Interactions between platelets and hematopoietic cells

by

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Summary

In addition to their primary role in hemostasis, platelets are increasingly recognized as important participants in numerous biological processes. Their ability to adhere to and communicate with different immune cells, endothelial cells, and cancer cells makes them a natural nexus that participates in development of different diseases, including cancer. Thus, one could also surmise interactions between platelets and hematopoietic stem and progenitor cells.

Previous studies have shown that bone marrow function recovers more quickly after transplantation with mobilized peripheral blood stem cells than with bone marrow-derived hematopoietic stem cells. A major difference between the two techniques is that mobilized peripheral blood stem cells are exposed to activated platelets during harvesting. As platelets communicate with a myriad of blood cells and carry cargoes of hundreds of proteins and other biologically active compounds, I wanted to investigate potential interactions between platelets and hematopoietic progenitor cells, including leukemic cells from acute myelogenous leukemia (AML).

Using flow cytometric analysis and colony forming unit (CFU) assessment, our group show that platelet releasate inhibits proliferation, conserves erythroid phenotype, and increases levels of erythroid progenitors in cultivated mobilized peripheral blood stem and progenitor cells. Expression of CD14 antigen and monocyte-associated mRNAs also increased, suggesting that platelet releasate induced monocytopoiesis.

Upon activation, platelets degranulate and release the content of their alpha granules, dense granules, and lysosomes. Activated platelets also shed platelet microparticles (PMP), membranous vesicles that contain platelet cargo. These microparticles are internalized by many different cells, including cancer cells, and are known to alter their biological

behavior. Using flow cytometry and fluorescence microscopy, we show that these microparticles are internalized by AML cells, with a subsequent transfer of miR-125a and miR-125b and a downregulation of the pro-apoptotic protein PUMA. This microRNA transfer could explain the anti-apoptotic properties of PMPs that we also observed following treatment with several apoptosis inductors, where daunorubicin is of particular interest, as it is a mainstay in the treatment of AML.

Thus, multiple potential interactions between platelets and hematopoietic progenitor cells and leukemic cells are identified. The results must be confirmed by more advanced *in vitro* and translational models before their clinical relevance can be fully appreciated, but the findings may benefit *ex vivo* production of monocytes and erythrocytes and support the use of therapeutic platelet inhibition in AML patients.

List of papers

Paper I

Cacic, D.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Releasate Augments *in vitro* Monocytopoiesis and Erythropoiesis. Manuscript.

Paper II

Cacic, D.; Reikvam, H.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Protect Acute Myelogenous Leukemia Cells against Daunorubicin-Induced Apoptosis. Cancers 2021, 13, 1870.

Paper III

Cacic, D.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Decrease Daunorubicin-Induced DNA Damage and Modulate Intrinsic Apoptosis in THP-1 Cells. Int. J. Mol. Sci. 2021, 22, 7264.

Abbreviations

AGM	Aorta-gonad-mesonephros
AML	Acute myelogenous leukemia
ASCO	American Society of Clinical Oncology
ATRA	All-trans retinoic acid
BFU-E	Burst forming unit erythroid
BSA	Bovine serum albumin
CAD	Caspase-3 dependent DNase
CFU-E	Colony forming unit erythroid
CFU-G	Colony forming unit granulocyte
CFU-GEMM	Colony forming unit granulocyte, erythroid, megakaryocyte, macrophage
CFU-GM	Colony forming unit granulocyte-macrophage
CFU-MK	Colony forming unit megakaryocyte
CFU-S	Colony forming unit spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTC	Circulating tumor cell
DC	Dendritic cell
DISC	Death-inducing signaling complex

DPC	Days post conception
ELN	European Leukemia Network
ELP	Early lymphoid progenitor
EMT	Epithelial-to-mesenchymal transition
ETP	Early thymic progenitor
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FCR	Fc receptor
FPKM	Fragments per kilobase million
GMDP	Granulocyte, monocyte, dendritic cell progenitor
GMP	Granulocyte-monocyte progenitor
GVHD	Graft-versus-host disease
HDAC	High-dose AraC
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HUVEC	Human endothelial vein endothelial cell
IDAC	Intermediate-dose AraC
IMDM	Iscove′s Modified Dulbecco′s Medium
iPSC	Induced pluripotent stem cell

LIC	Leukemia initiating cell
LMPP	Lymphomyeloid primed progenitor
LSC	Leukemia/leukemic stem cell
LTC IC	Long-term culture initiating cell
M-CSF	Macrophage colony-stimulating factor
M-CSFR	Macrophage colony-stimulating factor receptor
MEP	Megakaryocyte-erythroid progenitor
MK	Megakaryocyte
MLP	Multipotent lymphoid progenitor
MNC	Mononuclear cell
MOMP	Mitochondrial outer membrane permeabilization
MoP	Monocyte progenitor
MPP	Multipotent progenitor
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
NeP	Neutrophil progenitor
NGS	Next-generation sequencing
NOD	None-obese diabetic
NSG	NOD-SCID-gamma
PBMC	Peripheral blood mononuclear cell

PBSCT	Peripheral blood stem cell transplantation
PDX	Patient-derived xenograft
PMN	Polymorphonuclear cell
PMP	Platelet microparticles
PS	Phosphatidylserine
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
TPM	Transcripts per million
UPR	Unfolded protein response
VAF	Variant allele frequency
VTE	Venous thromboembolism
vWF	von Willebrand factor

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1 Introduction

1.1 Hematopoiesis

1.1.1 Background

Hematopoietic stem cells (HSCs) are bone marrow cells capable of self-renewal and differentiation into all hematopoietic cell types. They thereby sustain long-term hematopoiesis and the estimated production of approximately 4.9 x 10^{11} blood cells per day [1]. For over 60 years, hematopoietic stem cell transplantation (HSCT), known more simply as "bone marrow transplantation", has utilized the capabilities and functions of these cells in the treatment of hematological malignancies [2]. However, our knowledge of a number of aspects in the development and regulation of hematopoiesis remains incomplete, as described below.

1.1.2 Embryogenesis

Our understanding of embryogenic hematopoiesis is largely based on animal studies, especially murine models. Fetal hematopoiesis is described as having two "waves" or bursts of development termed primitive and definitive hematopoiesis. Clonogenic assays in mice have identified cells of mesodermal origin with restricted erythroid and macrophage potential (hence the term "primitive") as early as embryonic day 7.0 (E7), marking the start of primitive hematopoiesis [3]. A second wave of hematopoiesis, known as definitive hematopoiesis, begins on E8.25 [4]. By E9.5, all hematopoietic cells originate from the yolk sac and can generate most of the cell types found in mature hematopoiesis, but are not yet HSCs *per se* because they cannot sustain long-term HSC functions [4-6].

The first definitive HSCs with the ability to repopulate bone marrow are generally believed to be derived from the hemogenic endothelium of the

aorta-gonad-mesonephros (AGM) at E10.0–E10.5 [6-8] and from the liver, yolk sac, and placenta at E10.5–E11.5 [8, 9]. HSCs from the placenta, yolk sac, and AGM presumably colonize the fetal liver, which is the main site for hematopoiesis until late gestation (Figure 1) [10].

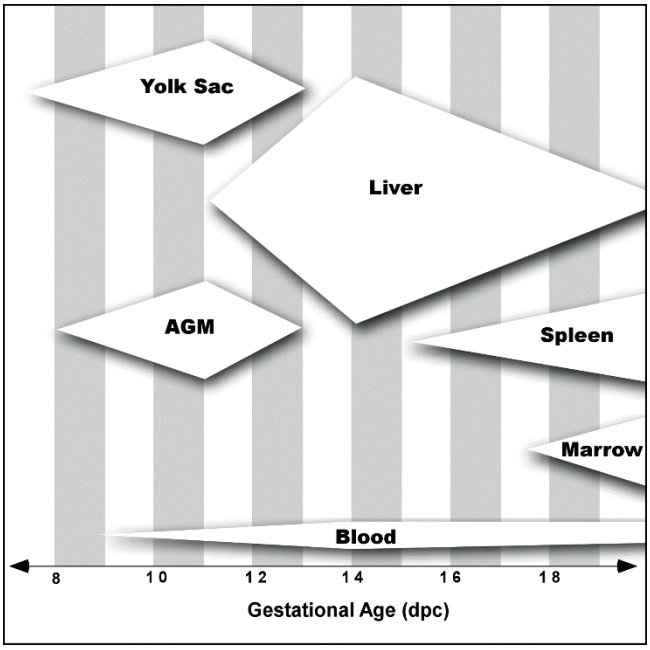

Figure 1. Distribution of hematopoietic progenitor cells in murine embryos. Gestational age is noted in days post conception (dpc). Note that the liver is the main hematopoietic organ until late gestation. Reprint of Christensen et al. [11].

