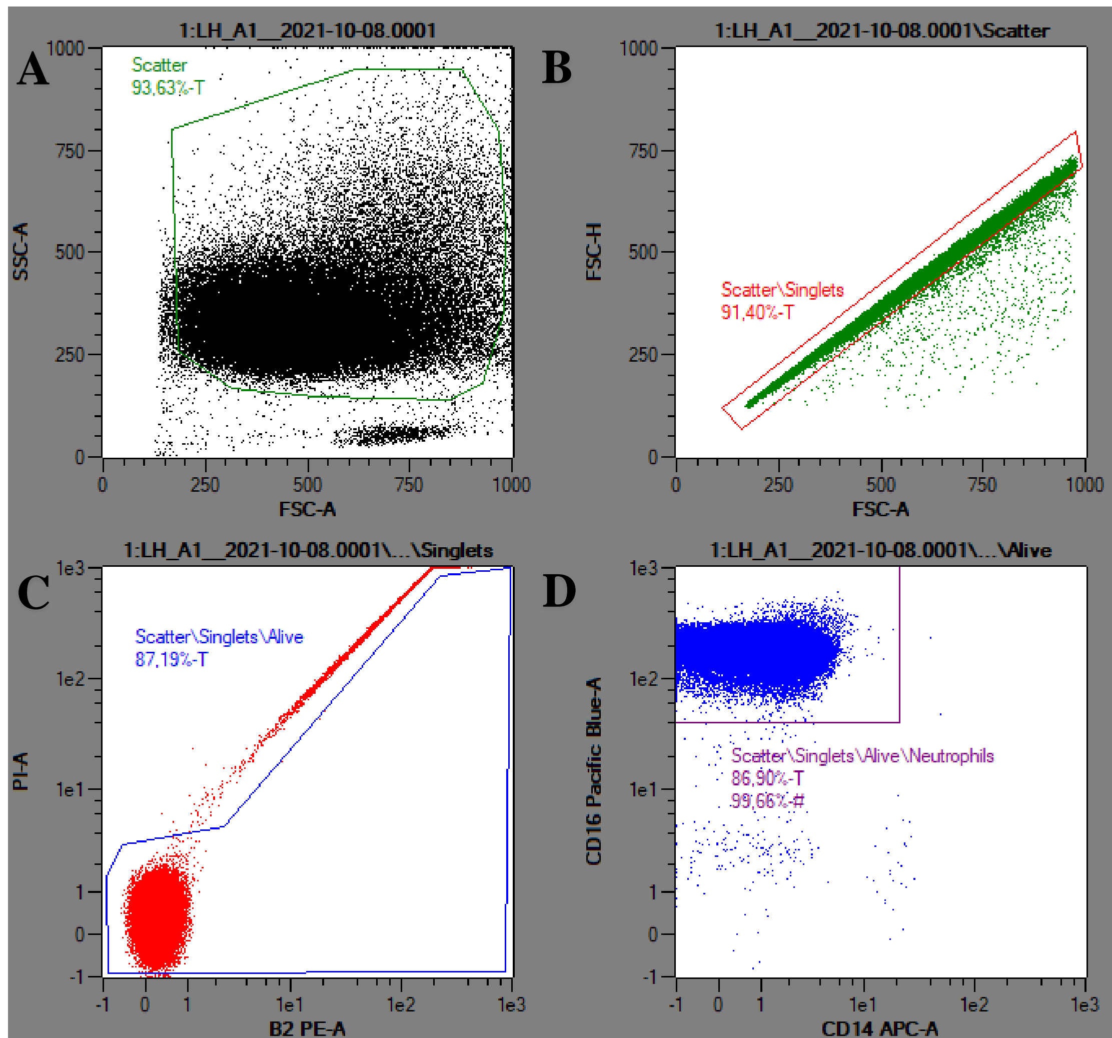


Fig. 6 Determination of the purity of neutrophil granulocytes via flow cytometry. *Flow cytometry is a technique that examines the fluorescence and light scattering characteristics of individual cells or particles suspended in a saline buffer following irradiation by laser beams, enabling the quantification of cellular subpopulations. (A) The biggest cells with more granularity and complexity of the cellular interior were included. (B) Cell doublets and triplets were excluded. (C) Necrotic cells were excluded. (D) $CD16^{pos}CD14^{neg}$ cells were included into analysis.*

2.2.4. FACS assay

2.2.4.1. Toxicity and activity test via flow cytometry

Flow cytometry is a technology that analyzes the fluorescence and light scatter pattern of individual cells or particles suspended in the saline buffer after being irradiated by laser beams, allowing the quantification of cellular subpopulations. When passing beams of visible light in 2 different directions, forward scatter (FSC) and side scatters (SSC) are formed, revealing characteristics such as cell size, complexity, and granularity. Furthermore, after previous preparation with antibody-bound fluorophores, the cytometer can measure the cellular fluorescence pattern, allowing the differentiation of cell groups in mixed samples[154]. The objective of this experiment is to measure neutrophil activation and evaluate the impact of CDK inhibitors on this parameter in addition to neutrophil vitality.

For this purpose, neutrophils must be isolated from the other cell groups present in the sample. Their activation and vitality are measured, and finally, the effect of CDKIs on those parameters is analyzed. In a flow cytometer, cells pass individually in a row through the flow chamber and are irradiated by a selection of lasers with different wavelengths. The scattering pattern is created from 2 perpendicular beams of visible light. The frontal beam generates the FSC, whose value is proportional to cell size. A beam of visible light perpendicular creates the SSC beam to the one that formed FSC, revealing the patterns of granularity and complexity of the cellular interior, its value being higher in cells with more granules[154].

Furthermore, cell doublets and triplets are excluded. The next step is to exclude cell groups from the sample, not neutrophils, from the specific pattern of cell surface markers, using cell fluorescence measurement. First, cells whose surface markers CD45[227] and CD16[185] are poorly expressed are excluded because CD45 is a leukocyte marker and CD 16 is a neutrophil marker. Then, cells with high expression of CD 193[92] and CD 14[126] on their surface are excluded, as these are eosinophil and monocyte markers, respectively.

Subsequently, the activation of neutrophils (defined as $CD45^{pos}CD16^{pos}$ $CD193^{neg}$ $CD14^{neg}$ cells) is evaluated as they have been adequately isolated from

other groups of cells. First, the expression of CD18 is quantified. Also known as integrin β-2, CD18 is a leukocyte surface protein essential for adhesion to the endothelium and extravasation into inflamed tissue[223]. Posteriorly, the expression of CD62L, or L-selectin, a parameter inversely proportional to the level of leukocyte activation, is measured. L-selectin is rapidly shed from the cell surface after leukocyte activation by a proteolytic mechanism that cleaves the receptor in a membrane-proximal extracellular region. This process may facilitate migration across the endothelium before entry into tissues. Activated leukocytes decrease their expression and undergo shedding[35]. And finally, the vitality of cells and toxicity of CDK inhibitors are evaluated. Annexin V, linked to FITC dye, is used for detecting apoptotic cells by the cytometer. Annexin V is a cellular protein that binds to phosphatidylserine, located on the inner side of the lipid bilayer facing the cytosol. However, upon undergoing apoptosis, the cellular activity of flippase decreases, allowing the translocation of phosphatidylserine to the outer layer of the plasma membrane, which, in turn, binds to Annexin V[229].

Zombie NIR is a hydrophilic and polarized amine-reactive fluorescent dye that cannot permeate into intact cell membranes. During the process of necrosis, the integrity of the plasma membrane is lost, allowing Zombie NIR to bind to peptides of cytosolic proteins and, consequently, the detection of necrotic cells by cytometry[18].

The initial steps of the FACS assay are identical to the ROS assay until the cell suspension coating is in the wells. From that point, the white 96-well microplate with the cell suspension and CDKs inhibitors was loosely covered with a lid and incubated in an incubator at 37°C, 5% CO_2 for 2 hours. During this interval, 100 µL of FACS buffer was added through a 40µm nylon mesh filter to a new 96-well plate with a V-bottom and centrifuged (500g, RT, 10 minutes). The purpose of this procedure is to humidify the filter to make the transfer of the cell suspension to the new 96-well plate with a V-bottom more efficient. Then 100 µL of FACS buffer was discarded, and the samples were transferred from the wells of the 96-well white plate to a 96-well plate with a V-bottom through the 40µm nylon mesh filter and centrifugated at 450g and RT for 6 minutes. More 100 µL of FACS buffer was added through the 40µm Nylon Mesh filter to a new 96-well plate with a V-bottom and centrifuged at 450g and RT for 6 minutes.

After that, the supernatant was removed, and the pellets were each resuspended in 40 µL Annexin V binding buffer (ABB). Then 10 µL Zombie NIR (diluted 1: 1000 in ABB) were added to each well, and the plate was incubated at 4-8 °C for 15 minutes in the dark. After the incubation period, 10 µL of the anti-CD mix (Tab. 12) was added to each well, and the plate was incubated at RT for 15 minutes in the dark.

Tab. 12 Composition of the Anti-CD mix

Dye	Fluorophore conjugate	Volume per well [µL]
Annexin V-binding-Buffer	-	7.71
Anti-human CD45-antibody solution	Brilliant Violet 510 conjugated mouse anti-human CD45 antibody	0.08
Annexin V FITC solution	FITC conjugated Annexin V	0.31
Anti-human CD14- antibody Solution	PE/Cy7 conjugated mouse anti-human CD14 antibody	0.38
Anti-human CD16- antibody solution	Pacific Blue conjugated mouse anti-human CD16 antibody	0.38
Anti-human CD18- antibody Solution	APC conjugated mouse anti-human CD18 antibody	0.38
Anti-human CD62L- antibody solution	PerCP/Cyanine5.5 conjugated mouse anti-human CD62L antibody	0.38
Anti-human CD193- antibody Solution	PE-conjugated mouse anti-human CD193 antibody	0.38

The plate was then centrifuged at 450 g and RT for 6 minutes, the supernatant discarded, the pellets resuspended in 200 µL ABB, and then centrifuged again at 450 g and RT for 6 minutes. Similarly, the supernatant was discarded, and the pellets were resuspended in 200 µL FACS buffer and incubated at room temperature for 15 minutes in the dark. After another centrifugation at 450 g and room temperature for 6 minutes and removal of the supernatant, the pellets were resuspended in 100 µL of fixation buffer and incubated again at room temperature for 15 minutes in the dark. Then 100 µL FACS buffer was added to each well, carefully mixed, and centrifuged at 450 g and RT for 6 minutes. The supernatant was discarded, and the pellets were resuspended in 100 µL FACS buffer. The 96-well plate was stored at 4 °C in the dark until the measurement time (max. 1 week). On the measurement day, the samples were resuspended, and the measurement was started. The experiment was performed with duplicate

determinations for each concentration and control. The analysis mask on the flow cytometer was used as follows (Fig.7,8).

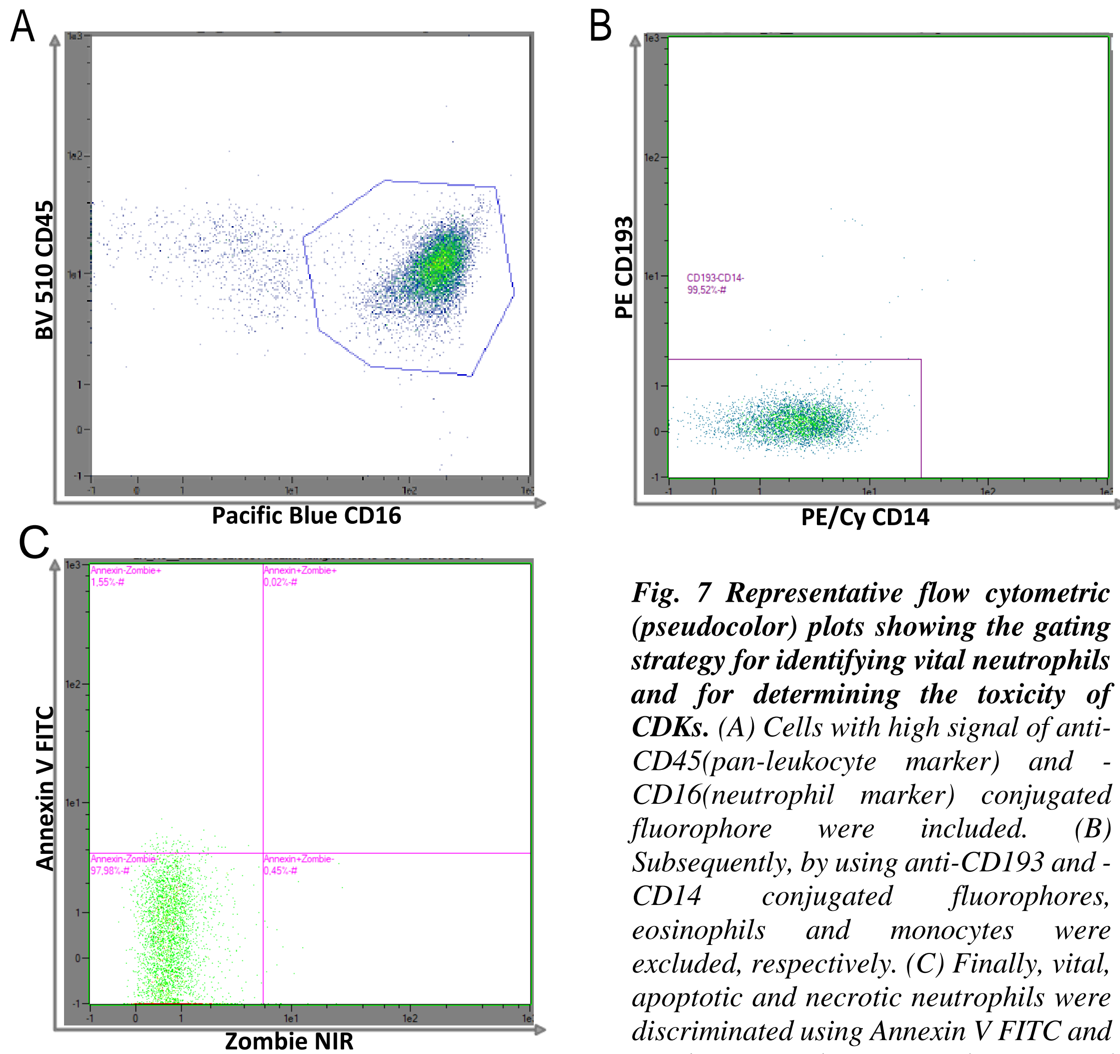


Fig. 7 Representative flow cytometric (pseudocolor) plots showing the gating strategy for identifying vital neutrophils and for determining the toxicity of CDKs. *(A) Cells with high signal of anti-CD45(pan-leukocyte marker) and -CD16(neutrophil marker) conjugated fluorophore were included. (B) Subsequently, by using anti-CD193 and -CD14 conjugated fluorophores, eosinophils and monocytes were excluded, respectively. (C) Finally, vital, apoptotic and necrotic neutrophils were discriminated using Annexin V FITC and Zombie NIR to determinate the toxicity of CDKIs.*