1.1.3 Hematopoietic stem cells

The term "stem cell" and the idea that blood cells are derived from a common progenitor cell can be traced back to as early as the 19th century [12]. However, the details of this hierarchical system for the development of hematopoietic cells are still not clear. The original mapping of the transition from hematopoietic stem cells to differentiated peripheral blood cells was performed using morphological analysis [12]. In older textbooks, one can see models where hematopoiesis is described from the morphology of clonogenic bone marrow cells cultured in

semisolid medium (illustrated in Figure 2A) [13]. This concept of hematopoiesis was first based on the identification by Till and McCulloch in 1961 of a pluripotent spleen colony-forming unit, the CFU-S [14], which was isolated from allogeneic bone marrow cells that colonized the spleens of X-ray irradiated mice, producing morphologically distinct erythroid and myeloid progenitors. Additional colony forming progenitor cells have since been identified, which will be elaborated later.

Later models, as described in a review by Laurenti and Göttgens, use fluorescence-activated cell sorting to identify specific progenitor subsets based on immunophenotype [15]. In mice, the expression of IL-7R divides early progenitors into restricted common myeloid (IL-7R^{-}) and common lymphoid (IL-7R^{+}) progenitors [16, 17]. Similar work with human cells defined specific immunophenotypes for committed progenitors with limited or restricted differentiation abilities [18, 19]. This followed the basic dogma that HSCs differentiate in a stepwise manner into distinct fates, as illustrated in Figure 2B. With the emergence of advanced techniques such as single-cell RNA sequencing, it is evident that cell fate is not as categorical as first assumed. Rather, cell differentiation is controlled by certain genetic transcription programs and appears to be continuous through gradual priming. Thus, hematopoiesis is now described as a continuum that begins with initially primed progenitor cells derived from HSCs, which then develop gradually into unipotent progenitors and finally into terminally differentiated cells (Figure 2C) [15, 20-22]. However, uncertainty remains about whether lineage fate is first determined in stem and multipotent progenitor cells by the establishment of specific committed progenitors, the "CLOUD-HSPC" theory, or through a gradual lineage bias [15, 22-24].

A major caveat to our understanding of hematopoiesis is that it derives mostly from *in vitro* assays and transplantation models, which deprive HSCs of their normal microenvironment and induce supraphysiological

stress. This issue is especially evident when assessing the relative contribution of each progenitor to hematopoiesis, as we have no means of investigating how these manipulations affect specific stages of hematopoiesis. However, *in vivo* animal models can provide additional information. Using a Sleeping Beauty transposon tagging system in murine hematopoietic stem and progenitor cells, Sun et al. showed that murine hematopoiesis *in vivo* was mostly driven by multipotent progenitor cells, not HSCs [25]. Even after 45 weeks, very little overlap was observed between granulocytes and lymphoid cells, indicating that the differentiated cells derived from a more restricted progenitor cell downstream of an HSC or multipotent progenitor with shared myeloid and lymphoid potential. Rodriguez-Fraticelli et al. used a similar tagging system to examine the dynamics of murine hematopoiesis and found that, in the first two weeks, nucleated erythroblasts, megakaryocyte progenitors, granulocytes, monocytes, and B cell progenitors were replenished by unipotent progenitors [26]. This time frame has been corroborated using Tie2 genetic knock-in mice [27]: after two weeks, contributions by shared multipotent progenitors increased, except for the megakaryocyte progenitor lineage, where approximately half of which entirely bypassed a multipotent progenitor stage and stemmed instead directly from HSCs.

The gold standard for testing whether a cell type is a hematopoietic stem cell is the ability of a single flow-sorted human cell to regenerate bone marrow function in immunodeficient mice, though not all presumed HSCs will engraft in murine models [15]. Cells are considered stem cells if they are capable of sustaining complete hematopoiesis for at least 16 weeks and engraft in at least two serial transplantations [15]. A certain immunophenotype of hematopoietic cells, $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$, is normally considered to be a stem cell, but only regenerates normal bone marrow function in 1 of 10.5 cases of single cell transplantation when harvested from cord blood [28].

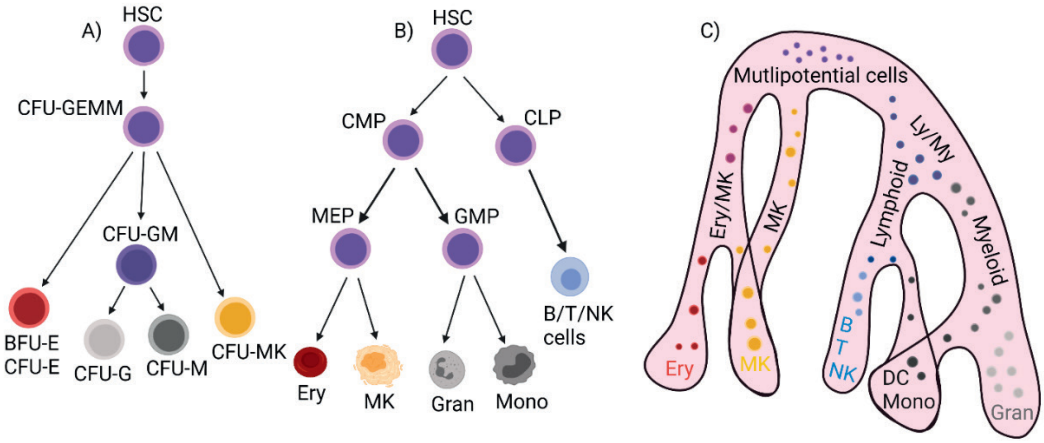

Figure 2. Simplified scheme of the old and new view of hematopoiesis. The understanding of hematopoiesis has evolved from a categorical stepwise transition (A, B) to a continuum (C) of progressively committed progenitors [15]. Ery, erythrocyte. MK, megakaryocyte, DC, dendritic cell, Mono, monocyte. Gran, granulocyte. Ly, lymphoid cell. My, myeloid cell. For complete list of abbreviations of hematopoietic progenitors, see Figure 7 or "Abbreviations". Created with BioRender.com.

Hematopoietic stem cells are believed to represent a small niche of between 50,000 and 200,000 active cells that replicate only once every 2–20 months [29]. Other models estimate only 11,000 cells replicating every 40 weeks [30]. Calling the $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ cell fraction "true" HSCs is also somewhat inaccurate. When adding the mitochondrial stain Rho, $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+Rho^{lo}$ cells constitute only 1 of 145,000 mononuclear cells (MNCs) in mobilized peripheral blood, whereas 1 of 100 MNCs are $CD34^+$ [31]. However, only 2.3% of this fraction has long-term repopulating capability, the hallmark of HSCs [31]. Thus, a patient weighing 80 kg who receives a transplant of 5×10^6 $CD34^+$ cells per kg would receive 4×10^8 $CD34^+$ cells, of which only approximately 6,300 would be of the described immunophenotype and have HSC functionality. This calculation does not consider into account that there may be other cells with unknown immunophenotypes and with long-term repopulating abilities. Still, in a more functional approach using cellular barcodes to track clonal behavior, only 0.007% of $CD34^+$ cord blood

cells were found to have long-term repopulating properties in xenotransplant models [32].

1.1.4 *HSC function and regulation*

The understanding of how transcription factors regulate cellular development from stem cells to mature cells has been greatly advanced by a new technique of cellular engineering, namely the generation of induced pluripotent stem cells (iPSCs), which resulted in the Nobel Prize in medicine and physiology in 2012 [33]. Human fibroblasts transduced with a single transcription factor, *OCT4*, can be reprogrammed to $CD45^+$ multipotent hematopoietic cells that, with the correct cytokine stimulus, are capable of producing clonogenic progenitors [34]. In addition, transduction of cells from hemangiogenic endothelium with seven transcription factors (*ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1*, and *SPI1*) is sufficient to produce HSC-like cells with the capacity to repopulate bone marrow, although with limitations when compared to *bona fide* cord blood-derived $CD34^+$ cells [35].