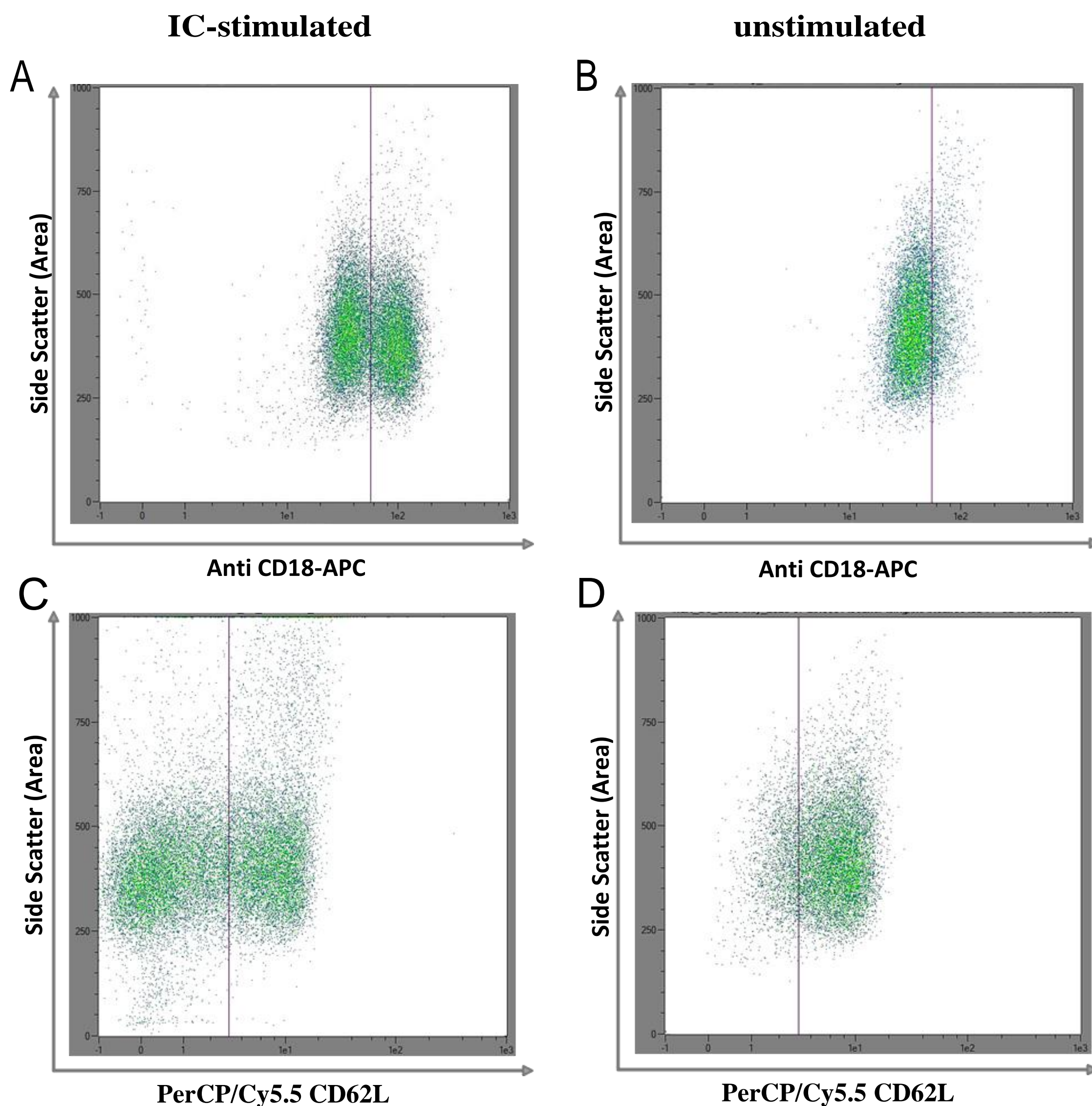


Fig. 8 Representative flow cytometric (pseudocolor) plots differences between IC-stimulated and unstimulated CD18 and shedding of CD62L. *Sub-gates discriminating neutrophils with high and low expression of CD18 molecules in an IC-stimulated sample (A) and unstimulated sample (B) reveal increased CD18 expression in activated neutrophils. Sub-gates identifying neutrophils with high expression and low expression/shedding of CD62L molecules in an IC-stimulated sample (C) and unstimulated sample (D) demonstrate increased shedding of CD62L in activated neutrophils.*

2.2.5. Statistical analysis

For the statistical analysis, a Friedman test was performed. It is a non-parametrical method with one-way repeated measures analysis of variance by ranks. It is used to detect differences in treatments across multiple test attempts. This test analyzes whether each CDKI is significant concerning the IC-stimulated positive control[72].

In addition, the Dunn's multiple comparison post hoc test was performed to identify which specific concentrations of CDKI were significant relative to the positive control. Each concentration was compared to the IC-stimulated positive control. It is a non-parametric method that can pinpoint which specific means are significant from the others[61].

Data are shown as Tukey’s box-and-whiskers plots, with the lower quartile indicating the 25th percentile, the middle line indicating the median, and the upper quartile indicating the 75th percentile[228]. The whiskers in a box plot represent maximum values by adding the 75th percentile to 1.5 times the interquartile range, and minimum values by subtracting 1.5 times the interquartile range from the 25th percentile. Values beyond these whiskers are depicted individually on the plot and termed as outliers.[170].

Mann-Whitney U test was performed to compare the IC-stimulated control with the unstimulated only cells control without prior normalization about CD18 expression or CD62L shedding [145].

To conduct ROS assay analysis, four controls, in duplicate, were carried out: a positive control and three negative controls. The mean of each duplicate negative control and positive control was calculated for each 62 measurements and plotted on a graph. The area under the curve (AUC) of the negative and positive controls of each donor was measured, and the positive control AUC was defined as 100%. If the AUC of each negative control was less than 50% of the AUC of the positive control or the purity of neutrophil granulocytes via flow cytometry was less than 85%, the sample was excluded from statistical analysis.

For FACS assay analysis, duplicate tests were performed with a positive control and three negative controls. The mean of each duplicate negative and positive control was calculated, and the data were normalized to the positive control (IC-stimulated neutrophils) to facilitate the statistical analysis. The sample was considered eligible when negative control's CD18 expression or CD62L shedding was less than 50% of the IC group's normalized CD18 expression or CD62L shedding. In this way, it was possible to adjust the individual differences in the expression of each blood donor's markers and compare the relative inhibition of each CDKI concerning the positive control.

3. RESULTS

3.1. ROS release underlies donor-dependent heterogeneity

The production of ROS measured by luminescence differs considerably between different blood donors. Comparing them and defining whether CDKIs influence neutrophil activation creates a challenge. For this purpose, negative and positive controls were first defined. The positive control contained IC-stimulated PMNs; the three negative controls consisted of only PMNs or PMNs in the presence of either only human COL7 E-F or only anti-COL7 IgG1. Control measurements were performed in duplicate, and the mean was calculated. Then the values obtained referring to the release of ROS during the 2h were plotted on a graph (Fig. 9). The AUC was calculated, the positive control AUC was defined as 100% and compared in percentage with the area of the other samples. If the AUC of each negative control was less than 50% of the AUC of the positive control, and the purity of neutrophil granulocytes via flow cytometry was at least 85%, the experiment was considered satisfactory, and the comparison of the AUCs of each CDKI at the four different concentrations concerning the positive control was performed. This process diminished the heterogeneity related to 7 blood donors' biological (D) differences.

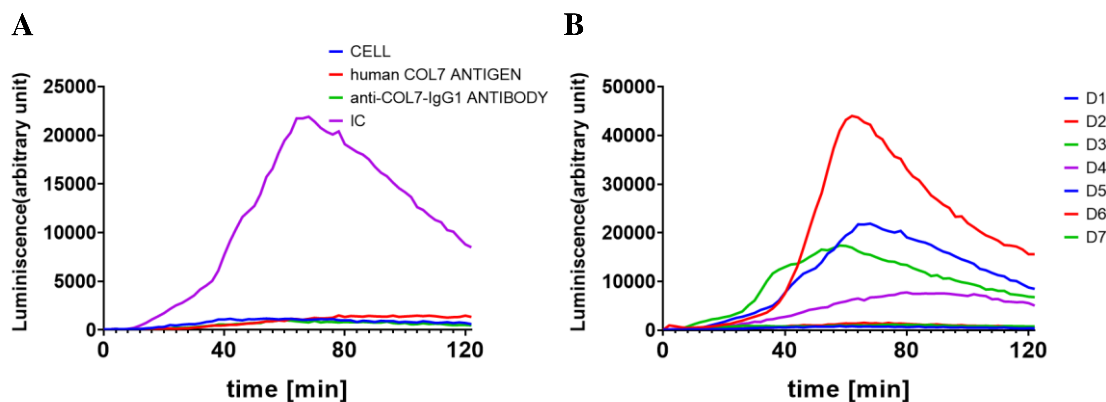


Fig. 9 Luminescence signal over time of stimulated neutrophils. *Human PMNs were isolated from 7 blood donors and incubated under different conditions, and the luminescence was measured over 2 h. (A) The positive control (IC) of one representative sample shows a higher increase and higher peak than the three negative controls (PMNs incubated with only human anti-COL7 IgG1 or with only human COL7 E-F or with only PMNs). (B) The height and exact position of the luminescence peak and the detailed shape of the curve of the IC-control are individual for each blood donor (D1 – D7), which requires a normalization of the*

obtained data

3.2. Four of the eleven tested compounds reduce the ROS production significantly in stimulated human PMNs

This experiment aimed to investigate the effect of CDKI on neutrophil activation, and ROS production of the stimulated neutrophils is an adequate parameter for this purpose due to its tissue-destroying potential. As described in 2.2.1, human PMNs were isolated from fresh human whole blood and incubated with IC and CDKIs at four different concentrations. The luminescence was measured over 2 h. For the analysis, the mean values of the duplicates of each time point were calculated and added up to an AUC value. All AUCs were normalized relative to the positive control, whose AUC was defined as 100%.

Seven out of eleven inhibitors show significance in the Friedman test (Fig. 10). Four of those eleven substances Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), BAY 1251152 (CDK9I, PTEFbI), and OTS964 (TOPKI, CDK11I) show significance in the Dunn's test for the 10µM inhibitor concentration. THZ2 (CDK7I) also shows significance in the Dunn's test for the concentration of 1µM and 100nM (Fig. 10). Four of the eleven inhibitors show no significant effect on the reduction of ROS release (Fig. 11).

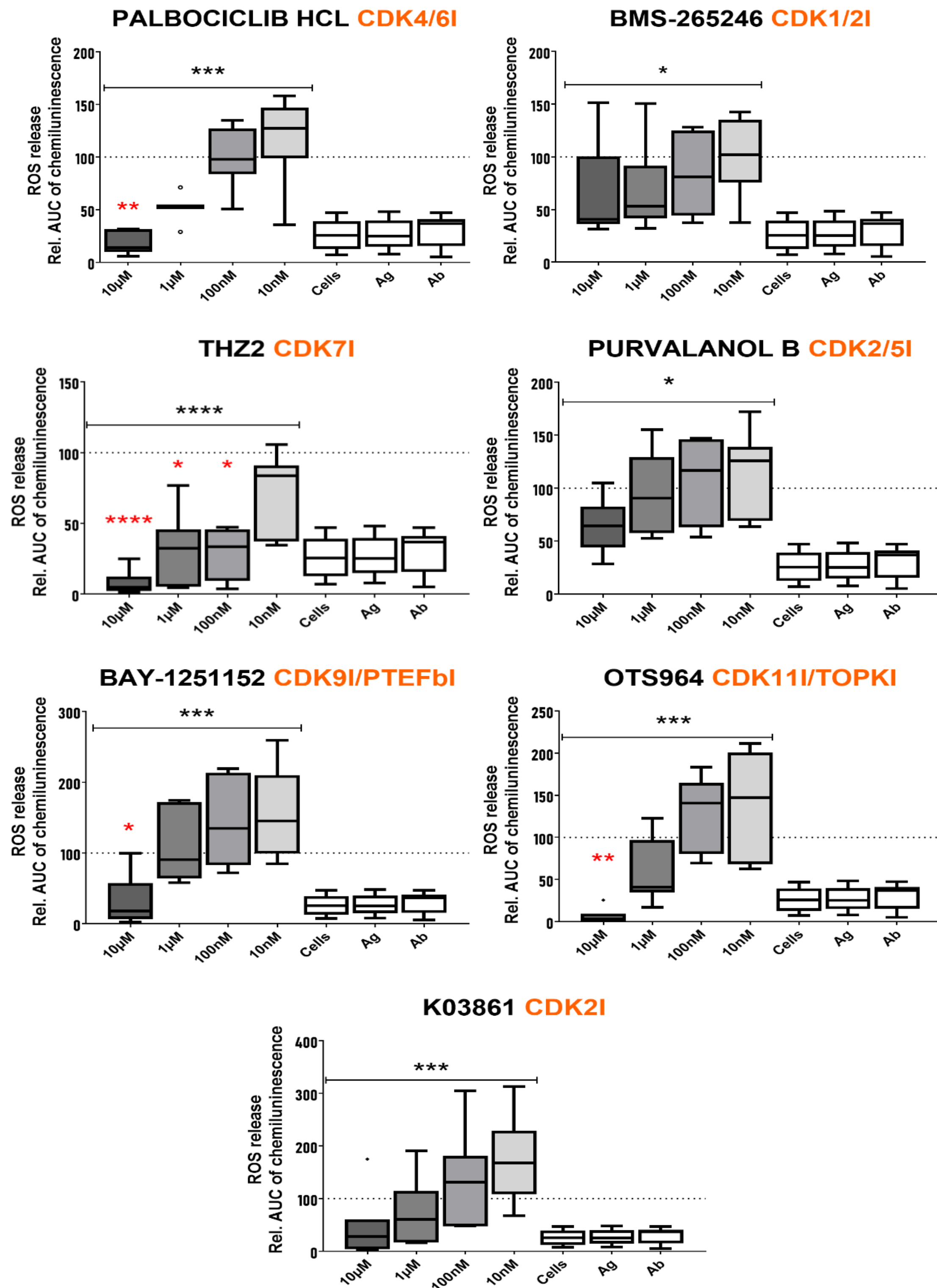


Fig. 10 Seven of the CDKIs reduce the ROS release. *Human PMNs were isolated from fresh whole blood and then stimulated with ICs in the presence of either one of the kinase inhibitors. The ROS release was measured indirectly via the reaction with luminol. Depicted is the difference in the AUC values of the chemiluminescence over 2 h. Data were normalized with the positive control (IC-stimulated PMNs). Data are shown as Tukey's box-and-whisker plots. n=7. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by a Dunn's multiple comparison (indicated by*

*red stars). The dashed line indicates the normalized positive control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

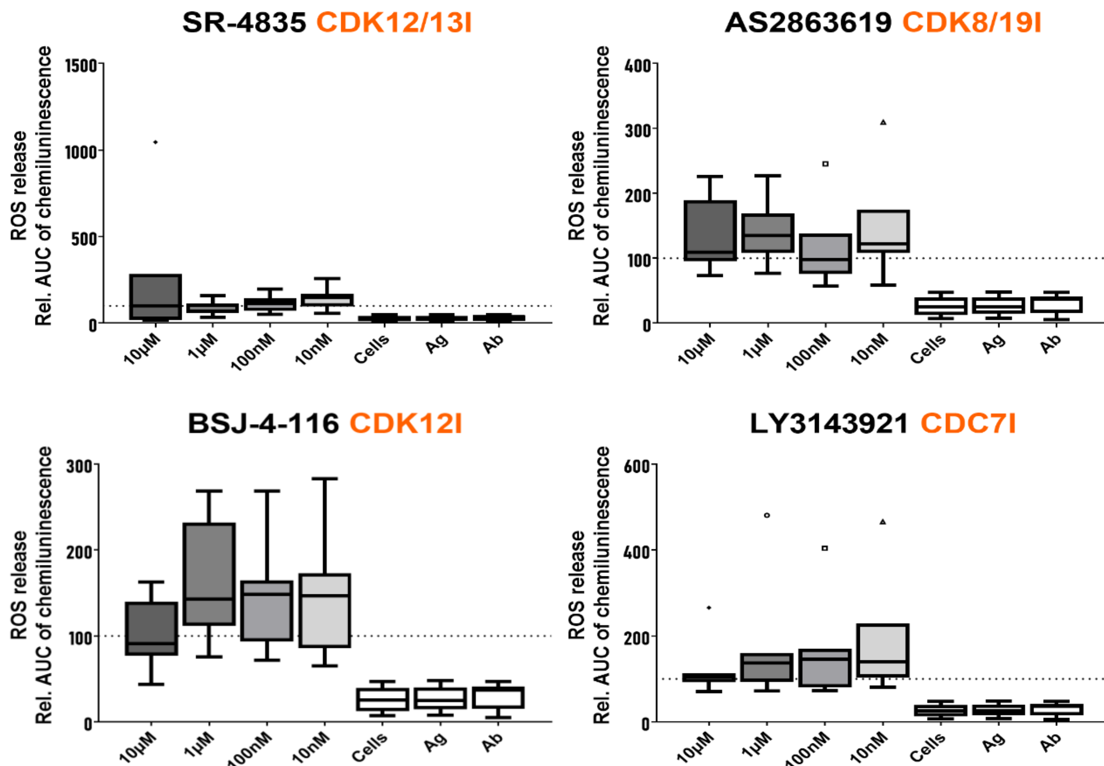


Fig. 11 Four of the eleven inhibitors show no significant effect on ROS-reduction in IC-stimulated PMNs. *Human PMNs were isolated from fresh whole blood and then stimulated with ICs in the presence of either one of the CDKI. The ROS release was measured indirectly via the reaction with luminol. Depicted is the difference in the AUC values of the chemiluminescence over 2 h. Data were normalized with the positive control (IC-stimulated PMN). Data shown as Tukey's box-and-whisker plots. n=7. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple comparison (indicated by red stars). The dashed line indicates the normalized positive control.*

3.3. Flow cytometric measurement underlies donor-dependent heterogeneity

As mentioned in 2.2.4, $CD18^{pos}$ and $CD62L_{neg}$ are indicators of neutrophil activation and, therefore, valuable parameters to be analyzed to assess the possible inhibition of CDKIs on neutrophils. Furthermore, an increased expression of CD18 and CD62L shedding contributes to a more inflammatory phenotype in antibody transfer-induced murine models[22, 23].

The mechanism of action of CDKIs is the induction of the intrinsic apoptosis pathway, which, in turn, would possibly interfere with neutrophilic activation, as neutrophils would enter apoptosis before being activated. Apoptotic cells emit find me and eat me signals, being later phagocytosed in a process called efferocytosis, not generating more proinflammatory cytokine production[151]. Thus, it is a desirable effect in the control of autoimmunity and inflammatory diseases. However, necrosis can intensify inflammation, unlike apoptosis, since necrotic cells can induce the production of proinflammatory cytokines in other cells of the immune system[19].

To further investigate the potential therapeutic properties of the CDKs, a flow cytometric analysis (n=5) was performed. In this experiment, freshly isolated human PMNs were stimulated via IC in the presence of the CDKs at four chosen concentrations for 2 hours. Duplicate measurements were taken. After the incubation, the PMNs were treated with a defined panel of antibody-conjugated fluorophores and fluorescent dyes to identify subpopulations positive or negative for specific marker molecules. In a flow cytometer, the subpopulations of the neutrophils were determined. To rule out the possible toxic effects of the inhibitors, the non-vital neutrophils were stained using Annexin V-conjugated FITC and Zombie NIR.

Similar to the ROS release assay, the PMNs of each blood donor reacted differently to the same experiment (Fig. 12), so the data were normalized to the positive control (IC-stimulated neutrophils) to facilitate the statistical analysis of the data. The sample was considered eligible when negative control's CD18 expression or CD62L shedding was less than 50% of the IC group's normalized CD18 expression or CD62L shedding. In this way, it was possible to adjust the individual differences in the expression of each blood donor’s markers and compare the relative inhibition of each CDKI concerning the positive control.

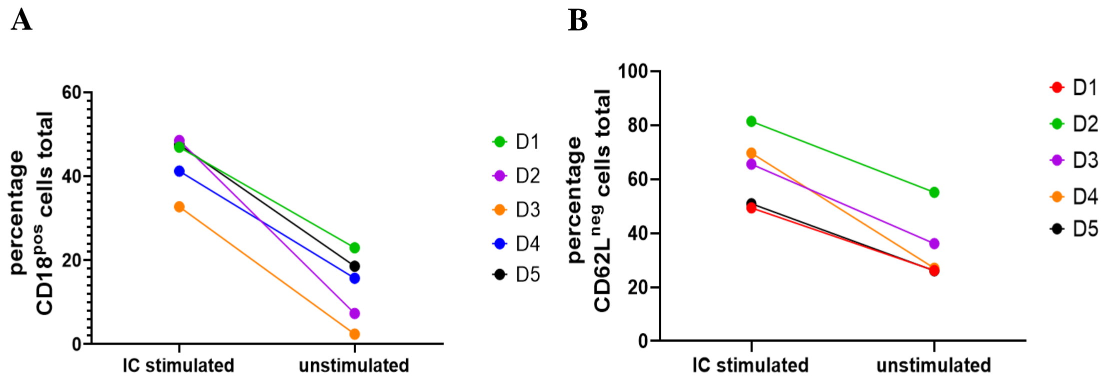


Fig. 12 The IC-stimulated controls and unstimulated controls reflect the heterogeneity related to blood donors' biological (D) differences. *Human PMNs were isolated from fresh whole blood and stimulated with ICs over 2 h. Afterward, they were stained with a fluorophore-conjugated mouse Anti-CD mix. PMNs were analyzed in a flow cytometer and sub-gated to identify vital $CD45^{pos}CD16^{pos}CD193^{neg}CD14^{neg}$- (A) $CD18^{pos}$ and (B) - $CD62L_{neg}$ neutrophils. Colored points depict mean values of the double determinations, and both controls of one biological replicate are linked via a black line. n=5. Mann-Whitney U test was applied. p=0,0625 for both groups.*

3.4. Three CDKIs exhibit influence on the expression of CD18 on stimulated neutrophil granulocytes

Three of the eleven inhibitors Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), and OTS964 (TOPKI, CDK11I) showed significance in the Friedman test regarding the reduction of $CD18^{pos}$ cells (Fig. 13). Two of those three inhibitors Palbociclib (PD-0332991) HCl (CDK4/6I) and THZ2 (CDK7I) show significance in the Dunn’s test. THZ2 (CDK7I) shows significance in the Dunn’s using 10µM inhibitor concentration. Palbociclib (PD-0332991) HC1 shows significance in the Dunn’s Test for the concentration of 10nM. Eight of the eleven CDKIs show no sign of significance neither in the ANOVA nor the multiple comparisons, and LY3143921 hydrate (CDC7I) even increased the expression of CD18 (Fig. 14).

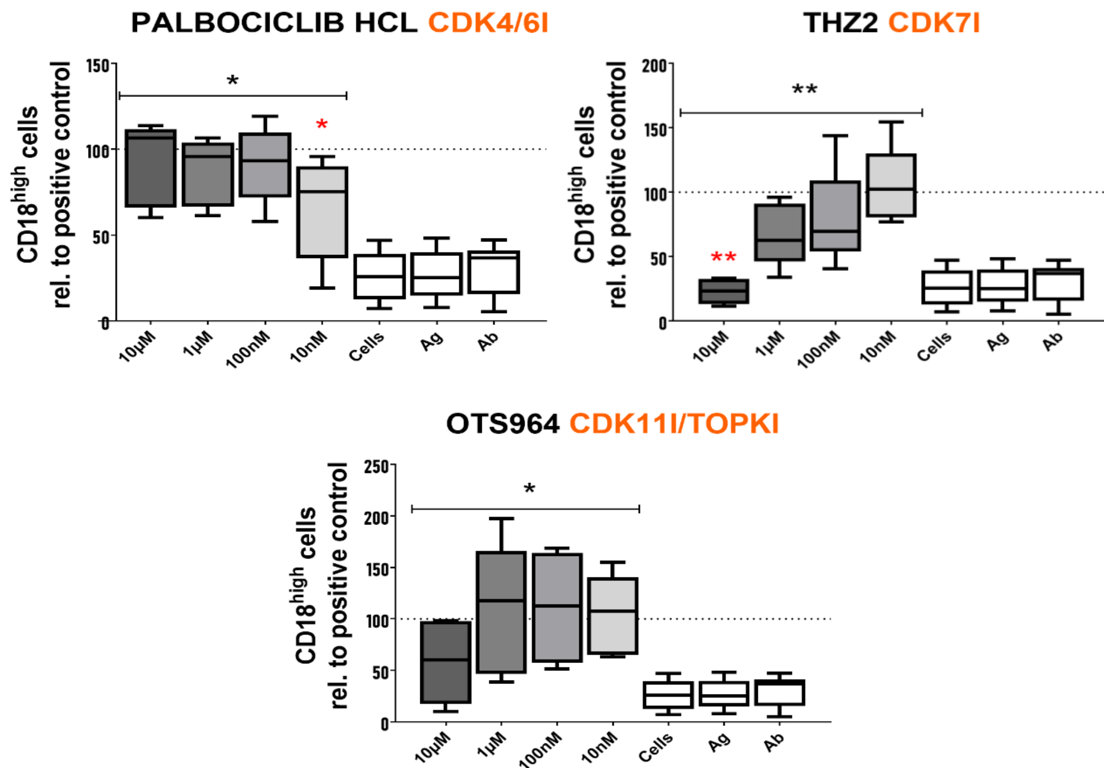


Fig. 13 IC-induced increase in CD18 expression is affected by three CDKIs. *Human PMNs were isolated from fresh whole blood and stimulated with ICs over 2 h. Afterward, they were stained with a fluorophore-conjugated mouse anti-Human antibody solution. PMNs were analyzed in a flow cytometer and sub-gated to identify vital $CD45^{pos}CD16^{pos}CD193^{neg}CD14^{neg}$-$CD18^{pos}$. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple comparison (indicated by red stars). The dashed line indicates the normalized positive control. *p<0.05, **p<0.01.*

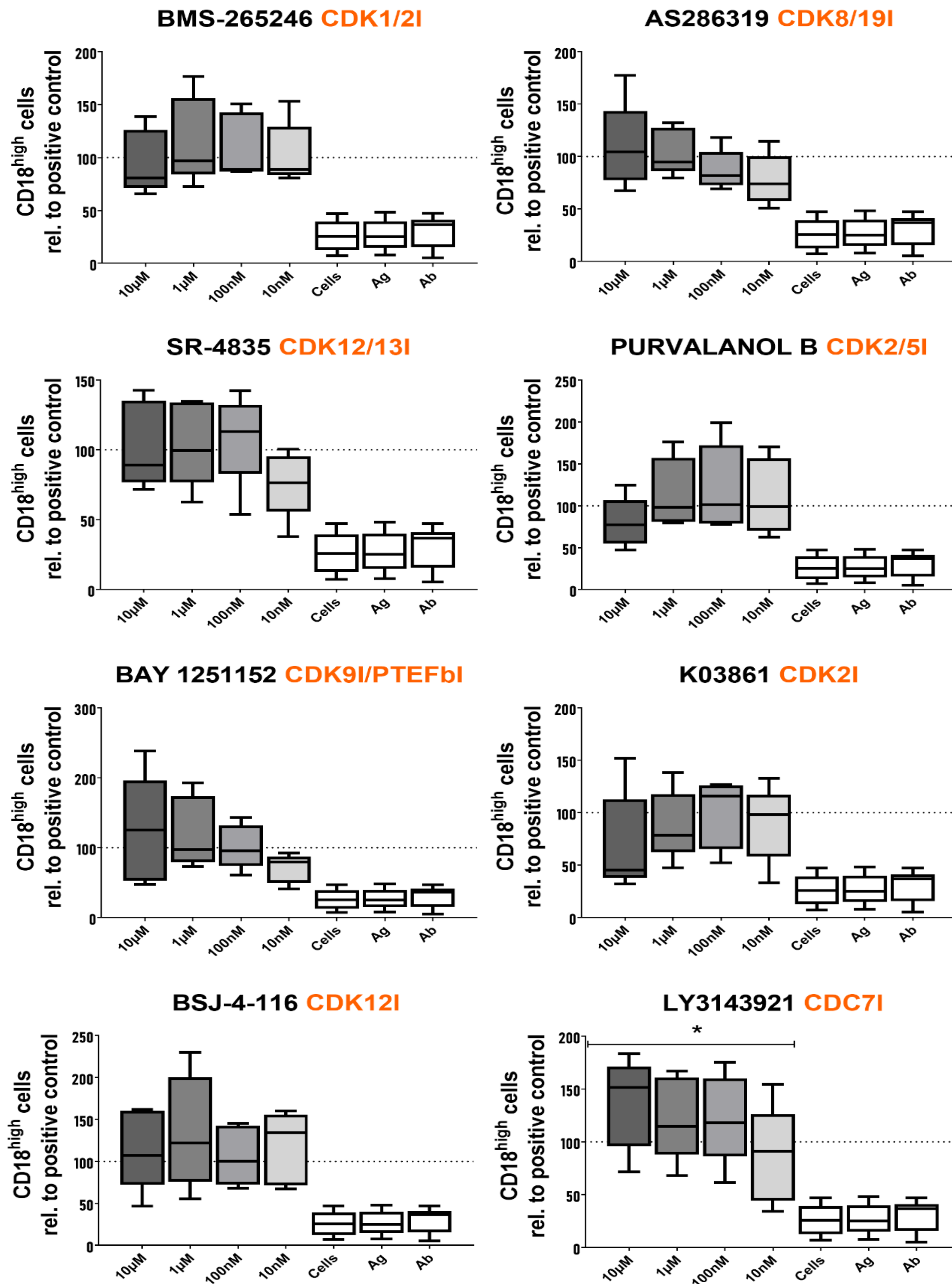


Fig. 14 Some CDKIs do not affect the IC-induced increase in CD18 expression or can even intensify it. *Human PMNs were isolated from fresh whole blood and stimulated with ICs over 2 h. Afterward, they were stained with a fluorophore-conjugated mouse anti-Human antibody solution. PMNs were analyzed in a flow cytometer and sub-gated to identify vital $CD45^{pos}CD16^{pos}CD193^{neg}CD14^{neg}$-$CD18^{pos}$. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple comparison*

*(indicated by red stars). The dashed line indicates the normalized positive control. *$p<0.05$.*

3.5. None of the CDKIs exhibit a reduction on $CD62L_{neg}$ -levels on IC-stimulated neutrophils

The absence of CD62L indicates the ability of neutrophilic granulocytes to migrate into the tissue[35]. Consequently, a reduction in the population of IC-stimulated $CD62L_{neg}$ neutrophils after incubation with CDKIs would indicate inhibition of chemotactic activity.

None of the inhibitors significantly impact the reduction of $CD62L_{neg}$ cells (Fig. 15). The opposite effect was observed. Seven of the eleven inhibitors SR-4835 (CDK12/13I), Purvalanol B (CDK2I/5), BAY 1251152 (CDK9I, PTEFbI), OTS964 (TOPKI, CDK11I), K03861 (CDK2I), and BSJ-4-116 (CDK12I), and LY3143921 hydrate (CDC7I) stimulated the CD62L shedding (Fig. 16).