While many transcriptional programs are shared across different cell lineages [36], several genes and transcription factors are known to be indispensable for lineage commitment and HSC function. In murine models, deletion of *Znf90* inhibits proliferation and self-renewal of HSCs without altering lineage fate [37], whereas deletion of *Zeb2* impairs the development of plasmacytoid dendritic cells and monocytes [38]. Furthermore, the transcription factor *XBP1* is selectively required by among bone marrow cells for the survival and development of human eosinophil progenitors [39]. Other transcription factors have a more general regulatory function. For example, *Gfi1* knockout mice lack mature neutrophils and have reduced lymphocyte count in both peripheral blood and bone marrow [40]. Additional crucial transcription factors will be discussed in subsequent paragraphs on the development of the most common blood cells.

The regulation of transcription programs involved in defining lineage fate is still unclear. Binding sites for transcription factors such as *GATA1* and *TAL1,* which drive the formation of myeloerythroid progenitor cells, have lower levels of methylation in myeloid progenitors than in lymphoid progenitors, suggesting DNA methylation may play a role in regulating lineage fate, though a similar mechanism was not specifically identified in lymphoid progenitors [41]. mRNA methylation has provided further insight into the regulation of lineage fate and self-renewal. Knockout of *Mettl3*, a crucial gene for m6A methylation, resulted in a 40% reduction in bone marrow cellularity and pancytopenia in mice, especially evident for myeloid and erythroid cells [42]. The authors also found that the catalytic activity of the METTL3 protein is essential for the symmetric commitment of HSCs via its regulation of oncogenic *Myc* mRNA.

Hematopoietic stem cells are mostly dormant in G0 cell phase, with entry into the cell cycle regulated by CDK6 [43]. Inhibition of DEGS1, the final enzyme of sphingolipid synthesis, affects self-renewal and lineage commitment of HSCs [44] and sphingosine-1-phosphate receptor 3 is a central regulator in myelopoiesis suggesting that the lipidome also plays a role in HSC lineage determination [45]. Glycolysis is the main energy source for HSCs as they have few active mitochondria and low mitochondrial activity [46, 47]. Some degree of mitochondrial activity, however, is required to maintain normal stem cell function [48]. The reliance of HSCs on glycolysis is likely to protect them from reactive oxygen species (ROS), which causes loss of function and DNA damage [49], although the mutational frequency in mature granulocytes is actually found to be not significantly higher than that in their parental HSCs [50]. Loss of *Mtch2*, a mitochondrial regulator gene, accelerates mitochondrial respiration and drives HSCs into the cell cycle [51], with a concomitant rise in ROS levels [52]. In murine models, knockout of *Vdr,* the vitamin D receptor gene, results in increased HSC quiescence and a reduced ability to accumulate ROS [53]. Compared to $CD34^-$

$CD15^+$ cells (terminally differentiated granulocytes), $CD34^+CD15^-$ cells (hematopoietic progenitor cells) have an upregulated number of proteins involved in cell signaling, vessel trafficking, metabolism, and transcriptional regulation [54]. A striking loss of DNA methylation occurs during granulopoiesis, when 99.2% of differentially methylated CpG sites become hypomethylated in the transition from common myeloid and granulocyte-myeloid progenitors (CMPs and GMPs) to granulocytes (defined as the stages from promyelocytes to PMNs) [55]. In summary, HSCs are quiescent cells with only minimal activity to protect cellular health. Upon maturation, the cells swiftly increase mitochondrial respiration, gene expression, and protein production, with many genes becoming hypomethylated.

Co-culture of hematopoietic progenitor cells with bone marrow stromal cells, such as mesenchymal stem cells (MSCs), or endothelial cells, increases proliferation of early and late hematopoietic progenitor cells, demonstrating their importance in HSC regulation [56-59]. The bone marrow milieu is hypoxic, more so in the sinusoidal region than in the endosteal region [60]. HSCs were originally thought to reside primarily in the sinusoidal region of the bone marrow and they are regulated by a variety of chemokines and growth factors, including CXCL12, SCF, and TPO; where the first two are produced by endothelial cells and perivascular stromal cells, but there are several other growth factors and cell types involved in their regulation [61]. However, several distinct HSC niches have been revealed in mice, where myeloid-platelet-biased (vWF^+) HSCs are interconnected with megakaryocytes, while lymphoid-biased (vWF^-) HSCs are more associated with arterioles [62]. The regulatory role of megakaryocytes (MKs) is apparent, as their depletion leads to the formation of more unrestricted vWF^+ HSCs.

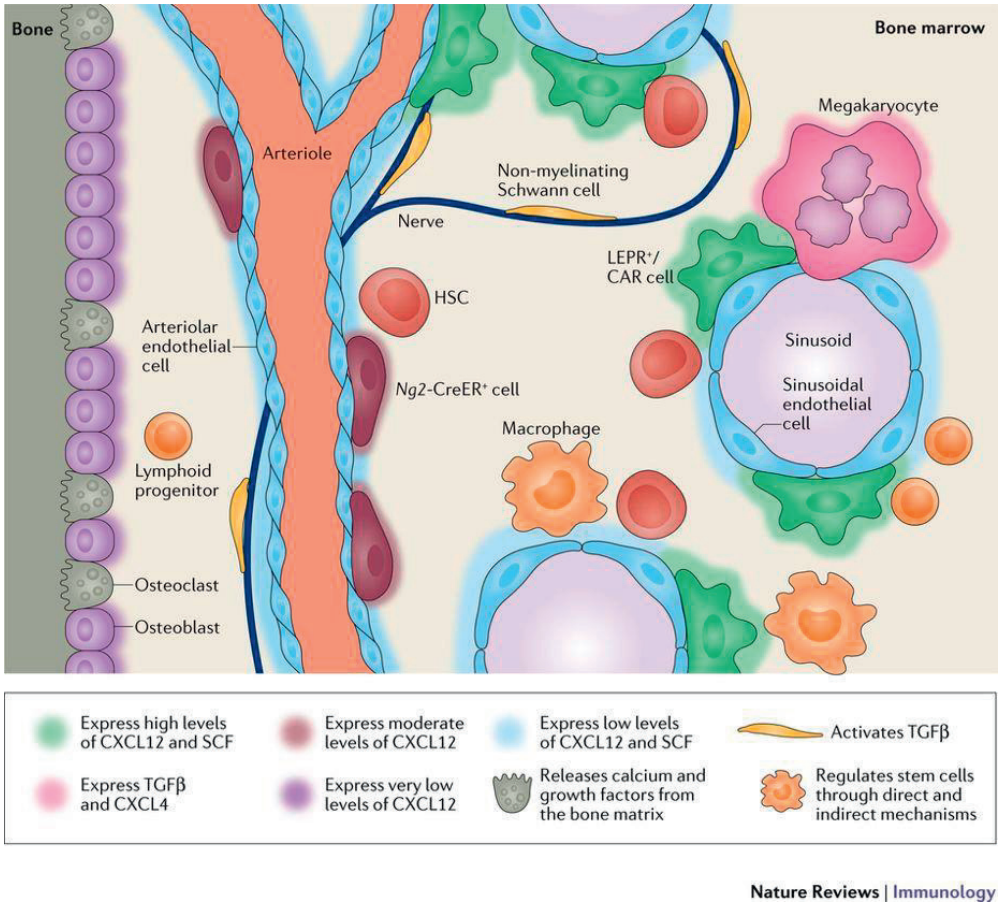

Figure 3. Overview of hematopoietic stem cell regulation. HSCs reside in different niches and are regulated by a number of different bone marrow cells. Reprint of Crane et al. [61].