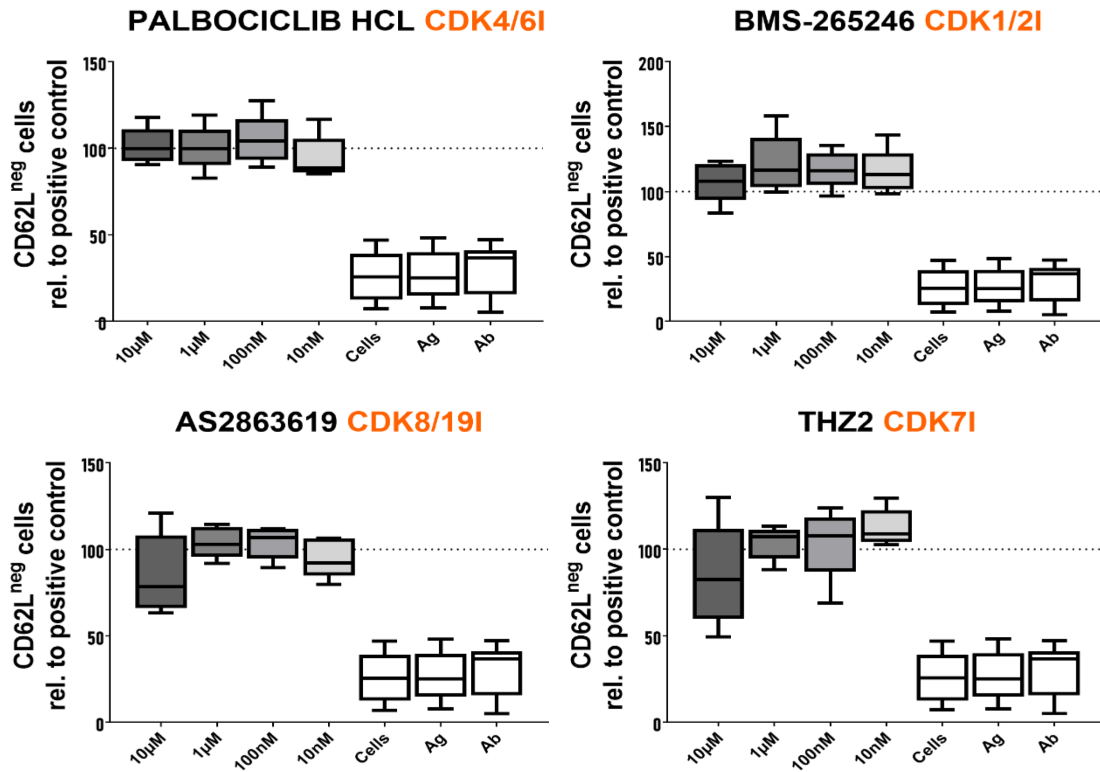


Fig. 15 Some CDKIs do not affect the reduction of CD62L-shedding. *Human PMNs were isolated from fresh whole blood and stimulated with ICs over 2 h. Afterward, they were stained with a fluorophore-conjugated mouse anti-Human antibody solution. PMNs were analyzed in a flow cytometer and sub-gated to identify vital* $CD45^{pos}CD16^{pos}CD193^{neg}CD14^{neg}$*-*$CD18^{pos}$*. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple comparison (indicated by red stars). The dashed line indicates the normalized positive control.*

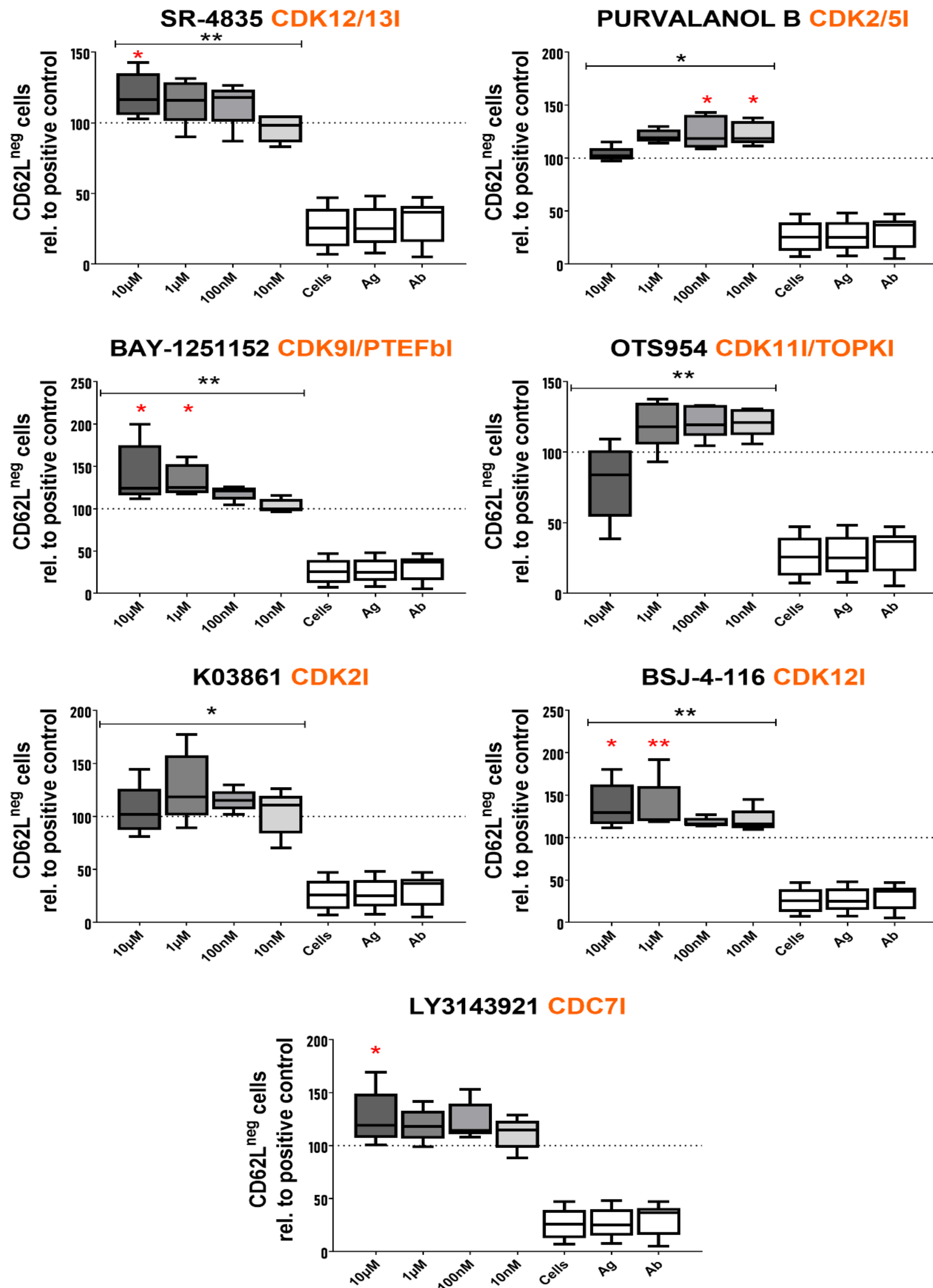
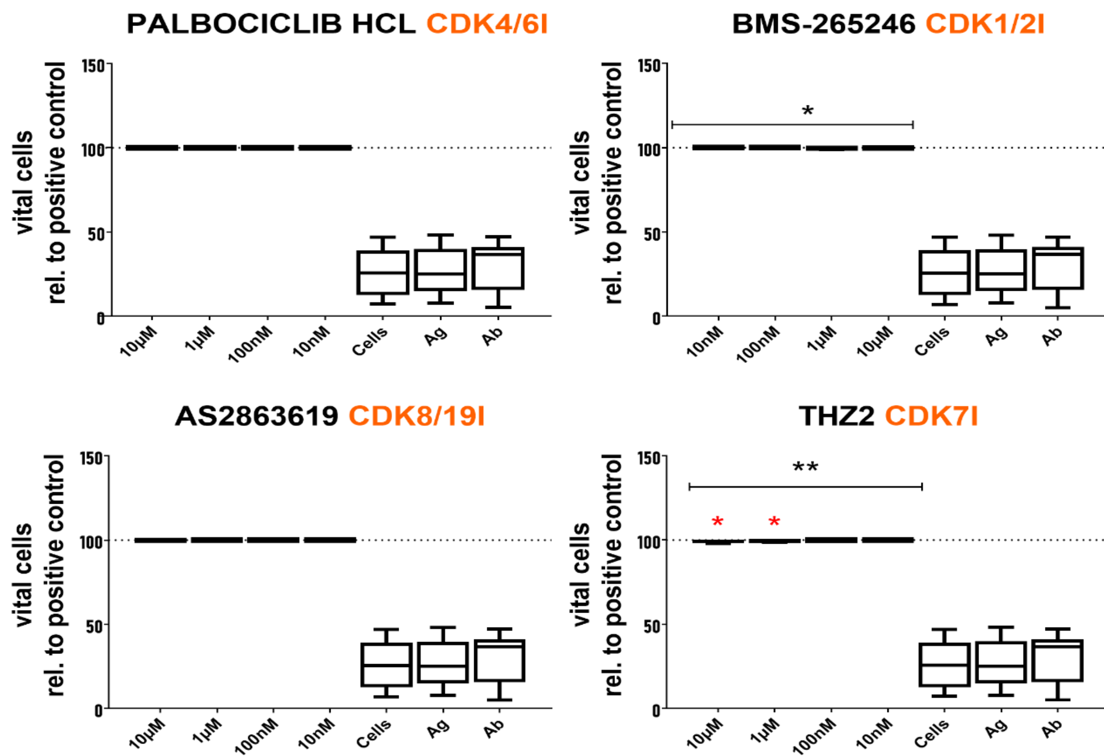


Fig. 16 Some CDKIs increased the CD62L-shedding. *Human PMNs were isolated from fresh whole blood and stimulated with ICs over 2 h. Afterward, they were stained with a fluorophore-conjugated mouse anti-Human antibody solution. PMNs were analyzed in a flow cytometer and sub-gated to identify vital* $CD45^{pos}CD16^{pos}CD193^{neg}CD14^{neg}$*-*$CD18^{pos}$*. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by*

*Dunn's multiple comparison (indicated by red stars). The dashed line indicates the normalized positive control. *p<0.05, **p<0.01.*

3.6. None of the CDKIs show cytotoxic effects on stimulated neutrophil granulocytes

The cytotoxicity of CDKIs was evaluated using Annexin V-conjugated FITC and Zombie NIR. All eleven inhibitors show a proportion of vital cells close to 100 % compared to the IC- stimulated positive control (Fig.17). Four CDKIs BMS-265246 (CDK1/2I), THZ2 (CDK7I), OTS964 (TOPKI, CDK11I), and BSJ-4-116 (CDK12I) show significance in the Friedman test. In the Dunn's test, THZ2 (CDK7I), OTS964 (TOPKI, CDK11I), and BSJ-4-116 (CDK12I) show the significance for the 10 µM concentration and one inhibitor THZ2 (CDK7I) for the one more concentration, 1 µM. Seven CDKIs show no significance in the Friedman's or Dunn's test.



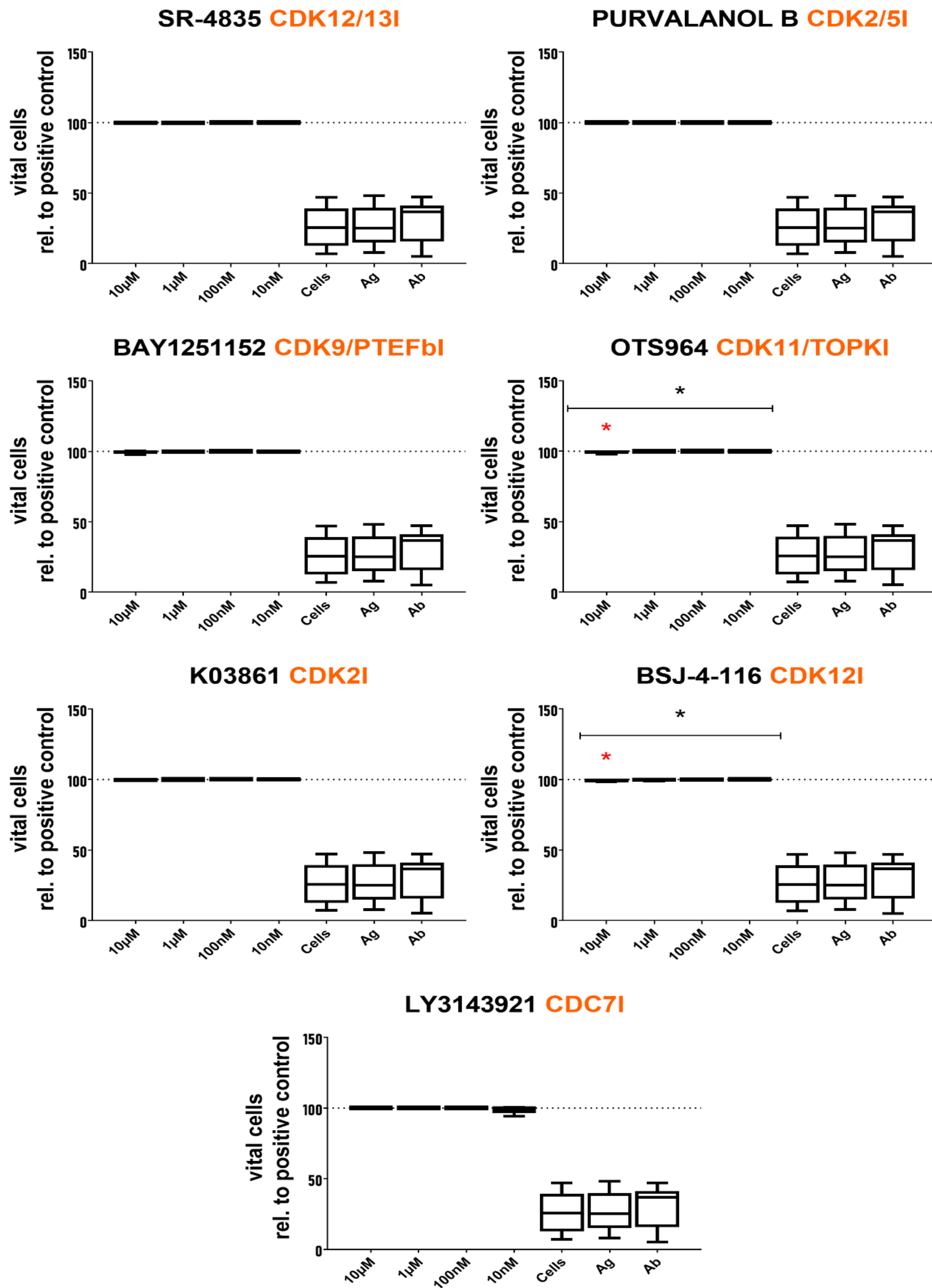


Fig. 17 None of the substances show a cytotoxic effect on the IC-stimulated neutrophils. *Human PMNs were isolated from fresh whole blood and then stimulated with ICs over 2 h in the presence of one CDKI. Afterward, they were stained with FITC conjugated Annexin V and Zombie NIR for a flow cytometry measurement to identify non-vital neutrophils. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple*

*comparison (indicated by red stars). The dashed line indicates the normalized positive control. *p<0.05, **p<0.01*

3.7. Four of the CDKIs show apoptotic effects on stimulated neutrophil granulocytes

Neutrophils are recruited rapidly to sites of inflammation, where they phagocyte, release preformed granular enzymes, proteins, and ROS[215], and when their lifespan is significantly extended. Aged circulating neutrophils are homing to the liver, spleen, and bone marrow and phagocyted by resident macrophages to end their short lives. Most of the longer-lived post-migrated and inflammatory neutrophils eventually undergo apoptosis, inducing their clearance by phagocytic macrophages in an efferocytosis process. This process helps to keep the delicate balance between the generation and resolution of inflammation, enabling immunity while avoiding excessive inflammation[152].

Thence, substances with the ability to induce apoptosis in activated neutrophils may have the potential to be used clinically for various diseases with an inflammatory background. The apoptotic effect of the kinase inhibitors was determined using Annexin V-conjugated FITC. Four BMS-265246 (CDK1/2I), THZ2 (CDK7I), OTS964 (TOPKI, CDK11I), and BSJ-4-116 (CDK12I) of the eleven CDKIs show a significant apoptotic effect on activated neutrophils compared to the IC- stimulated positive control in the Friedman test (Fig. 18). In the Dunn's post hoc test, THZ2 (CDK7I), OTS964 (TOPKI, CDK11I), and BSJ-4-116 (CDK12I) show the significance for the 10 µM concentration and one substance (THZ2 (CDK7I) for one more concentration, 1 µM. Seven CDKIs show no significance in the Friedman's or Dunn's test (Fig. 19).

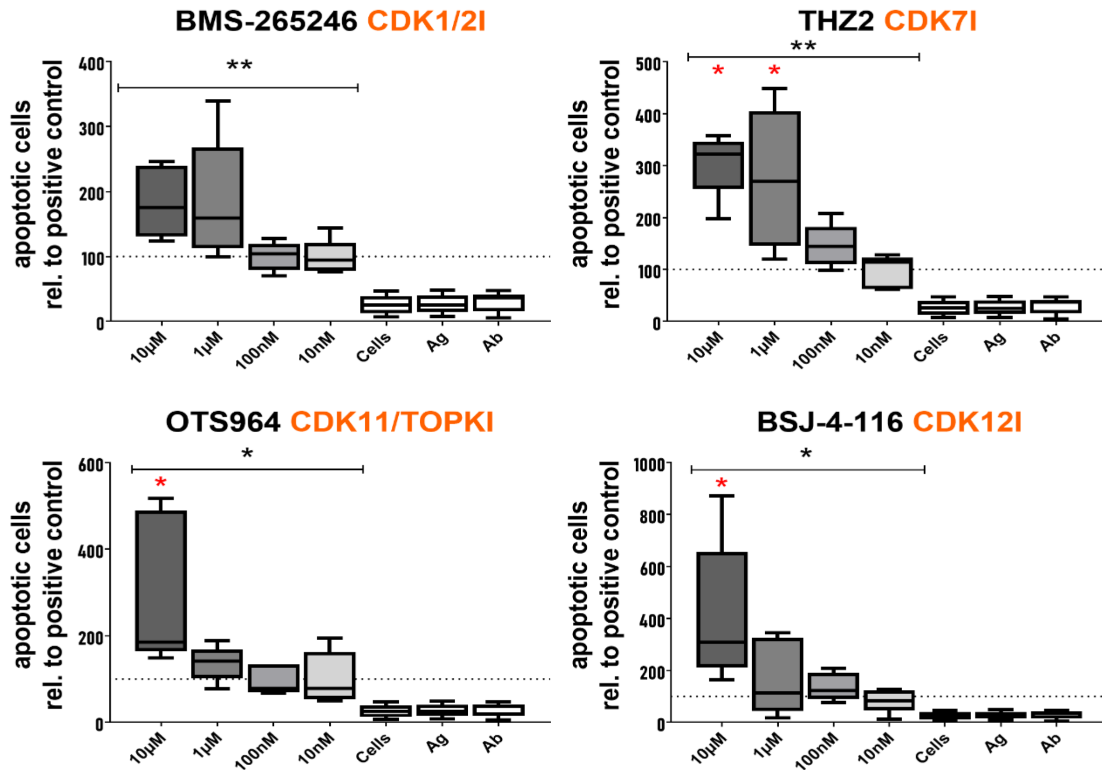


Fig. 18 Four CDKIs show an apoptotic effect on the IC-stimulated neutrophils. *Human PMNs were isolated from fresh whole blood and then stimulated with ICs in the presence of one CDKI. Afterward, they were stained with FITC conjugated Annexin V for a flow cytometry measurement to identify apoptotic neutrophils. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by Dunn's multiple comparison (indicated by red stars). The dashed line indicates the normalized positive control. *p<0.05, **p<0.01.*

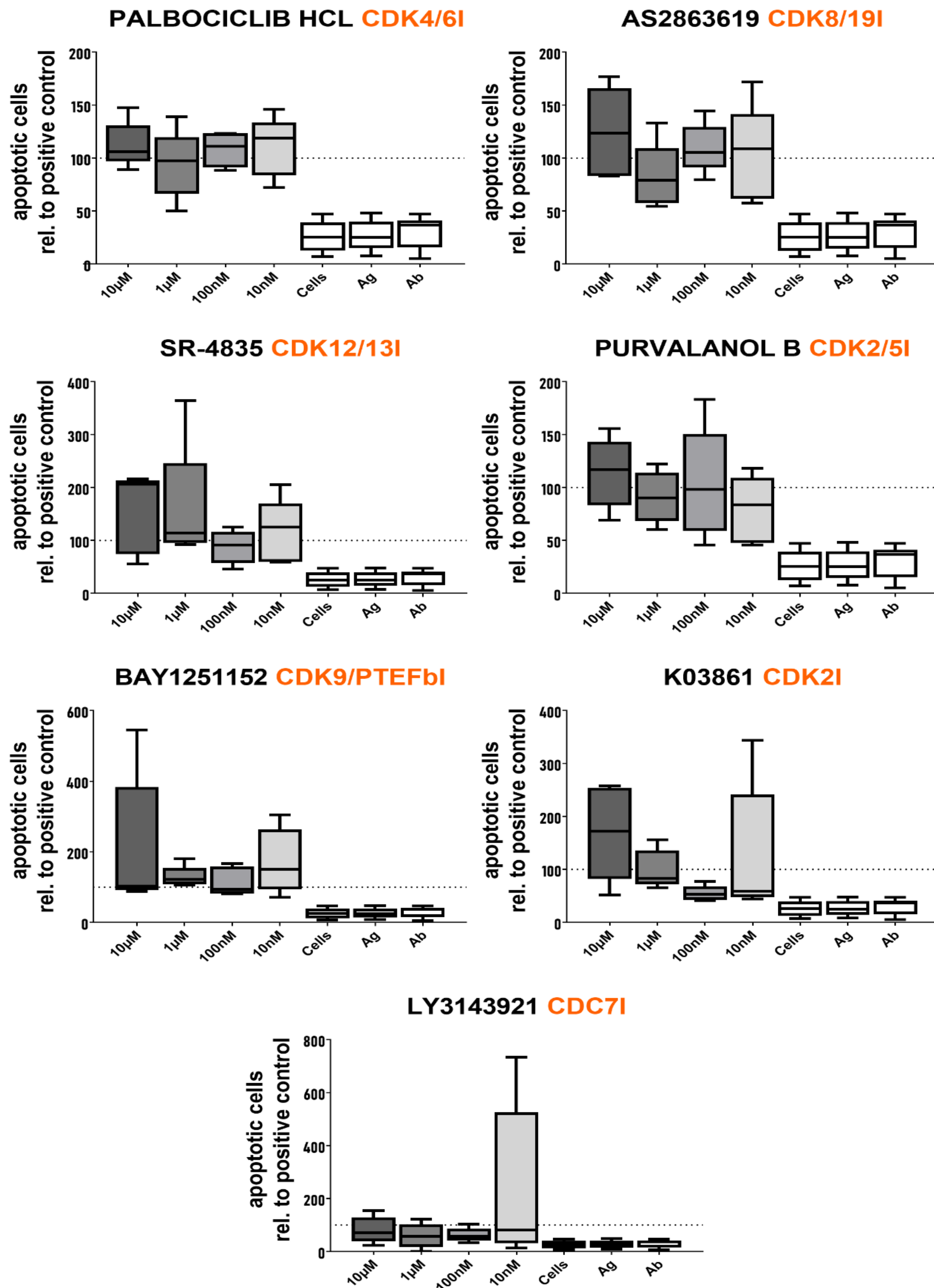


Fig. 19 Seven CDKIs show no apoptotic effect on the IC-stimulated neutrophils. *Human PMNs were isolated from fresh whole blood and then stimulated with ICs in the presence of one CDKI. Afterward, they were stained with FITC conjugated Annexin V for a flow cytometry measurement to identify apoptotic neutrophils. Data were normalized with the positive control (IC-stimulated PMNs). Data shown as Tukey's box-and-whisker plots. n=5. Friedman (ANOVA of ranks; indicated by black stars) was applied, followed by a Dunn's multiple comparison (indicated by red stars).*

The dashed line indicates the normalized positive control.

4. DISCUSSION

CDKIs are endogenously produced kinases that block cell cycle progression under unfavorable conditions, leading to apoptosis. This process aims to ensure orderly sequential progression through the cell cycle [159]. The treatment of several autoimmune diseases is based on immunosuppression, significantly contributing to increased morbidity and mortality. For instance, patients with pemphigoid diseases have a mortality rate that is more than doubled compared to age- and sex-matched controls[127], and iatrogenic immunosuppression contributes significantly to increased it [66]. Due to the increasing incidence of PD, the inadequate treatment options, and the reduced life score, effective and safe therapeutic strategies for patients with pemphigoid diseases are urgently needed [140]. In this sense, the selected group of inhibitors would be applicable in treating several pathological conditions, including auto-inflammatory, infectious diseases, and cancers. CDKIs have been tested *in vitro* or animal models for autoinflammatory diseases such as rheumatoid arthritis and autoimmune encephalomyelitis, with satisfactory results, making it possible to imagine an eventual use in EBA[206, 244].

CDKs are overexpressed in several malignancies, and CDKIs show promise in treating several cancers due to their selectivity, minimizing adverse effects[82]. The combination of cyclin-dependent kinase 4 and 6 (CDK 4/6) inhibitors plus endocrine therapy improved the survival outcomes and became the standard of care in treating metastatic hormone-positive breast cancer[179].

In addition, the maintenance of the inflammatory response of neutrophils can hinder or even lead to complications after myocardial infarction. In this sense, CDK9I (AT7519 and flavopiridol) in zebrafish animal models revealed the potential of such treatments to promote the repair of the damaged myocardium[110].

The virus can alter the expression and function of host CDKs to influence cell cycle progression, thence, presumably, CDKIs would be useful in infectious

diseases [62, 164]. For instance, HBV infection increases the activity of the G1-phase CDK4[79].

In this study, the impact of eleven preselected CDKIs on neutrophil activation was investigated. The aim of this research was to enhance our comprehension of the relationship between CDKs and ICs-dependent signal transduction pathways in neutrophils. Building on these insights, it seeks to explore the potential utilization of CDKIs in animal models and subsequently in patients with diseases where neutrophils play a pivotal role as effector cells, such as in EBA.

Before starting the discussion of the experimental data obtained, some facts must be considered. Although the CDKIs analyzed in this work are highly selective, CDKs are responsible for the regulation of several cellular processes and the interaction of CDKs with other cellular enzymatic processes is poorly understood, which can generate surprising results. Moreover, the study sample (5-7 blood donors) suggests a trend that may not be confirmed in further studies with a higher n or *in vivo*. In spite of that, the data obtained summarize several questions that can help us understand the role of CDKs in the ICs-dependent signal transduction pathway in neutrophils and define their applicability as future therapeutic targets.

Four CDKIs Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), BAY 1251152 (CDK9I, PTEFbI), and OTS964 (TOPKI, CDK11I) significantly reduce ROS production in stimulated human PMNs. They show significance in the Dunn's 10μM inhibitor concentration test. THZ2 (CDK7I) also shows significance in the Dunn's Test for the concentration of 1μM and 100nM.

Neutrophils express most strongly CDK7 and CDK9[132], so THZ2 (CDK7I) and BAY 1251152 (CDK9I, PTEFbI) were expected to have an inhibitory effect on ROS release by inducing massive apoptosis. Surprisingly, this was not the case in this study. Only THZ2 (CDK7I) induced significant apoptosis for the concentration of 10μM and 1 μM, but it was not massive, suggesting some as yet undescribed mechanism. CDK7 and CDK9 mediate gene transcription by stimulating RNAP-II, thence not only the transcription of Mcl-1 and other genes related to the maintenance of cell survival but also theoretically all RNAP-II-

dependent processes are affected by CDKIs 7 and 9[239], so it could explain the significant inhibition in ROS release.

CDK7 phosphorylates the C-terminal domain (CTD) of RNAP-II at Ser_5 to facilitate transcription initiation[239], and phosphorylates CDK9, which phosphorylates the (CTD) of RNAP-II at Ser_2 to drive transcription elongation[128]. Besides, CDK7 phosphorylates CDK2/cyclin E complex which in turn phosphorylates also RNAP-II[208]. Furthermore, CDK7 phosphorylates CDK4/6 that induces a gradual increase in CDK2 activity that is required to initiate CDK2-Rb positive feedback CDK4/6-independent cell-cycle progression, and further phosphorylation of RNAP-II[115]. Following CDK7 inhibition, the CDK4 and CDK6 activity is rapidly lost[202].

If there is a positive correlation between transcription by stimulating RNAP-II and ROS release, then it is assumed that THZ2 (CDK7I) more strongly inhibits ROS release than BAY 1251152 (CDK9I, PTEFbI) because, theoretically, a CDK7I would have a more pronounced effect on RNAP-II than a CDK9I by adding the inhibition of RNAP-II plus the inhibition of CDK2, CDK4/6 and CDK9 activation. The data from experiments supported this assumption. While BAY 1251152 (CDK9I, PTEFbI), significantly inhibited ROS release only at a concentration of 10 µM, THZ2 (CDK7I) did so at the concentration of 10 µM, 1 µM and 100nM. Perhaps the transcription process, through the stimulation of RNAP-II and the release of ROS, may indeed be interconnected and independent of apoptosis induction. Using human rheumatoid fibroblast-like synoviocyte cells, BS-181(CDK7I) treatment as well as CDK7 knockdown effectively suppressed IL-1β, IL-6, IL-8 and RANKL transcript levels, IL-1β/IL-6 secretion, prevented NF-κB signaling pathway activation and restrained p65 nuclear translocation[93], showing that the action of CDK7 is far beyond than though.

Furthermore, THZ2 (CDK7I) showed significance regarding the reduction of $CD18^{pos}$ cells at 10µM, supporting the hypothesis that CDK7 could be involved in the mechanisms of neutrophils activation and the expression of surface proteins related to their activation. Nevertheless, none of the CDKIs had an effect on the reduction in CD62L-shedding. The present state of research fails to sufficiently elucidate these findings as a direct correlation between various signaling

pathways in neutrophil activation and apoptosis has yet to be established. Surprisingly, Palbociclib (PD-0332991), a CDK4/6I, has a pronounced effect on reducing ROS release, even if only at higher doses (10μM) since a CDK4/6I prevents cell cycle progression from the G1 to the S phase of the cell cycle[143], and granulopoiesis is accompanied by irreversible cell-cycle arrest. The maturation of neutrophils occurs in the following sequence: myeloblast, promyelocytes, myelocytes, metamyelocytes, band cells, and segmented neutrophil cells. From the metamyelocyte stage, the non-proliferative phase begins; cells no longer divide[14]. Presumptively, neutrophils should not express CDKs 4/6; however, mature neutrophils have CDK 4/6 at low concentrations, and express also CDK1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 at various levels[132]. An explanation for this effect on reducing ROS release would be the stimulatory action of CDK4/6 on CDK2 which, in turn, phosphorylates RNAP-II. CDK2 controls the G1/S and S/G2 transitions in dividing cells[45], in addition to regulating the phosphorylation of several transcription factors[45], including RNAP-II[54]. Thus, a CDK4/6I would have the ability to inhibit the action of RNAP-II. Nevertheless, Palbociclib (PD-0332991) did not induce apoptosis; so, suggesting that the process of transcription by stimulating RNAP-II and ROS release may be connected and are independent of apoptosis induction.

During inflammation, neutrophils produce neutrophil extracellular traps (NETs), which facilitates the immobilization of pathogens that are too large to be engulfed[165]. It was demonstrated that CDK4/6 are required for NET formation[10]. During the process of NEToses formation as well as in mitosis, nuclear envelope disintegration occurs[10], and this could explain the expression of CDK 4/6 by non-proliferating cells such as mature neutrophils since it would be necessary to reactivate cell cycle regulators. Additionally, NEToses formation also requires the production of ROS by the enzyme NADPH oxidase[74] through mitogen-activated protein kinases (MAPKs)[86], which leads to chromatin decondensation[173]. For that reason, another possible explanation for the pronounced effect of Palbociclib (PD-0332991) HC1(CDK4/6I) on reducing ROS release lies in the intrinsic relationship between CDKs 4/6 and NET formation, which in turn is dependent on the production of ROS.

Typically, activated CDK4/6 complexes phosphorylate the retinoblastoma gene product (Rb)[162], which leads to a cascade of phosphorylations whose result is DNA synthesis and entry into the S phase and, consequently, allowing cell division[88].

Moreover, CDK4/6 not only regulate cell cycle phase transitions of proliferating cells but also can play an essential role in other processes. CDK4 phosphorylation of histone acetyltransferase-GCN5 can lead to acetylation of peroxisome proliferator-activated receptor-gamma coactivator-PGC-1α and is associated with decreased glucose metabolism in liver cells [131]. In another work, using human cancer cells and patient-derived xenografts in mice, the cyclin D3–CDK6 kinase phosphorylates and inhibits the catalytic activity of two main enzymes in the glycolytic pathway, 6-phosphofructokinase, and pyruvate kinase M2. Inhibition of cyclin D–CDK6 in tumor cells reduces flow through the pentose phosphate and serine pathways, thereby depleting NADPH's antioxidants and glutathione. This, in turn, increases the levels of ROS and causes apoptosis of tumor cells[232].

OTS964 a CDK11I and T-lymphokine-activated killer-cell-originated protein kinase inhibitor (TOPKI) at the 10μM inhibitor concentration could significantly and intensely inhibit ROS release. In addition, also for the 10μM inhibitor concentration, OTS964 (TOPKI, CDK11I) significantly increased the proportion of apoptotic neutrophils, but it was not massive.