1.1.5 *Megakaryocytopoiesis*

Megakaryocytic development is controlled by a gene module containing *GATA2* and *NFE2* [24]. MKs are believed to be derived from a shared progenitor with erythroid cells and/or from a unipotent progenitor cell generated by the common myeloid progenitor (CMP) or one of its progenitors [26, 63-65]. Germline mutations in the transcription factors *RUNX1*, *GATA1*, *FLI1*, *GFI1b*, and *ETV6* result in dysfunctional megakaryocytopoiesis (reviewed by Daly [66]), underlining their

prominent role in megakaryocytopoiesis. The bidirectional lineage fate of the megakaryocyte-erythroid progenitor (MEP) is strongly influenced by *RUNX1* and its target gene, the master erythropoiesis regulator *KLF1* [67]. *FLI1* is hemizygously deleted in patients with Jacobsen syndrome, resulting in macrothrombocytopenia. *FLI1* expression is negatively correlated with levels of *ETS1* and positively correlated with *ETV6*, and its knockout in induced megakaryocytes is associated with impaired maturation and platelet release [68]. Knockout of miR-22 leads to impaired megakaryocyte development in mice and human cell lines through the upregulation of another erythropoiesis regulator, *GFI1*, an effect that is likely downstream of the MEP differentiation stage [69]. Murine models have shown that *Gfi1b*, the paralog of *Gfi1*, regulates megakaryocytopoiesis via the Wnt/β-catenin signaling pathway through multiple targets [70]. Germline mutations in the *ETV6* gene are associated with thrombocytopenia, where developing MKs have a marked defect in cytoskeletal reorganization and a reduced proplatelet releasing capacity [71]. Furthermore, *TRIB3* negatively regulates megakaryocytopoiesis [72]. Thus, megakaryocytopoiesis is intricately regulated by multiple genes and transcription factors. A "master switch" for megakaryocytopoiesis has not yet been identified and indeed may not exist, at least at the genetic level.

Regulation of megakaryocytopoiesis and the production of platelets, i.e., thrombopoiesis, by cytokines is somewhat simpler. MK development is primed by thrombopoietin (TPO) via an increase in metabolic activity [73]. *Tpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice show greatly reduced platelet numbers but production is partially restored by treatment with adenoviral vectors expressing *SDF-1* or *FGF-4*, which increase adhesion and migration of megakaryocyte progenitors through bone marrow endothelial cells [74]. Insulin growth factor 1 also stimulates megakaryocyte development, proplatelet formation, and platelet release, independently of TPO [75]. IL-21 accelerates platelet turnover as it can enhance megakaryocytopoiesis and macrophage platelet clearance [76].

Moreover, the characteristic inflammatory thrombocytosis seen in cancer is due to increased levels of TPO in response to increased IL-6 levels [77].

MKs are distributed throughout the bone marrow, but are almost always in close proximity to a blood vessel [78], as shear stress is important for MK maturation [79]. Under normal conditions, extensions of the MK plasma membrane called proplatelets protrude into the bone marrow sinusoids where they bud off into circulation as platelets [80]. The fission of proplatelets is preceded by sorting of granules and organelles and is a result of microtubule depolymerization and reorganization [81], and regulated by the protein kinase CK2b [82]. Thus, not surprisingly megakaryocyte transcriptome varies distinctively with increased ploidization and maturation to prepare for proplatelet generation [83]. However, upon higher demand, megakaryocytes can burst to rapidly produce platelets [80]. The role of mechanistic forces in platelet formation is especially evident when developing systems for the *ex vivo* production of platelets, as addition of turbulence and vorticity increase yield of platelets [84].

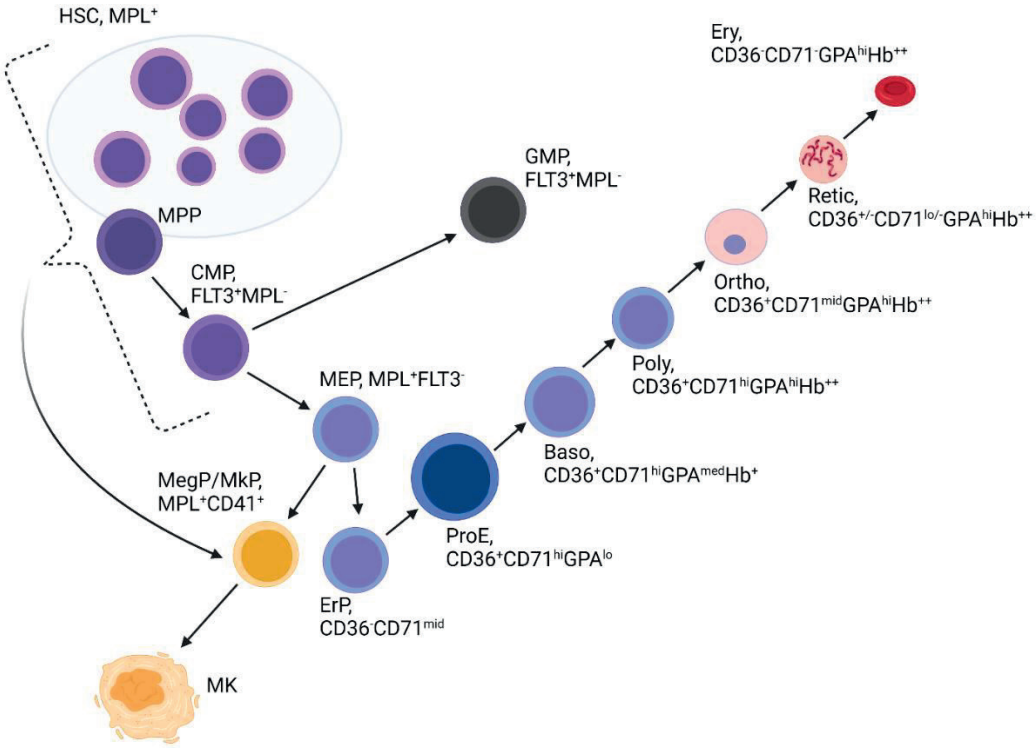

Figure 4. Proposed model of megakaryocytopoiesis and erythropoiesis. Megakaryocytes are derived from either a unipotent progenitor in early hematopoiesis or a bipotent progenitor in a pathway shared with erythropoiesis [63]. As illustrated, megakaryocyte-erythroid progenitors (MEPs) can be identified by gain of expression of the MPL receptor and loss of expression of FLT3. Immunophenotype of erythroid progenitors are derived from multiple publications [85-87]. MegP/MkP, megakaryocyte progenitor. ErP, erythrocyte progenitor. ProE, proerythroblast. Baso, basophilic erythroblast. Poly, polychromatic erythroblast. Orto, ortochromatic erythroblast. Retic, reticulocyte. For complete list of abbreviations of hematopoietic progenitors see Figure 7 or "Abbreviations". Created with BioRender.com.

1.1.6 *Monocytopoiesis*

Mature monocytes are divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) monocytes, with classical and non-classical types showing distinct transcriptional signatures [88]. Monocytes are generally believed to originate from the granulocyte-myeloid progenitor (GMP) and from the lymphoid-primed multi-potential progenitor (LMPP), perhaps through

an intermediate GMP-like stage [36, 89-92]. GMP-derived monocytes originate from increasingly restricted progenitors, such as the monocyte-dendritic progenitor (MDP), granulocyte-monocyte-dendritic cell progenitor (GMDP) [93], and a CLEC12A^{hi}CD64^{hi} monocyte progenitor (MoP) [92]. The monocyte/dendritic cell pathway is controlled by the *IRF7*/*IRF8* gene module [24]. During maturation, expression of the transcription factors *IRF8*, *SPI1* (*PU.1*), *CEBP*, and *KLF4* plateaus in the MoP stage while expression of the chemokine receptors *CX3CR1* and *CCR2* increases until terminal maturation [92]. Macrophage colony-stimulating factor (M-CSF) and its receptor (M-CSFR or CSF1R) are believed to be central regulators of monocytes. However, as shown in Figure 5, monocytes express high levels of receptors for other cytokines as well.

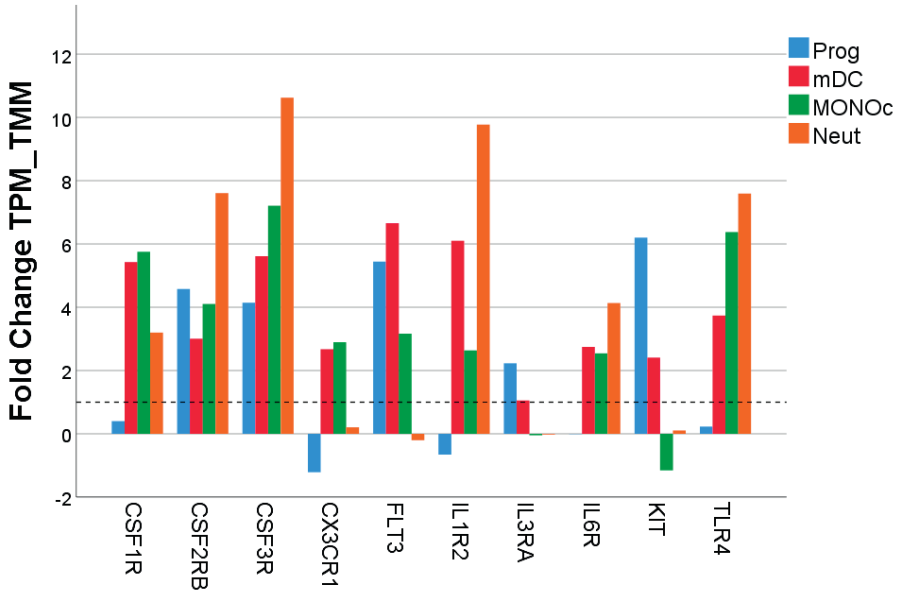

Figure 5. Expression of regulatory cytokine receptors in myeloid cells. Histograms are generated from RNASeq data from Monaco et al. [94]. Y-axis shows the log2 fold change for each cell type calculated with respect to the remaining cell types in PBMCs. The trimmed mean of M values (TMM) of transcripts per million (TPM) gene expression values, or TPM_TMM, was calculated by the authors as the change in expression relative to a core set of genes. Prog,

progenitor ($CD34^+CD45^{lo}$). mDC, myeloid dendritic cell. MONOc, classical monocyte. Neut, neutrophil.

1.1.7 *Granulopoiesis*

The half-life of neutrophils in circulation, measured with isotope labeling, is just 13–19 h [95]. Thus, replenishing these cells requires an enormous production, as they account for most of the nucleated cells in human peripheral blood. Neutrophils are derived from the GMP fraction of hematopoiesis [89]. A unipotent neutrophil progenitor (NeP), the $CD66b^+CD117^+CD38^+CD34^{+/-}$ fraction of GMP, has been identified in humans and constitutes 1%–3% of $CD45^+$ cells in the bone marrow [96]. Adding the transferrin receptor (CD71) further discriminates an otherwise similar progenitor, the $Lin^-CD66b^+CD117^+CD71^+$ subset. However, both immunophenotypes are positive for CD66b, CD117, and/or CD34, resembling the traditional antigen expression pattern of myeloblasts or promyelocytes. Maturation of neutrophils occurs through multiple steps, including segmentation of the nucleus and production of cytoplasmic granules. Maturation is controlled by master regulators such as *GFI1*, *SPI1,* and the CCAAT-enhancer-binding protein family [24, 97] acting through several genes and microRNAs, such as *EVI2B* [98], miR-182 [99], *CARD10* [100], *ELA2*/*ELANE* [101], and *MPO* [102]. Four characteristic cytoplasmic granules, with differing protein content (azurophilic granules, specific granules, gelatinase granules, and secretory vesicles), are generated at different time points during maturation, as illustrated in Figure 6 [103].

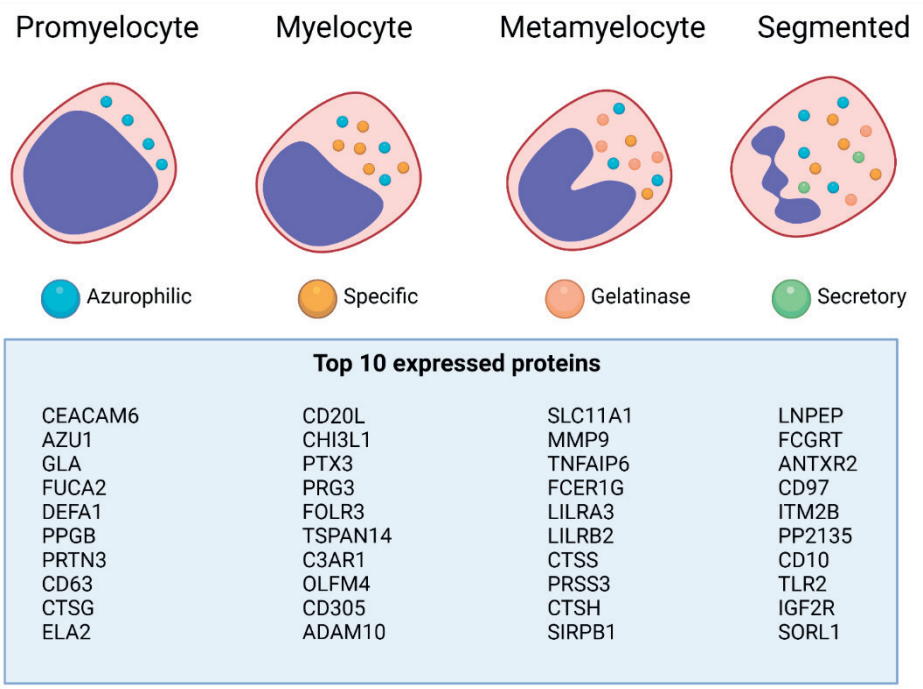

Figure 6. Simplified diagram of the origin of different neutrophil granules.Though, contrary to the illustration, granule production possibly begin prior to the myeloblast/promyelocyte stage and there may be some overlap in production of granules between multiple stages [97]. An analysis of the top ten expressed proteins reveals it is little overlap in protein content between granule types, though results of a functional analysis using the uniprot.org database [104] show that all granule types share functions related to cytotoxicity, chemotaxis, adhesion, and migration. The tight correlation between the transcriptome at different neutrophil maturation stages and granule protein content suggest that proteins are not selectively sorted before packing [103]. Created with BioRender.com.

1.1.8 *Erythropoiesis*

Erythroid colony-forming units were first described in 1971 using murine fetal liver cells with a plasma culture technique [105]. Later, they were categorized into "burst forming unit-erythroid" (BFU-E) and "colony forming unit-erythroid" (CFU-E) [106], which remained the earliest known erythroid progenitor cells for a long time.

The immunophenotype of the more immature BFU-E was later revealed as $CD45^{+}GPA^{-}IL3R^{-}CD34^{+}CD36^{-}CD71^{lo}$, an expression pattern which

shows little lineage restriction, suggesting its origin is close to the fraction known today as the common myeloid progenitor or CMP [107]. However, only with the recent entry of transcriptomics and flow-sorting techniques have we begun to understand erythroid development from early hematopoietic stem cells to mature erythrocytes. We now think that erythrocytes stem from a common progenitor shared with megakaryocytes, as $CD34^{+}CD38^{+}CD135^{-}CD45RA^{-}$ [89] and $CD34^{+}CD38^{mid}CD45RA^{-}CD135^{-}CD110^{+}CD36^{-}CD41^{-}$ [63] megakaryocyte-erythroid progenitors are known to produce erythroid cells.

CRISPR-mediated knock-out of transcription factors *HOXC6*, *NFATC1*, *GSX2*, or *MXD3* in $CD34^{+}$ hematopoietic stem and progenitor cells in *in vitro* systems results in a significant reduction in $CD71^{+}$ and $CD41^{+}$ cells, supporting the theory of a common ancestor cell for erythrocytes and MKs with shared transcriptional regulation [108]. An early restricted erythroid progenitor is found in the $CD71^{mid/+}CD105^{+}$ fraction of MEP [109].

The steps in erythropoiesis can easily be discriminated by a panel of antigens or morphological features, as shown in Figure 4. Early in the commitment to the erythrocyte lineage, progenitors lose CD34 and gain CD36, while expression of glycophorin A increases continuously until late in maturation [85, 107]. CD71 is transiently expressed at a high level but decreases during maturation [85]. *KLF1*, *NFE2*, *GFI1B*, *YBX1*, and *GATA1* are the most abundant transcription factors in erythrocyte progenitors [110], and the levels of transcription factors and proteins in general peak in the early progenitor stage [85]. Details of the regulation of transcription factors in erythropoiesis are shown in Table 1.