The expression of CDK11 in neutrophils has not been described, so it is not expected that a CDK11I inhibits ROS release *in vitro*[132]. CDK11 probably has a role in regulating histone gene expression. Histones and several neutrophil granule proteins are associated with the DNA framework when Neutrophil extracellular traps (NETs) are formed[76]. The main form of NET (suicidal NETosis), which leads to neutrophil death, generates the following morphological changes: the disintegration of the nuclear membrane, chromatin decondensation, disappearance of the plasma membrane, and the spill of DNA-based NETs into the extracellular space[74]. The migration to the nucleus of neutrophil elastase and myeloperoxidase, histone modification, and decondensation are the pivotal events of NETosis, which in turn usually need the

stimulation of neutrophils and the generation of ROS by NADPH oxidase[84]. Perhaps OTS964 (TOPKI, CDK11I) inhibits NETosis, which would lead to inhibition of ROS release, which is very important in the NET formation process. CDK11 has two protein isoforms, CDK11p110 and CDK11p58[50]. CDK11p58 is weakly expressed only in the G2/M-phase of the cell cycle[95].

On the other hand, in mice, CDK11p110 is abundantly expressed in all tissues, independently of the cell cycle time, and is involved in regulating RNA transcription and processing[134]. Perhaps human neutrophils could express CDK11, which has not been described yet. Furthermore, like the expression of CDK 4/6 by non-proliferating cells such as mature neutrophils, maybe CDK11 would be necessary to reactivate cell cycle regulators.

T-lymphokine-activated killer-cell-originated protein kinase (TOPK), also known as PDZ-binding kinase (PBK), is a mitotic serine/threonine protein kinase and it is overexpressed in various actively proliferative cells, such as sperm cells and malignant tumor cells[1, 78, 99]. However, despite being a group of non-proliferating cells, neutrophils express TOPK[161], which involves many biological functions, including apoptosis inhibition, cell growth promotion, and anti-oxidation[249]. During mitosis, PBK/TOPK binds to the CDK1/cyclin B1 complex at the mitotic spindle and is then phosphorylated, thus suggesting a possible role in the regulation of cellular proliferation and cell-cycle progression[175]. Furthermore, Src, a protein involved in the activation of neutrophils by IC [108], can directly bind and phosphorylate PBK/TOPK, thereby enhancing its activity and PBK/TOPK stability, which allows it to avoid degradation after ubiquitination [241]. As mentioned in chapter 1.5., crosslinking of activating Fc receptors for IgG (FcγRs) by IC induces the phosphorylation of receptor-associated γ-chains by Src kinase family members which ultimately leads to the activation of downstream MAP kinases and BTK[188]. PI3K and p38 mitogen-activated protein kinase (MAPK) are critical to stimulate the chemotaxis[53]. Besides, BTK plays a role in producing reactive oxygen species[243]. Therefore, a TOPKI is expected to inhibit ROS production due to its influence on the Src family and BTK. Furthermore, TOPK-deficiency mice produce less ROS[144], emphasizing this assumption.

OTS964 (TOPKI, CDK11I) has shown promising effects in treating patients with malignancies by inducing apoptosis of cancer cells since this cell type overexpresses TOPK[176], and its expression level inversely correlates with patient prognosis[60]. A study using promyelocytes showed that knockdown of PBK/TOPK significantly increased Bax expression, decreased Bcl-2 expression, promoted cleavage of caspase-3 and -9, and increased apoptotic cell death in the human acute promyelocytic leukemia NB4 and HL-60 cell lines. Furthermore, ROS release was significantly inhibited due to induced mitochondrial dysfunction[136]. Nevertheless, this effect of a PBK/TOPK inhibitor in autoimmune diseases and the mechanism of action in non-proliferative cells have not been described yet.

It is noteworthy that BSJ-4-116 (CDK12I) only at a concentration of 10 µM induced significant, but not massive apoptosis. Neutrophils do not express CDK12[132], and it was not expect the induction of apoptosis in neutrophils by BSJ-4-116 (CDK12I). Similar to CDK9, CDK12/cyclin K complex phosphorylates RNAP-II at Ser_2, which is thought to be a critical step in the transition from transcriptional initiation to elongation[39]. Inhibition of CDK12 decreases the expression of Survivin and Mcl-1, thus potentially inducing apoptosis[221] Nevertheless, and interestingly, RNAP-II transcription is not globally impaired in cells without CDK12/cyclin K complex. The inhibition of CDK12 does not affect the global transcription level and phosphorylation at Ser_2[25]. However, it diminishes RNAP-II processivity, resulting in shortened transcripts of DNA replication genes. This effect aligns with impaired transcription elongation, ultimately leading to either cell death or cancer.[40]. The maintenance of the level of global transcription and phosphorylation at Ser_2 may explain why BSJ-4-116 (CDK12I) did not interfere with ROS release but induced apoptosis and corroborate the hypothesis that CDK7I and CDK9I can inhibit ROS release by inhibiting the transcription of some yet unidentified proteins that influence ROS release. A question arises from the data presented in this study: do neutrophils not express CDK12?

Given the data of this work and the current scientific literature, it may be plausible to speculate that CDKIs can hinder neutrophil activation through two distinct mechanisms: either by inhibiting RNAP-II or by inhibiting NET formation.

As previously described, CDK7 and CDK9 mediate gene transcription by activating RNAP-II[47], consequently maintaining Mcl-1 levels and preventing apoptosis. [87]. So, the initial premise was that CDK7/9I would inhibit neutrophil activation by theoretically having the ability to induce apoptosis massively. In spite of that, THZ2 (CDK7I), a CDK7I significantly reduced ROS release and CD18 expression on the surface of neutrophils without triggering extensive apoptosis. These findings indicate that inhibiting RNAP-II through transcriptional CDKIs not only hinders Mcl-1 transcription but also affects various other factors crucial for neutrophil activation. It's noteworthy that the cell suspension was exposed to CDKIs for 2 hours, and despite significant apoptosis induction, it's possible that the duration was insufficient for a widespread apoptosis induction.

The expression of Mcl-1 drops drastically after 9h of cell culture, and only 20% of activity remains after 20h[233]. But why was CD62L shedding not also inhibited? Most likely, it does not follow signaling pathways that are influenced by CDKs. Unfortunately, the correlation between neutrophil activation and the mechanisms of action of CDKIs has not been elucidated yet. As a result, further studies should be carried out to solve this question with certainty. CDKs which directly regulate cell-cycle transitions and cell division could have a residual function in non-proliferative cells. During NET formation, envelope disintegration and mitosis occur, and this process is required for NET formation. Nevertheless, DNA synthesis, condensation of chromosomes, or cytokinesis do not occur after neutrophil activation, indicating that cell-cycle re-entry is not happening[10]. Moreover, NETosis formation also requires the production of ROS by the enzyme NADPH oxidase[74] through mitogen-activated protein kinases (MAPKs) [86], which leads to chromatin decondensation[173]. The data from this work suggest that neutrophils can express, even in tiny amounts, CDKs, which are relevant in the control of cell division in proliferating cells to induce the formation of NET that ultimately leads to cell death. Furthermore, it was implied that non-transcriptional CDKIs could affect neutrophil activation due to the interdependence between NET formation and neutrophilic activation, which could explain their inhibition of ROS release and CD18 expression on the neutrophilic surface. Unfortunately, this process is still poorly understood.

To summarize, CDKIs have emerged as potential anti-inflammatory drugs, capable of influencing the resolution of inflammation. The results of this work confirmed this assertion and supported the hypothesis that NET formation plays o role much important as though. The exact mechanism of action of CDKs and their temporal relationship with other proteins remain to be explored. Hereupon, this work hopes to deepen this knowledge. Studies with oncologic patients have been performed with promising results, but the use of CDKIs for inflammatory diseases is still beginning. Of the 11 CDKIs analyzed in this work, THZ2 (CDK7I) and OTS964 (TOPKI, CDK11I) stand out as the most promising candidates for further testing the efficacy of topical or systemic therapies in the EBA antibody transfer-induced murine model.

5. SUMMARY

Neutrophils, the most abundant leukocytes, play a crucial role in the initial defense against invading microbes by detecting them through various receptor systems. Nevertheless, pathological activation of neutrophils is involved in many chronic and autoimmune diseases, such as epidermolysis bullosa acquisita (EBA) where activation of neutrophils ultimately elicits skin blisters. Current treatment primarily relies on immunosuppression, contributing to increased morbidity and mortality. Hence effective and safe therapeutic strategies are urgently needed. Cyclin-dependent kinase inhibitors (CDKIs) block endogenously cell cycle progression under unfavorable conditions, leading to apoptosis, prompting interest in their potential for treating autoinflammatory and autoimmune diseases, including EBA. This study explores the impact of eleven specific CDKIs on neutrophil activation induced by immune complexes (ICs) *in vitro*, utilizing assays for reactive oxygen species (ROS) release and fluorescence-activated cell sorting (FACS). Among the CDKIs tested, four CDKIs Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), BAY 1251152 (CDK9I, PTEFbI), and OTS964 (TOPKI, CDK11I) significantly reduce ROS production in stimulated human polymorphonuclear leukocytes (PMNs). Two CDKIs Palbociclib (PD-0332991) HCl (CDK4/6I) and THZ2 (CDK7I) showed significance regarding reducing $CD18^{pos}$ cells, although no CDKI demonstrated significant effects on the reduction of $CD62L_{neg}$ cells. All eleven inhibitors maintained a proportion of viable cells close to 100% compared to the IC-stimulated positive control.

To summarize, CDKIs have emerged as promising candidates for anti-inflammatory therapeutics, owing to their capacity to modulate the resolution of inflammatory processes, and this work confirms this impression *in vitro* for EBA. Furthermore, of the 11 CDKIs analyzed, THZ2 (CDK7I) and OTS964 (TOPKI, CDK11I) stand out as the most promising for further testing of the efficacy of systemic application in the murine model of EBA induced by antibody transfer.

6. ZUSAMMENFASSUNG

Neutrophile, die am häufigsten vorkommenden Leukozyten, spielen eine entscheidende Rolle bei der anfänglichen Verteidigung gegen eindringende Mikroben, indem sie diese über verschiedene Rezeptorsysteme erkennen. Dennoch ist die pathologische Aktivierung von Neutrophilen an vielen chronischen und Autoimmunerkrankungen beteiligt, beispielsweise an der Epidermolysis bullosa acquisita (EBA), bei der die Aktivierung von Neutrophilen letztlich zur Bildung von Hautblasen führt. Die derzeitige Behandlung beruht in erster Linie auf der Immunsuppression, was zu einer erhöhten Morbidität und Mortalität beiträgt. Daher werden wirksame und sichere Therapiestrategien dringend benötigt. "Cyclin-dependent kinase inhibitors" (CDKIs) blockieren das endogene Fortschreiten des Zellzyklus unter ungünstigen Bedingungen, was zu Apoptose führt, was Interesse an ihrem Potenzial zur Behandlung von autoinflammatorischen und Autoimmunerkrankungen, einschließlich EBA, weckt. Diese Arbeit untersucht die Wirkung von elf spezifischen CDKIs auf die IC-induzierte Neutrophilenaktivierung *in vitro* unter Verwendung von Freisetzung reaktiver Sauerstoffspezies- (ROS) und FACS-Assays. Unter den getesteten CDKIs, vier CDKIs Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), BAY 1251152 (CDK9I, PTEFbI), und OTS964 (TOPKI, CDK11I) reduzieren die ROS-Produktion in stimulierten humanen polymorphkernigen Leukozyten (PMNs) signifikant. Zwei CDKIs Palbociclib (PD-0332991) HCl (CDK4/6I) und THZ2 (CDK7I) zeigten Signifikanz bezüglich der Reduktion von $CD18^{pos}$-Zellen. Jedoch war kein CDKI bei der Reduktion von $CD62L_{neg}$-Zellen signifikant. Alle elf Inhibitoren hielten den Anteil lebensfähiger Zellen nahe bei 100 % im Vergleich zur IC-stimulierten Positivkontrolle.

Zusammenfassend haben sich CDKIs als potenzielle entzündungshemmende Medikamente herausgestellt, was durch diese Arbeit weiter *in vitro* für EBA weiter bestätigt werden konnte. Darüber hinaus sind von den 11 analysierten CDKIs THZ2 (CDK7I) und OTS964 (TOPKI, CDK11I) die vielversprechendsten für weitere Tests der Wirksamkeit der systemischen Anwendung im Antikörpertransfer-induzierten Mausmodell der EBA.

7. DETAILED SUMMARY

7.1. Introdution

Epidermolysis bullosa acquisita (EBA) is a rare autoimmune disease, accounting for approximately 5% of autoimmune bullous diseases. An annual incidence varies between 0.08 to 0.5 cases per million individuals[121]. Knowledge about pathophysiology comes primarily from animal models, given the rarity of EBA in humans. In murine models, purified IgG antibodies from the sera of type 7 collagen(COL7) mCOL7-immunized rabbits are transferred, or mice are immunized with a recombinant peptide fragment from the immunogenic NC-1 domain of mCOL7. In the second model, after a few weeks, mice begin to produce antibodies against mCOL7 and then show the clinical features of EBA[21]. Accordingly, the depletion of T[209] or B cells, or dendritic cells and macrophages before the immunization phase results in no disease development[101]. Autoantibodies against COL7 deposit on the dermal-epidermal junction (DEJ)[186]. After the deposition of immune complexes (IC) in DEJ, the complement system is activated, mainly the alternative pathway[156]. Then, the recruitment of leukocytes occurs due to attraction to the lipid mediator leukotriene B4 (LTB4) and other chemoattractants[5]. Also, several cytokines are produced, modulating the inflammatory response[118]. So, the IC deposited in DEJ bind in an Fc-dependent manner to neutrophils triggering the signaling cascade, which culminates in the release of Reactive oxygen species (ROS), proteases, and the production of more proinflammatory cytokines amplification of the inflammatory response, and tissue destruction[118]. The production of autoantibodies is directed to type 7 collagen (COL7) located in the basement membrane zone of the epidermis, specifically in the dense-sublamina[109]. COL7 is the main anchoring component of sublamina-dense fibrils. Therefore, its damage results in the loss of dermo-epidermal adhesion, skin fragility, and the formation of vesicles, blisters, and erosions[121].

Treatment of EBA is based on local care that includes the prevention of physical trauma and infection, the use of non-adherent dressings, topical corticosteroids, and, if necessary, the use of systemic medications such as corticosteroids,

dapsone, cyclosporine, colchicine, intravenous immunoglobulin, and sometimes additionally plasmapheresis[139]. Unfortunately, EBA patients respond poorly to therapy and iatrogenic immunosuppression contributes significantly to increased mortality [127] which could be more than doubled compared to a control of the same age and sex [66]. Due to the inadequate treatment options and the increasing incidence of pemphigoid diseases, effective and safe therapeutic strategies for patients with pemphigoid diseases are urgently needed[140].

Previously unpublished work by the research group Lübecker Institut für Experimentelle Dermatologie (LIED) was the starting point to support this research. After stimulation of neutrophils with IgG1 IC, 141 selective inhibitors were analyzed at a concentration of 1μM, of which 31 showed inhibitory substances effecting ROS release. In parallel, the kinase activity in IC-activated human neutrophils using the novel PamGene technology was analyzed. Interestingly, one major group of kinases, namely cyclin-dependent kinases (CDK), is activated by ICs. Based on the results, a selection of CDKs was made, and suitable inhibitors were selected for this thesis. A favorable IC_{50} value and a good specificity for the corresponding kinase were significant for selecting the substances for *in vitro* analysis and a possible later application in animal models.

Cyclin-dependent kinases (CDKs) are protein kinases that need a separate subunit - a cyclin - that provides domains essential for enzymatic activity[142]. CDKs are responsible for regulating several cellular processes, including life cycle, transcription, metabolism, communication, and apoptosis. During cell division, CDKs ensure that each cell correctly replicates its DNA and divides it evenly among the resulting cells. If the cell division is defective, the cells will go into apoptosis. In summary, CDKs 1, 2, 4 and 6 regulate the cell cycle[135] while CDK7, 8, 9, 12 and 13 phosphorylate the C-terminal tail of RNA polymerase II (CTD), regulating gene transcription (Fig. 3)[105]. CDK7 also activates CDKs1, 2, 4 and 6, thus indirectly influencing the cell cycle[49, 69]. CDK3 promotes the transition from quiescence (G0) into G1 by phosphorylating retinoblastoma gene product (Rb)[190]. CDK5, 10, 11, 14–18 and 20, on the other hand, have diverse functions that are tissue specific. For instance, CDK10 and CD11 control the transcription of multiple transcription factors, hormone receptors, associated regulators, and splicing factors[135].