Hemoglobinization increases rapidly during the basophilic erythroblast stage [85]. In late-stage erythropoiesis, orthochromatic erythroblasts actively sort proteins and expel their nucleus and much of their cytoplasm to form reticulocytes [85]. Reticulocytes then rapidly clear the

rest of their organelles, morphing into mature erythrocytes within a few days [111, 112].

Table 1. Transcription factors in erythropoiesisTop 20 transcription factors up or downregulated between different stages in early erythropoiesis. Cellular subsets are analyzed with RNASeq and genes are listed in order of decreasing absolute change in fragments per kilobase million (fpkm). Upregulated genes are written in red color while downregulated genes are written in normal font [107].

#	CD34 to BFU-E	BFU-E to CFU-E	CFU-E to ProE
1	JUND	ATF4	GFI1B
2	TSC22D3	HMGB2	ATF4
3	HMGB2	HMGA1	YBX1
4	ATF4	HMGB1	E2F4
5	HMGB1	YBX1	MYC
6	HMGA1	GTF3A	TFDP1
7	NR4A1	FOS	MYBL2
8	MAFF	MYC	HMGA1
9	ELF1	XBPI	GTF3A
10	NFE2	MAZ	SREBF1
11	HOPX	JUND	HMGB2
12	KLF1	LYL1	MAZ
13	ZNF394	KLF1	KLF1
14	FOSL2	CAMTA1	TCF3
15	MAZ	NFE2	NFE2
16	LYL1	TFDP1	STAT5A
17	JUNB	TP53	MYB
18	XBP1	HES6	UBTF
19	TP53	MAX	XBPI
20	GATA1	MBD3	TALI

1.1.9 *NK-poiesis*

NK cells are heterogenic innate lymphoid cells with up to six different cell subsets with a distinct transcriptome in human blood and spleen

[113]. NK cells were originally described as being constricted to lymphoid origin with a committed progenitor from the common lymphoid progenitor (CLP) fraction, which has been identified as $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ [114]. However, 1.49% of human bone marrow cells express both CD56, thought to be a defining marker of NK cells, and myeloid antigen CD33, suggesting that NK cells can originate from both lymphoid and myeloid progenitor cells [115, 116]. The $CD56^-CD117^+M\text{-}CSFR^+$ fraction and the $CD56^{lo}CD36^+$ fraction of $CD34^+$ cultured cord blood cells, which both produce NK cells, are derived from the granulocyte-monocyte progenitor [115, 116].

1.1.10 *Lymphopoiesis*

The earliest multipotent lymphoid progenitors, believed to be distinct from HSCs or MPPs, are the rare and closely related LMPPs, which retain a small myeloid potential, and multi-lymphoid progenitors (MLPs) [90]. However, several multipotent lymphoid progenitors, identified by the expression of CD127, have been identified with different functionality and differentiation potential regarding T cells [117]. The restriction in early hematopoiesis between the myelo-lymphoid and erythro-megakaryocyte lineages appears as early as the $CD49f^+$ HSC/MPP subset [21]. Early lymphopoiesis is regulated by the transcription factors *HES1*, *RUNX3*, *POU2F2*, *LEF1*, *IKZF1*, *IRF8*, and *TCF4*, which are highly upregulated in the lymphoid-restricted MLP fraction [90]. T cell progenitors are produced in the bone marrow, but mature in the thymus, where one has identified early $CD7^-$ thymocytes with stem-cell-like and T cell biased transcriptome, prior to restriction from B, NK, and myeloid cells, thus resembling the functionality of LMPPs or earlier progenitors [118]. Commitment to B cells from MLPs is regulated by the transcription factors *BCL11A*, *BCL6*, *SOX4*, and *TEAD1* [36]. Furthermore, the earliest committed B cell progenitors are pre-proB cells ($CD19^+CD10^-CD34^+$), more frequent in fetal

hematopoiesis, and proB cells ($CD19^+CD10^+CD34^+$) both derived from the early lymphoid progenitor (ELP) fraction [119].

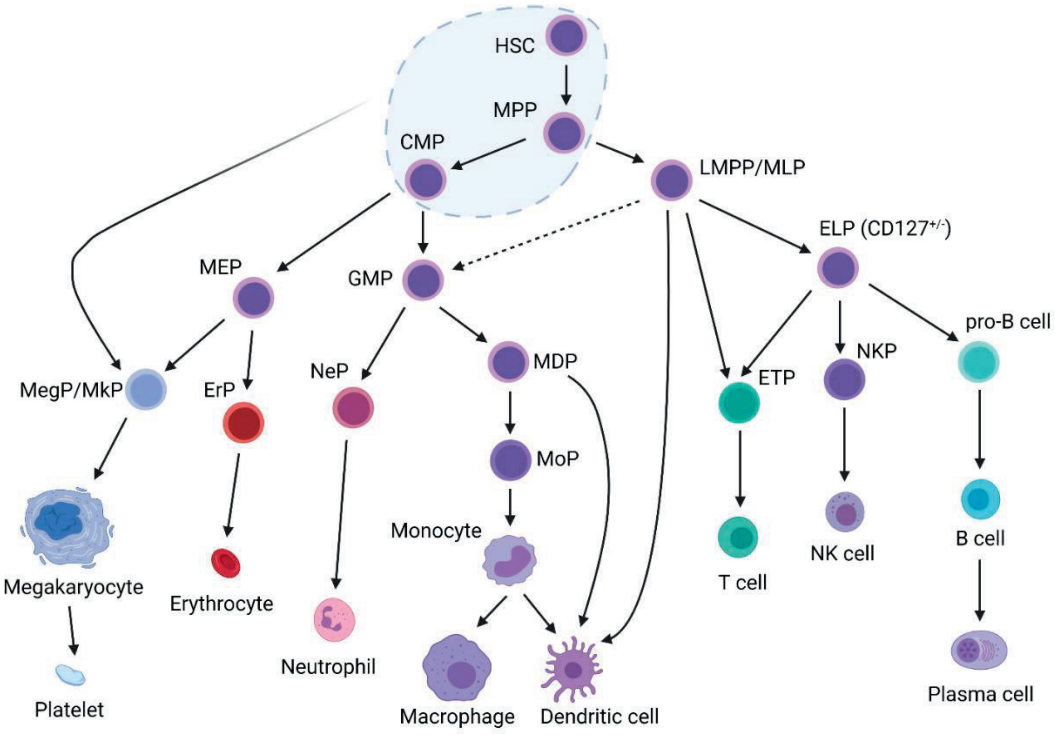

Figure 7. Simplified roadmap of the origin of common blood cells. HSC, hematopoietic stem cell. MPP, multipotent progenitor. CMP, common myeloid progenitor. LMPP, lymphoid-primed multi-potential progenitor. MLP, multi-lymphoid progenitor. MEP, megakaryocyte-erythroid progenitor. GMP. Granulocyte-monocyte progenitor. ELP, early lymphoid progenitor. MegP/MkP, megakaryocyte progenitor. ErP, erythroid progenitor. NeP, neutrophil progenitor. MDP, monocyte-dendritic cell progenitor. NKP, NK cell progenitor. ETP, early thymic progenitor. MoP monocyte progenitor. Created with BioRender.com.

1.1.11 *Hematopoietic stem cell transplantation*

Hematopoietic stem cell transplantation (HSCT) is an organ transplantation used to treat various hematolymphoid diseases. The first transplantations in humans, using bone marrow from allogenic donors,

were published in 1957 [120]. This technique requires the aspiration of a large amount of bone marrow (approximately 1 L) and general anesthesia because of discomfort for the donor during aspiration [121].

Peripheral blood stem cell transplantation (PBSCT) was introduced approximately 30 years later [122]. This procedure, which uses hematopoietic stem and progenitor cells mobilized from the bone marrow and harvested from peripheral blood by apheresis, now accounts for approximately 75% of allogenic and almost 100% of autologous hematopoietic stem cell treatments [122].

Other than their technical execution, the two methods have several differences:

During the harvesting procedure of peripheral blood stem cells, platelets become activated and release microparticles and growth factors [123, 124]. The composition of the product is also different, as the graft of mobilized peripheral blood cells contains more cytotoxic T cells and NK cells and fewer regulatory T cells than bone marrow aspirate [125-128].

Meta-analyses comparing the two techniques have shown faster engraftment, but also more graft-versus-host disease (GVHD) with PBSCT [129-131]. The mechanism of this has somewhat eluded researchers for several years.

An early study found that $CD34^+$ cells from cord blood and bone marrow had lower expression of attachment molecules or integrins, such as CD41 and CD61, than mobilized peripheral $CD34^+$ cells [132]. The same study found that short incubation with platelet microparticles (PMPs) abrogated this difference. The increased integrin expression led to decreased time to engraftment in a xenotransplant model [132]. Fucosylation of $CD34^+$ cord blood cells has yielded similar results [133]. Integrins function as "anchors" by which $CD34^+$ cells attach to endothelial cells in bone marrow blood vessels, thereby increasing homing capacity. Whether the increased expression of CD41 and CD61

in mobilized peripheral $CD34^+$ cells is due to the transfer of antigens from PMPs, through membrane fusion or simple binding, was at a time controversial [134]. However, recent evidence suggests that PMPs are in fact internalized by $CD34^+$ progenitor cells and can also stimulate megakaryocytopoiesis directly through the transfer of miR-1915-3p [135]. Furthermore, the uptake of platelet microparticles has been proven in numerous types of cells [136-141]. These microparticles can transfer proteins, mRNAs, microRNAs, and other small RNAs and have direct effects on proliferation, apoptosis, and tissue-specific cell functions [135-137, 142].

GVHD is a syndrome in patients treated with allogeneic stem cell therapy where donor lymphocytes react to host HLA antigen [143]. The pathophysiology of acute GVHD is shown in Figure 8. GVHD can cause damage to multiple tissues and organs, and is the main cause of non-relapse death after HSCT. "Acute" and "Chronic" GVHD are categorized by whether onset of symptoms occurs before or after 100 days of transplant. PBSCT grafts contain a higher ratio of T cells and NK cells to $CD34^+$ cells and fewer regulatory T cells than bone marrow aspirate, cell types that are central to the pathophysiology of GVHD [144, 145]. Bone marrow aspirate, however, also contains mesenchymal stem cells, which have an immunomodulatory effect in GVHD [146, 147]. Thus, the differences in the risk of GVHD between transplantation by PBSCT or bone marrow aspirate may be explained by the different composition of the products. The potential role of platelets in GVHD remains unknown even though they are known to interact with multiple types of immune cells.

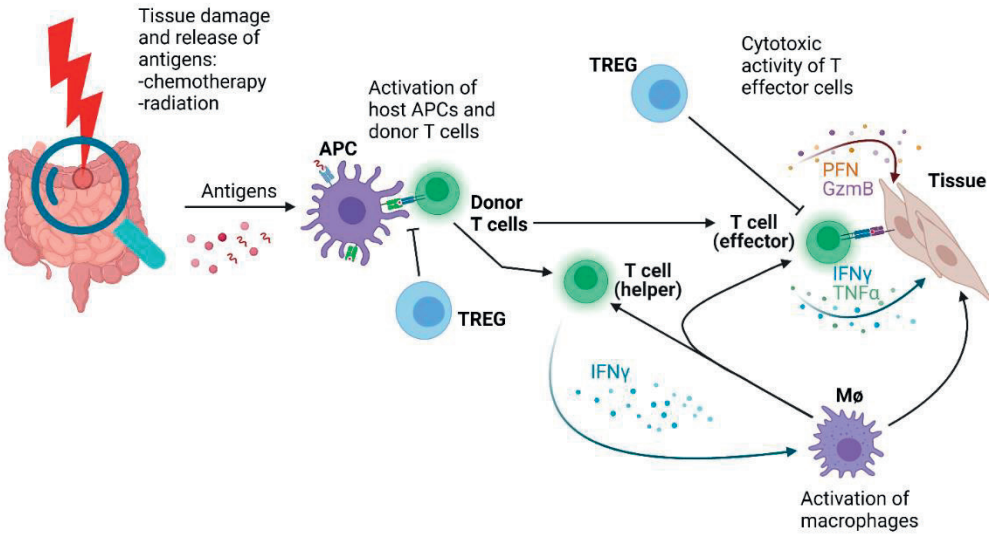

Figure 8. Simplified model of the pathophysiology of acute GVHD. Conditioning regimes induce tissue damage and inflammation, which then attract antigen presenting cells. These activate donor T helper cells, which react to host HLA antigens. Furthermore, T helper cells activate different effector cells, causing tissue damage. TREG, regulatory T cells. PFN, perforins. GzmB, granzyme B. IFNγ, interferon gamma. TNFα, tumor necrosis factor alpha. Created with BioRender.com.

1.2 Platelets

1.2.1 Platelets in health and disease

Platelets were first recognized over a hundred years ago for their important role in blood coagulation [148]. Since then they have also been recognized as significant contributors in a wide range of biological processes, including tissue regeneration, immunology, and cancer [149-155]. Platelets are anucleate membrane-bound cell fragments derived from megakaryocytes, with a discoid shape typically 2–3 μm in diameter and 350–800 nm in height [156].

Platelets contain alpha granules, dense granules, and lysosomes. Alpha granules contain several hundred proteins, including P-selectin,

integrins, growth factors, coagulation factors, and von Willebrand factor (vWF) [157]. Dense granules are generally believed to only contain small molecules that promote coagulation, such as ADP, serotonin, and calcium, but mass spectrometry has revealed at least 40 different proteins in these granules including a handful of cell signaling proteins [158]. Lysosomes are necessary for autophagy and maintenance of normal platelet function [159]. More recent proteomic analysis across all platelet granule types has discovered over 800 proteins, underscoring the diverse functionality of platelets [160]. When activated, platelets release the contents of their granules in a partially selective manner, as different stimuli produce different secretomes [161, 162]. Activation of the coagulation system, which results in the generation of thrombin, also cleaves and activates IL-1a on the platelet surface, promoting an inflammatory response ("thromboinflamation") and inducing emergency megakaryocytopoiesis [80, 163].

As reviewed by Broos et al. [164], upon tissue injury, platelets adhere to the vessel wall and become activated after binding extracellular molecules such as vWF, fibrinogen, and collagen. Activated platelets aggregate, creating a platelet plug. Activation also leads to exposure of phosphatidylserine (PS) at the platelet surface, forming a procoagulant catalytic surface that catalyzes secondary hemostasis and finally the formation of a fibrin clot. The ability to expose PS on the cell surface is reduced in mice with impaired alpha granule exocytosis, resulting in a prolonged bleeding time [165]. Moreover, mice with normal plasma levels but no expression of platelet vWF are not capable of producing occlusive thrombi under arterial-like shear stress [166], further underlining the role of platelet granules in coagulation.

Regular use of platelet inhibitors decreases the risk of different types of cancer [167, 168] and improves cancer-specific survival in colorectal cancer [169, 170]. This anti-cancer effect is believed to be platelet-related rather than drug-related [154]. In addition, the protective effect of platelet inhibition in colorectal cancer is more prominent in tumors

with few tumor-infiltrating lymphocytes, suggesting an immune modulatory effect [171].

A relationship between cancer and venous thromboembolism (VTE) has long been suspected [172] and risk of VTE varies in different types of cancer [173]. Elevated platelet count is also generally indicative of a poor cancer prognosis [174-176], as well as a risk factor for VTE [173]. The mechanism for the increased risk of thrombosis in cancer is surely multifactorial, but can be partially explained by the ability of cancer cells to activate platelets directly, through secretion of soluble mediators such as ADP and HMGB1, and indirectly, via increased thrombin generation [172, 177].

HMGB1 is released by cancer cells and activates platelets via TLR4 [178]. Ticagrelor, an inhibitor of ADP receptors P2Y12 and P2Y1, substantially reduced tumor growth in a murine ovarian cancer model, even when compared to aspirin [179]. Cancers formed by different cell types likely generate thrombin via different mechanisms, with varying dependency on Tissue factor [180]. In addition to being a potent platelet activator, thrombin also cleaves the GARP receptor on the platelet surface [181]. Thus, it utilizes additional platelet functions rather than mere activation by releasing latent TGF-β from the GARP-TGF-β complex. Cancer cells can also activate platelets directly via binding of receptor/ligands like clec2/podoplanin, P-selectin/P-selectin glycoprotein ligand-1, GpVI/galectin-3, and ligands for platelet FcγRIIa [182-185].