Based on these data, the effect of CDKs inhibition on IC-induced neutrophil activation *in vitro* was analyzed to validate new therapeutic options for treating EBA or other IC-induced autoimmune diseases and enhance our knowledge of the disease development.

7.2.Material and Methods

Neutrophils from healthy blood donors were isolated, activated with IC *in vitro*, and treated with selected CDK inhibitors. ROS and FACS (Fluorescence-activated cell sorting) assays were performed, comparing neutrophil activation in the presence of CDK inhibitors with positive and negative controls.

For this purpose, peripheral blood was drawn from healthy volunteer donors into 4 Monovettes containing EDTA as anticoagulant totaling 36 ml, and the aim was to isolate neutrophils in a cell suspension diluted to the desired level ($2*10^6$ cells /mL). Moreover, a white 96-well microtiter plate was coated with human Collagen VII E-F, followed by anti-human Collagen VII IgG1 to form IC. Subsequently, the cell suspension was added to bind with IC, thereby stimulating neutrophils and initiating the release of ROS, which could be measured on the GloMax® Discover after adding luminol. Four different concentrations of cyclin-dependent kinase inhibitors (CDKI) with IC were investigated in duplicates for each CDKI, and its effect on neutrophil activation could be measured using a ROS release assay.

For this purpose, the inhibitors were delivered in powder form and stored in this state at -20° C. until the beginning of the dilution. The inhibitors listed in table 7 were diluted initially in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and further dilution steps produced later concentrations of 1 mM, 0.1 mM, and 0.01 mM again in DMSO. The exception was Palbociclib whose initial dilution was 5 mM, its saturation point. Nevertheless, it followed later the same dilution scheme. Each solution was aliquoted into in 2 µL and stored in a freezer at -20°C. The 10 mM stock solution was frozen at -80°C for future experiments.

The experiments were always performed under the same conditions: cell suspension with a concentration of approximately $2*10^6$ cells/mL and at least 85% neutrophils to avoid contamination with other cell groups, which could falsify the results. For this reason, Flow cytometric measurement was performed, $CD14^{pos}$ cells and necrotic cells were excluded, and $CD16^{pos}$ cells were included into analysis because neutrophils strongly

express CD16 on their surface, while monocytes and macrophages, CD14[126, 185]. Flow cytometry is a technology that analyzes the fluorescence and light scatter pattern of individual cells or particles suspended in the saline buffer after being irradiated by laser beams, allowing the quantification of cellular subpopulations.

To further investigate the potential therapeutic properties of the CDKs, a flow cytometric analysis (n=5) was performed. The initial steps of the FACS assay are identical to the ROS assay until the cell suspension coating is in the wells. In this experiment, freshly isolated human PMNs were stimulated via IC in the presence of the CDKs at four chosen concentrations for 2 hours. Duplicate measurements were taken. After the incubation, the PMNs were treated with a defined panel of antibody-conjugated fluorophores and fluorescent dyes to identify neutrophils, which were defined as $CD45^{pos}CD16^{pos}CD193^{neg}$ $CD14^{neg}$ cells; and isolated from other groups of cells. To rule out the possible toxic effects of the inhibitors, the non-vital neutrophils were stained using Annexin V-conjugated FITC and Zombie NIR.

The objective of this experiment was to assess the activation of neutrophils and its impact on their vitality. Additionally, CDK inhibitors' effect on these parameters was compared to positive controls. First, the expression of CD18 was quantified. Also known as integrin β-2, CD18 is a leukocyte surface protein essential for adhesion to the endothelium and extravasation into inflamed tissue[223]. Posteriorly, the expression of CD62L, or L-selectin, a parameter inversely proportional to the level of leukocyte activation, was measured. L-selectin is rapidly shed from the cell surface after leukocyte activation by a proteolytic mechanism that cleaves the receptor in a membrane-proximal extracellular region. This process may facilitate migration across the endothelium before entry into tissues. Activated leukocytes decrease their expression and undergo shedding[35].

And finally, the vitality of cells and toxicity of CDK inhibitors were evaluated. Annexin V, linked to FITC dye, was used for detecting apoptotic cells by the cytometer. Zombie NIR is a hydrophilic and polarized amine-reactive fluorescent dye that cannot permeate into intact cell membranes. During the process of necrosis, the integrity of the plasma membrane is lost, allowing Zombie NIR to

bind to peptides of cytosolic proteins and, consequently, the detection of necrotic cells by cytometry[18].

7.3.Statistical analysis

To conduct ROS assay analysis, four controls, in duplicate, were carried out: a positive control and three negative controls. The positive control contained IC-stimulated Polymorphonuclear leukocytes (PMNs); the three negative controls consisted of only PMNs or PMNs in the presence of either only human COL7 E-F or only anti-COL7 IgG1. The mean of each duplicate negative control and positive control was calculated for each 62 measurements and plotted on a graph. The area under the curve (AUC) of the negative and positive controls of each donor was measured, and the positive control AUC was defined as 100%. If the AUC of each negative control was less than 50% of the AUC of the positive control or the purity of neutrophil granulocytes via flow cytometry was less than 85%, the sample was excluded from statistical analysis.

Friedman test was performed to analyze whether each CDKI was significant concerning the IC-stimulated positive control[72]. In addition, the Dunn's multiple comparison post hoc test was performed to identify which specific concentrations of CDKI were significant relative to the positive control. Each concentration was compared to the IC-stimulated positive control.

For FACS assay analysis, duplicate tests were performed with a positive control and three negative controls. The mean of each duplicate negative and positive control was calculated, and the data were normalized to the positive control (IC-stimulated neutrophils) to facilitate the statistical analysis. The sample was considered eligible when negative control's CD18 expression or CD62L shedding was less than 50% of the IC group's normalized CD18 expression or CD62L shedding. In this way, it was possible to adjust the individual differences in the expression of each blood donor’s markers and compare the relative inhibition of each CDKI concerning the positive control.

Mann-Whitney U test was performed to compare the IC-stimulated control with the unstimulated only cells control without prior normalization about CD18 expression or CD62L shedding [145].

7.4.Results

The production of ROS measured by luminescence differs considerably between different blood donors. However, the statistical process described earlier helped to diminish the heterogeneity related to 7 blood donors' biological differences.

Seven out of eleven inhibitors show significance in the Friedman test (Fig. 10). Four of those eleven substances Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), BAY 1251152 (CDK9I, PTEFbI), and OTS964 (TOPKI, CDK11I) show significance in the Dunn’s test for the 10µM inhibitor concentration. THZ2 (CDK7I) also shows significance in the Dunn’s test for the concentration of 1µM and 100nM (Fig. 10).

As mentioned in 2.2.4, $CD18^{pos}$ and $CD62L_{neg}$ are indicators of neutrophil activation and, therefore, valuable parameters to be analyzed to assess the possible inhibition of CDKIs on neutrophils. Furthermore, an increased expression of CD18 and CD62L shedding contributes to a more inflammatory phenotype in antibody transfer-induced murine models[22, 23].

Three of the eleven inhibitors Palbociclib (PD-0332991) HCl (CDK4/6I), THZ2 (CDK7I), and OTS964 (TOPKI, CDK11I) showed significance in the Friedman test regarding the reduction of $CD18^{pos}$ cells (Fig. 13). Two of those three inhibitors Palbociclib (PD-0332991) HCl (CDK4/6I) and THZ2 (CDK7I) show significance in the Dunn’s test. THZ2 (CDK7I) shows significance in the Dunn’s using 10µM inhibitor concentration.

None of the inhibitors significantly impact the reduction of $CD62L_{neg}$ cells (Fig. 15). The opposite effect was observed. Seven of the eleven inhibitors SR-4835 (CDK12/13I), Purvalanol B (CDK2I/5), BAY 1251152 (CDK9I, PTEFbI), OTS964 (TOPKI, CDK11I), K03861 (CDK2I), and BSJ-4-116 (CDK12I), and LY3143921 hydrate (CDC7I) stimulated the CD62L shedding (Fig. 16).

To rule out the possible toxic effects of the inhibitors, the non-vital neutrophils were stained using Annexin V-conjugated FITC and Zombie NIR. All eleven inhibitors maintained a proportion of viable cells close to 100% compared to the IC-stimulated positive control.

7.5.Discussion

In this study, the impact of eleven preselected CDKIs on neutrophil activation was investigated. The aim of this research was to enhance our comprehension of the relationship between CDKs and ICs-dependent signal transduction pathways in neutrophils. Building on these insights, it seeks to explore the potential utilization of CDKIs in animal models and subsequently in patients with diseases where neutrophils play a pivotal role as effector cells, such as in EBA.

Neutrophils express most strongly CDK7 and CDK9[132], so THZ2 (CDK7I) and BAY 1251152 (CDK9I, PTEFbI) were expected to have an inhibitory effect on ROS release by inducing massive apoptosis. Surprisingly, this was not the case in this study. Only THZ2 (CDK7I) induced significant apoptosis for the concentration of 10μM and 1 μM, but it was not massive, suggesting some as yet undescribed mechanism. CDK7 and CDK9 mediate gene transcription by stimulating RNA polymerase II (RNAP-II), thence not only the transcription of Myeloid cell leukemia factor-1 (Mcl-1) and other genes related to the maintenance of cell survival but also theoretically all RNAP-II-dependent processes are affected by CDKIs 7 and 9[239], so it could explain the significant inhibition in ROS release.

If there is a positive correlation between transcription by stimulating RNAP-II and ROS release, then it is assumed that THZ2 (CDK7I) more strongly inhibits ROS release than BAY 1251152 (CDK9I, PTEFbI) because, theoretically, a CDK7I would have a more pronounced effect on RNAP-II than a CDK9I by adding the inhibition of RNAP-II plus the inhibition of CDK2, CDK4/6 and CDK9 activation. The data from experiments supported this assumption. While BAY 1251152 (CDK9I, PTEFbI), significantly inhibited ROS release only at a concentration of 10 μM, THZ2 (CDK7I) did so at the concentration of 10 μM, 1 μM and 100nM. Perhaps the transcription process, through the stimulation of RNAP-II and the release of ROS, may indeed be interconnected and independent of apoptosis induction. Using human rheumatoid fibroblast-like synoviocyte cells, BS-181(CDK7I) treatment as well as CDK7 knockdown effectively suppressed IL-1β, IL-6, IL-8 and RANKL transcript levels, IL-1β/IL-6 secretion, prevented NF-κB signaling pathway activation and restrained p65 nuclear translocation[93], showing that the action of CDK7 is far beyond than though.

Furthermore, THZ2 (CDK7I) showed significance regarding the reduction of CD18^{pos} cells at 10μM, supporting the hypothesis that CDK7 could be involved in the mechanisms of neutrophils activation and the expression of surface proteins related to their activation. Nevertheless, none of the CDKIs had an effect on the reduction in CD62L-shedding. The present state of research fails to sufficiently elucidate these findings as a direct correlation between various signaling pathways in neutrophil activation and apoptosis has yet to be established.

Surprisingly, Palbociclib (PD-0332991), a CDK4/6I, has a pronounced effect on reducing ROS release, even if only at higher doses (10μM) since a CDK4/6I prevents cell cycle progression from the G1 to the S phase of the cell cycle[143], and neutrophils no longer divide[14]. Presumptively, neutrophils should not express CDKs 4/6; however, mature neutrophils have CDK 4/6 at low concentrations[132]. An explanation for this effect on reducing ROS release would be the stimulatory action of CDK4/6 on CDK2 which, in turn, phosphorylates RNAP-II. CDK2 controls the G1/S and S/G2 transitions in dividing cells[45], in addition to regulating the phosphorylation of several transcription factors[45], including RNAP-II[54]. Thus, a CDK4/6I would have the ability to inhibit the action of RNAP-II. Nevertheless, Palbociclib (PD-0332991) did not induce apoptosis; so, suggesting again that the process of transcription by stimulating RNAP-II and ROS release may be connected and are independent of apoptosis induction.

During inflammation, neutrophils produce neutrophil extracellular traps (NETs), which facilitates the immobilization of pathogens that are too large to be engulfed[165]. It was demonstrated that CDK4/6 are required for NET formation[10]. During the process of NEToses formation as well as in mitosis, nuclear envelope disintegration occurs[10], and this could explain the expression of CDK 4/6 by non-proliferating cells such as mature neutrophils since it would be necessary to reactivate cell cycle regulators. Additionally, NEToses formation also requires the production of ROS by the enzyme NADPH oxidase[74] through mitogen-activated protein kinases (MAPKs)[86], which leads to chromatin decondensation[173]. For that reason, another possible explanation for the pronounced effect of Palbociclib (PD-0332991) HC1(CDK4/6I) on reducing

ROS release lies in the intrinsic relationship between CDKs 4/6 and NET formation, which in turn is dependent on the production of ROS.

OTS964 a CDK11I and T-lymphokine-activated killer-cell-originated protein kinase inhibitor (TOPKI) at the 10µM inhibitor concentration could significantly and intensely inhibit ROS release. In addition, also for the 10µM inhibitor concentration, OTS964 (TOPKI, CDK11I) significantly increased the proportion of apoptotic neutrophils, but it was not massive.

The expression of CDK11 in neutrophils has not been described, so it is not expected that a CDK11I inhibits ROS release *in vitro*[132]. CDK11 probably has a role in regulating histone gene expression. Histones and several neutrophil granule proteins are associated with the DNA framework when Neutrophil extracellular traps (NETs) are formed[76]. The migration to the nucleus of neutrophil elastase and myeloperoxidase, histone modification, and decondensation are the pivotal events of NETosis, which in turn usually need the stimulation of neutrophils and the generation of ROS by NADPH oxidase[84]. Perhaps OTS964 (TOPKI, CDK11I) inhibits NETosis, potentially resulting in the suppression of ROS release, a critical aspect of the NET formation pathway. It is conceivable that human neutrophils could express CDK11, which has not been described yet. Furthermore, like the expression of CDK 4/6 by non-proliferating cells such as mature neutrophils, CDK11 might play a crucial role in reactivating cell cycle regulators.

T-lymphokine-activated killer-cell-originated protein kinase (TOPK), also known as PDZ-binding kinase (PBK), is a mitotic serine/threonine protein kinase and it is overexpressed in various actively proliferative cells, such as sperm cells and malignant tumor cells[1, 78, 99]. However, despite being a group of non-proliferating cells, neutrophils express TOPK[161], which involves many biological functions, including apoptosis inhibition, cell growth promotion, and anti-oxidation[249]. During mitosis, PBK/TOPK binds to the CDK1/cyclin B1 complex at the mitotic spindle and is then phosphorylated, thus suggesting a possible role in the regulation of cellular proliferation and cell-cycle progression[175]. Furthermore, Src, a protein involved in the activation of neutrophils by IC [108], can directly bind and phosphorylate PBK/TOPK, thereby

enhancing its activity and PBK/TOPK stability, which allows it to avoid degradation after ubiquitination [241]. As mentioned in chapter 1.5., crosslinking of activating Fc receptors for IgG (FcγRs) by IC induces the phosphorylation of receptor-associated γ-chains by Src kinase family members which ultimately leads to the activation of downstream MAP kinases and Bruton's tyrosine kinase (BTK)[188]. PI3K and p38 mitogen-activated protein kinase (MAPK) are critical to stimulate the chemotaxis[53]. Besides, BTK plays a role in producing reactive oxygen species[243]. Therefore, a TOPKI is expected to inhibit ROS production due to its influence on the Src family and BTK. Furthermore, TOPK-deficiency mice produce less ROS[144], emphasizing this assumption.

It is noteworthy that BSJ-4-116 (CDK12I) only at a concentration of 10 μM induced significant, but not massive apoptosis. Neutrophils do not express CDK12[132], and it was not expect the induction of apoptosis in neutrophils by BSJ-4-116 (CDK12I). Similar to CDK9, CDK12/cyclin K complex phosphorylates RNAP-II at Ser_2, which is thought to be a critical step in the transition from transcriptional initiation to elongation[39]. Inhibition of CDK12 decreases the expression of Survivin and Mcl-1, thus potentially inducing apoptosis[221] Nevertheless, and interestingly, RNAP-II transcription is not globally impaired in cells without CDK12/cyclin K complex. The inhibition of CDK12 does not affect the global transcription level and phosphorylation at Ser_2[25]. However, it diminishes RNAP-II processivity, resulting in shortened transcripts of DNA replication genes. This effect aligns with impaired transcription elongation, ultimately leading to either cell death or cancer.[40]. The maintenance of the level of global transcription and phosphorylation at Ser_2 may explain why BSJ-4-116 (CDK12I) did not interfere with ROS release but induced apoptosis and corroborate the hypothesis that CDK7I and CDK9I can inhibit ROS release by inhibiting the transcription of some yet unidentified proteins that influence ROS release. A question arises from the data presented in this study: do neutrophils not express CDK12?

Given the data of this work and the current scientific literature, it may be plausible to speculate that CDKIs can hinder neutrophil activation through two distinct mechanisms: either by inhibiting RNAP-II or by inhibiting NET formation.

As previously described, CDK7 and CDK9 mediate gene transcription by activating RNAP-II[47], consequently maintaining Mcl-1 levels and preventing apoptosis. [87]. So, the initial premise was that CDK7/9I would inhibit neutrophil activation by theoretically having the ability to induce apoptosis massively. In spite of that, THZ2 (CDK7I), a CDK7I significantly reduced ROS release and CD18 expression on the surface of neutrophils without triggering extensive apoptosis. These findings indicate that inhibiting RNAP-II through transcriptional CDKIs not only hinders Mcl-1 transcription but also affects various other factors crucial for neutrophil activation. It's noteworthy that the cell suspension was exposed to CDKIs for 2 hours, and despite significant apoptosis induction, it's possible that the duration was insufficient for a widespread apoptosis induction. The expression of Mcl-1 drops drastically after 9h of cell culture, and only 20% of activity remains after 20h[233].

CDKs which directly regulate cell-cycle transitions and cell division could have a residual function in non-proliferative cells. During NET formation, envelope disintegration and mitosis occur, and this process is required for NET formation. Moreover, NETosis formation also requires the production of ROS by the enzyme NADPH oxidase[74] through mitogen-activated protein kinases (MAPKs) [86], which leads to chromatin decondensation[173]. The data from this work suggest that neutrophils can express, even in tiny amounts, CDKs, which are relevant in the control of cell division in proliferating cells to induce the formation of NET that ultimately leads to cell death. Furthermore, it was implied that non-transcriptional CDKIs could affect neutrophil activation due to the interdependence between NET formation and neutrophilic activation, which could explain their inhibition of ROS release and CD18 expression on the neutrophilic surface. Unfortunately, this process is still poorly understood.

To summarize, CDKIs have emerged as potential anti-inflammatory drugs, capable of influencing the resolution of inflammation. The results of this work confirmed this assertion and supported the hypothesis that NET formation plays o role much important as though. The exact mechanism of action of CDKs and their temporal relationship with other proteins remain to be explored. Hereupon, this work hopes to deepen this knowledge. Studies with oncologic patients have been performed with promising results, but the use of CDKIs for inflammatory

diseases is still beginning. Of the 11 CDKIs analyzed in this work, THZ2 (CDK7I) and OTS964 (TOPKI, CDK11I) stand out as the most promising candidates for further testing the efficacy of topical or systemic therapies in the EBA antibody transfer-induced murine model.

8. BIBLIOGRAPHY

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9. ABBREVIATIONS

µL	Microliter
µm	Micrometer
µM	Micromolar
AIBD	Autoimmune blistering disease
ABB	Annexin V binding buffer
ANOVA	Analysis of variance
AP-1	Activator protein 1
Apaf-1	Apoptosis-promoting activating factor-1
APC	Antigen presenting cell
APC	Allophycocyanin
AUC	Area under the curve
BB	Blocking Buffer
Bcl-2	B cell lymphoma 2
Bcl-XL	B cell lymphoma-extra large
Bim	BCL-2-interacting mediator of cell death
BTK	Bruton's tyrosine kinase
BSA	Bovine serum albumin
CD	Cluster of Differentiation
Cdc42	Cell division control protein 42 homolog
CDK	Cyclin-dependent kinase

CDKI	Cyclin-dependent kinase Inhibitor
CHK1	Checkpoint kinase 1
COL7	Collagen type VII
CTD	C-terminal domain
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptors
D	Donors
DAG	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DEJ	Dermal-epidermal junction
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
EBA	Epidermolysis bullosa acquisita
FACS	Fluorescence-activated cell sorting
FcγR	Fc gamma receptor
FcR	Fc receptor
FcRn	Neonatal Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLF	N-Formylmethionyl-leucyl-phenylalanine

FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GPCR	G protein-coupled receptors
HBV	Hepatitis B virus
hCOL7	human collagen type VII
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBD	Inflammatory bowel disease
IC	Immune complex
IC_{50}	Half maximal inhibitory concentration
ICAM	Intracellular adhesion molecule
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inibition motif
5-KETE	5-oxo-eicosatetraenoic acid
LIED	Lübecker Institut für Experimentelle Dermatologie
LTB4	leukotriene B4
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase

Mcl-1	Myeloid cell leukemia factor-1
mg	Milligram
MHC	Major histocompatibility complex
mL	Milliliter
mM	Millimolar
MN	Mononuclear cells
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NC1/2	Non-collagenous domain 1/2
NET	Neutrophil extracellular trap
NF-κb	Nuclear factor-κB
NUAK1	NUAK family SNF1-like kinase 1
PAMPs	Pathogen-associated molecular patterns
PAF	Platelet-activating factor
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PBK	PDZ-binding kinase
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C

PLCγ	Phospholipase C gamma
PMN	Polymorphonuclear leukocytes
PMP	Polymorphprep
P-TEFb	Positive transcription elongation factor
PTPN22	Protein tyrosine phosphatase nonreceptor type 22
PYK2	Protein tyrosine kinase 2
RA	Rheumatoid arthritis
Rac1/2	Ras-related C3 botulinum toxin substrate ½
Rb	Retinoblastoma gene product
RBC	Red blood cell
RHO	Ras homologue
RNAP-II	RNA polymerase II
ROCK1/2	Rho-associated, coiled-coil-containing protein kinase
ROS	Reactive oxygen species
RT	Room temperature
SLE	Systemic lupus erythematosus
SLP-76	Leukocyte-specific phosphoprotein of 76 kDa
Src	Sarcoma
SSC	Side scatter
SV	Secretory vesicle
SYK	Spleen tyrosine kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

TOPK	T-lymphokine-activated killer-cell-originated protein kinase
TOPKI	T-lymphokine-activated killer-cell-originated protein kinase inhibitor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
WEE-1	Wee1-like protein kinase
WB	Washing Buffer

10. APPENDIX

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10.2. Plate layout for ROS Release and FACS assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	cells only/DMSO	cells only/DMSO	10 µM S2	10 µM S2	10 µM S4	10 µM S4	10 µM S6	10 µM S6	10 µM S8	10 µM S8	10 µM S10	10 µM S10
B	Antigen only/DMSO	Antigen only/DMSO	1 µM S2	1 µM S2	1 µM S4	1 µM S4	1 µM S6	1 µM S6	1 µM S8	1 µM S8	1 µM S10	1 µM S10
C	Antibody only/DMSO	Antibody only/DMSO	100 nM S2	100 nM S2	100 nM S4	100 nM S4	100 nM S6	100 nM S6	100 nM S8	100 nM S8	100 nM S10	100 nM S10
D	IC /DMSO	IC /DMSO	10 nM S2	10 nM S2	10 nM S4	10 nM S4	10 nM S6	10 nM S6	10 nM S8	10 nM S8	10 nM S10	10 nM S10
E	10 µM S1	10 µM S1	10 µM S3	10 µM S3	10 µM S5	10 µM S5	10 µM S7	10 µM S7	10 µM S9	10 µM S9	10 µM S11	10 µM S11
F	1 µM S1	1 µM S1	1 µM S3	1 µM S3	1 µM S5	1 µM S5	1 µM S7	1 µM S7	1 µM S9	1 µM S9	1 µM S11	1 µM S11
G	100 nM S1	100 nM S1	100 nM S3	100 nM S3	100 nM S5	100 nM S5	100 nM S7	100 nM S7	100 nM S9	100 nM S9	100 nM S11	100 nM S11

10.3. CDK Inhibitors

Palbociclib (PD-0332991) HCl	S1
BMS-265246	S2
AS2863619	S3
THZ2	S4
SR-4835	S5
Purvalanol B	S6
BAY 1251152	S7
OTS964	S8
K03861	S9
BSJ-4-116	S10
LY3143921 hydrate	S11

10.4. p-values ROS release assay

Compound	Friedman Test	Dunn's multiple comparison 10 µM	1 µM	0.1 µM	0.01 µM
Palbociclib (PD-0332991) HCl	0,0001	0,0029	0,1120	>0,9999	>0,9999
BMS-265246	0,0342	0,2519	0,2519	>0,9999	>0,9999
AS2863619	0,0576	0,9469	0,2519	>0,9999	0,1120
THZ2	<0,0001	<0,0001	0,0162	0,0274	>0,9999
SR-4835	0,0762	>0,9999	0,9469	>0,9999	0,3639
Purvalanol B	0,0101	0,0718	>0,9999	>0,9999	>0,9999
BAY 1251152	0,0008	0,0274	>0,9999	>0,9999	0,7052
OTS964	0,0003	0,0094	0,3639	>0,9999	>0,9999
K03861	0,0005	0,1120	>0,9999	>0,9999	0,1701
BSJ-4-116	0,0500	>0,9999	0,1120	0,7052	0,9469
LY3143921 hydrate	0,0550	>0,9999	0,7052	0,5128	0,0718

10.5. p-values flow cytometry $CD18^{pos}$

Parameter	Compound	Friedman Test	Dunn's multiple comparison 10 µM	1 µM	0.1 µM	0.01 µM
$CD18^{pos}$	Palbociclib (PD-0332991) HCl	0,0228	>0,9999	>0,9999	>0,9999	0,0204
	BMS-265246	0,4510	0,9206	>0,9999	>0,9999	>0,9999
	AS2863619	0,1040	>0,9999	>0,9999	0,9206	0,182
	THZ2	0,0043	0,0055	0,4384	0,6461	>0,9999
	SR-4835	0,0621	>0,9999	>0,9999	>0,9999	0,182
	Purvalanol B	0,0976	0,182	>0,9999	>0,9999	>0,9999

Parameter	Compound	Friedman Test	10 µM	1 µM	0.1 µM	0.01 µM
	BAY 1251152	0,0708	>0,9999	>0,9999	>0,9999	0,0656
	OTS964	0,0342	0,1112	>0,9999	>0,9999	>0,9999
CD18^{pos}	K03861	0,3644	0,4384	>0,9999	>0,9999	>0,9999
	BSJ-4-116	0,7505	>0,9999	0,9206	>0,9999	>0,9999
	LY3143921 hydrate	0,0320	0,182	>0,9999	0,9206	>0,9999

10.6. p-values flow cytometry CD62L$_{neg}$

Parameter	Compound	Friedman Test	Dunn's multiple comparison			
			10 µM	1 µM	0.1 µM	0.01 µM
	Palbociclib (PD-0332991) HCl	0,0976	>0,9999	>0,9999	>0,9999	0,182
	BMS-265246	0,1712	>0,9999	0,0656	0,4384	0,9206
	AS2863619	0,0976	0,9206	>0,9999	0,6461	>0,9999
	THZ2	0,0976	>0,9999	>0,9999	0,6461	0,182
	SR-4835	0,0087	0,0373	0,2874	0,9206	>0,9999
CD62L$_{neg}$	Purvalanol B	0,0244	0,9206	0,0656	0,0204	0,0373
	BAY 1251152	0,0020	0,0108	0,0204	0,9206	>0,9999
	OTS964	0,0053	>0,9999	0,6461	0,0656	0,182
	K03861	0,0418	>0,9999	0,0656	0,182	>0,9999
	BSJ-4-116	0,0070	0,0108	0,0027	0,2874	0,2874
	LY3143921 hydrate	0,0805	0,0108	0,0027	0,2874	0,2874

10.7. p-values flow cytometry vital parameter

Parameter	Compound	Friedman Test	Dunn's multiple comparison 10 µM	1 µM	0.1 µM	0.01 µM
Vital	Palbociclib (PD-0332991) HCl	0,8088	>0,9999	>0,9999	>0,9999	0,9413
	BMS-265246	0,0058	0,0247	0,2716	>0,9999	>0,9999
	AS2863619	0,2805	0,2716	>0,9999	>0,9999	>0,9999
	THZ2	0,0006	0,0021	0,0139	0,4014	>0,9999
	SR-4835	0,1311	0,5765	0,3313	>0,9999	>0,9999
	Purvalanol B	0,1133	>0,9999	>0,9999	>0,9999	0,6461
	BAY 1251152	0,2662	0,9206	0,4384	>0,9999	0,6461
	OTS964	0,0151	0,0373	>0,9999	>0,9999	>0,9999
	K03861	0,0621	>0,9999	>0,9999	0,2297	>0,9999
	BSJ-4-116	0,0228	0,0204	>0,9999	>0,9999	>0,9999
	LY3143921 hydrate	0,6834	>0,9999	0,6461	>0,9999	>0,9999

10.8. p-values flow cytometry apoptosis

Parameter	Compound	Friedman Test	Dunn's multiple comparison			
			10 µM	1 µM	0.1 µM	0.01 µM
Apoptosis	Palbociclib (PD-0332991) HCl	0,5936	>0,9999	>0,9999	>0,9999	0,9413
	BMS-265246	0,0026	0,0186	0,1138	>0,9999	>0,9999
	AS2863619	0,4830	0,5765	>0,9999	>0,9999	>0,9999
	THZ2	0,0010	0,0041	0,0139	0,2716	>0,9999
	SR-4835	0,2188	0,6836	0,5765	>0,9999	>0,9999
	Purvalanol B	0,1133	>0,9999	>0,9999	>0,9999	0,6461
	BAY 1251152	0,2311	0,9206	0,4384	>0,9999	0,6461
	OTS964	0,0151	0,0373	>0,9999	>0,9999	>0,9999
	K03861	0,0717	>0,9999	>0,9999	0,2297	>0,9999
	BSJ-4-116	0,0228	0,0204	>0,9999	>0,9999	>0,9999
	LY3143921 hydrate	0,6834	>0,9999	0,6461	>0,9999	>0,9999

10.9. Approvement by the ethics committee

Ethik-Kommission

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Aktenzeichen: 20-463
Datum: 01. Februar 2021 J.E./IK

Verkürztes Verfahren - Anzeige

TitelUntersuchung des Einflusses verschiedener Signaltransuktionsinhibitoren auf die Aktivierung von neurtrophilen Granulozyten
Hier: E-Mail von Katharina Boch vom 27. Januar 2021

Sehr geehrter Herr Prof. Ludwig,

mit Ihrem o.g. Schreiben informieren Sie die Ethik-Kommission über Ihr geplantes Vorhaben. Dieses Teilprojekt zur Studie 20-338 erhält ein separates Aktenzeichen.

Folgende Unterlagen lagen vor:

- Anschreiben vom 26. Januar 2021
- Studienprotokoll Version 1 vom 26. Januar 2021.

Die Ethik-Kommission bewertet das Vorhaben zustimmend.

Mit freundlichen Grüßen

Prof. Dr. med. Alexander Katalinic
Vorsitzender

// 11. ACKNOWLEDGEMENTS

I would first like to thank Ralf Ludwig and Katja Bieber for mentoring and the opportunity to join a research group internationally recognized for their contributions to the understanding of bullous skin diseases.

Secondly, to Claudia Kauderei and Carla Zünkeler for their scientific support and companionship during the long laboratory journey.

Thanks to all the other members of the Lübeck Institute of Experimental Dermatology, as they create a friendly working atmosphere that fosters curiosity and scientific innovation.

I am grateful for the generosity of all blood donors, as they provided the essential raw material for carrying out the experiments.

I would like to thank my wife, for loving me unconditionally, even when I was absent due to my doctorate. I am also profoundly thankful for the support of my mother throughout my experiments. Unfortunately, she passed away before witnessing her son achieve his dream of becoming a doctor.

I thank God for the gift of life. Thank you, my lord!