In addition to coagulation, platelet activation also mediates functional advantages for cancer cells. Platelets interact with circulating tumor cells (CTCs) in breast cancer patients in clusters or aggregates, where they are believed to facilitate the epithelial-to-mesenchymal transition (EMT) through secretion of TGF-β1 [183, 186]. In ovarian cancer cell lines, inhibition of the TGF-β1 receptor reversed the effect of platelets on EMT, reducing metastasis potential [187]. Cancer cells can evade NK

cytotoxicity through these cancer-platelet aggregates or coating of platelets, resulting in pseudotransfer of platelet MHC class 1 molecules, disrupting recognition by NK cells and directly impairing their cytotoxic function [188-190]. Releasate from activated platelets reduces NK cell cytotoxicity against tumor cells by downregulating expression of the natural killer group 2, member D gene (NKG2D) and the effect is abolished when neutralizing TGF-β1, which underlines the central role of this particular cytokine in cancer biology and cancer-platelet interaction [189]. Co-incubation of NK cells with platelet ectosomes also downregulates activation receptors and NK cell function, possibly through transfer of TGF-β1 [191]. Furthermore, the surface-bound GARP-TGF-β complex on platelets is known to be a key mediator of platelet-associated T cell suppression in cancer models [192].

Platelets secrete VEGF upon activation with ADP, and this release increases migration and capillary tube formation in endothelial cells [193]. The same study found that the cancer cell line MCF-7 could stimulate endothelial cells to release VEGF in a transwell culture system. In addition, endothelial-like cells have been successfully produced from $CD34^+$ cord blood cells using VEGF and FGF2 [194, 195]. PDGF stimulates fibroblasts to produce an extracellular matrix that increases the attachment and activation of endothelial cells [196]. These and other angiogenesis regulatory proteins are present at high levels in platelets [197], indicating the importance of platelets in angiogenesis, a major feature of cancer development and progression. The role of platelets in cancer is illustrated in Figure 9.

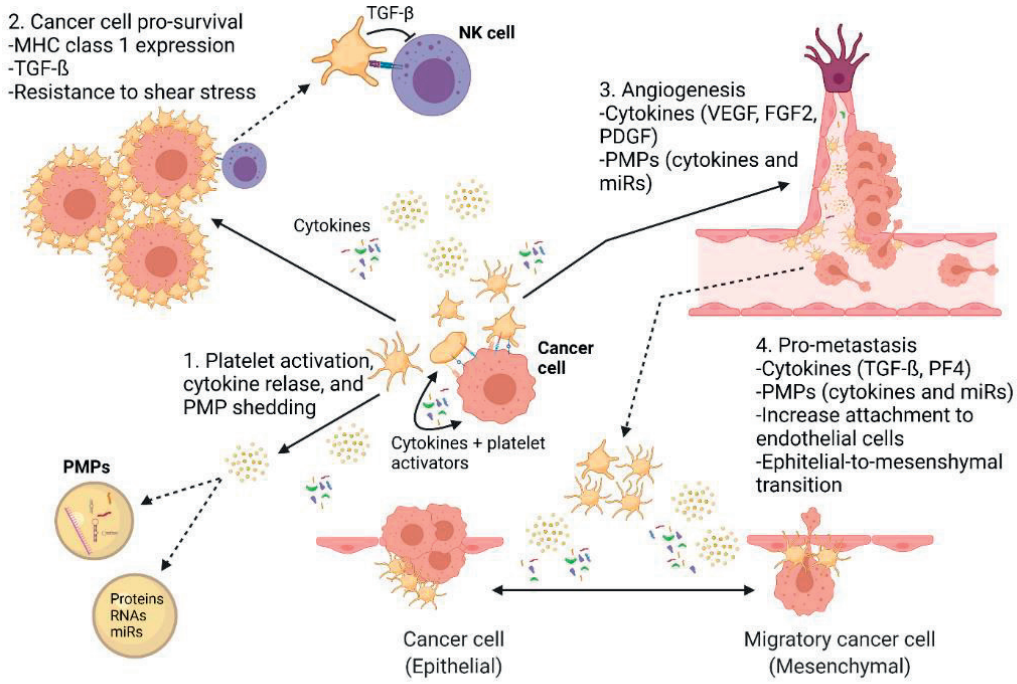

Figure 9. Effects of platelets in cancer. Cancer cells activate platelets, which protects them from immune cells and supports angiogenesis by releasing soluble factors and platelet microparticles. Platelets also facilitate metastasis by inducing epithelial-to-mesenchymal transition [198]. Created with BioRender.com.

1.2.2 *Growth factors, microparticles and microRNA*

In addition to containing numerous proteins, platelets also contain regulatory non-coding microRNAs [199, 200], and long RNA sequences, such as ribosomal RNAs and protein-coding transcripts inherited from parental megakaryocytes [201, 202]. Long RNA sequences are prone to time-dependent decay [201, 203], and correlation with the proteome is weak [202]. Thus, protein synthesis may be restricted to young or reticulated platelets [201]. The alpha granules of platelets contain several hundred different proteins that are secreted with some degree of selectively, meaning that release of the granule proteome is not simply

"on or off." Granule proteins include growth factors known to directly affect hematopoietic progenitor cells and cancer cells such as PDGF, FGF2, VEGF, and TGF-ß, which is further elaborated in Table 2. Platelets can also impact cancer progression through the transfer of proteins and microRNAs in platelet microparticles or PMPs [138-141], platelet-derived membranous vesicles with a diameter less than 1000 nm [204]. PMPs are budded off from platelets during platelet activation or apoptosis [205-207] and their formation, at least in activation, is dependent on signaling from the GTPase Rac1 [208, 209]. PMPs vary in size, composition, and function, but more than 80 percent are between 50 and 500 nm when the more strictly defined exosomes are included [205-207]. PMPs are the most abundant microparticles in plasma, with substantial contributions from other blood cells and endothelial cells [210-212]. Different activation stimuli result in quantitative and qualitative differences in PMP composition, suggesting that formation and packing involve active rather than stochastic processes [213]. PMPs were originally thought to promote coagulation, as they are 50–100 times more procoagulant than activated platelets [214]. However, recent evidence utilizing more sensitive methods has questioned earlier findings and suggests they may in fact support fibrinolysis [212]. Thus, the role of PMPs in coagulation seems to be bimodal, as reviewed by Puhm et. al [204].

MicroRNAs are small, approximately 22 bp, non-coding RNAs that regulate mRNA through degradation or blockage of translation [215]. Platelets can, to a limited extent, process early transcripts of microRNAs (pri- and pre-microRNAs) [216, 217]. The transfer of microRNAs from platelets has been shown to affect many different cell types. Internalization of PMPs regulates gene and protein levels and promotes angiogenesis in human umbilical vein endothelial cells (HUVECs) through an increase in the microRNA Let-7a [136, 137]. Transfer of miR-223 to vascular smooth muscle cells results in reduced proliferation through regulation of IGF-1R, and inhibition of miR-223 increases

atherosclerosis in mice [218]. PMPs are bound and internalized by monocytes, where they can have an anti-apoptotic effect, and binding is greatly diminished by an antibody against P-selectin [207]. In addition, specific microRNAs found in PMPs can alter functions crucial to cancer development and progression in a variety of cancer cells, including invasiveness, proliferation, and viability [138, 139, 141].

Patients with AML can be stratified into prognostic groups based on genetic and chromosomal aberrances [219], which will be elaborated later. A similar model has been developed in pediatric AML for the expression of specific microRNAs [220], underlining their importance in the biology of the disease. Several platelet-associated microRNAs (according to [216]) are known to be significant in leukemia [221-229]. Some microRNAs, including miR-125a and miR-125b, known to be present in platelets [230], are even associated with chemotherapy resistance in AML [231-233].

Table 2. Important growth factors for normal and leukemic progenitors. Several proteins found in alpha granules are known to have both *in vitro* and clinical relevance in human hematopoietic stem and progenitor cells (HSCs/HPCs) and AML.

Cytokine	HSC/HPC	AML
PDGF	Increased total cell count, total CD34 count, total $CD41^+CD61^+$ count, total CFU MK count, total CFU count, and LTC ICs [234-237]. Decreased relative frequency of $CD41^+$ cells and CFU MKs, when added to general mix of cytokines [238].	Dose-dependent and divergent effects on proliferation in patient samples [239]. Increase in proliferation in 17/60 samples [240]. Mutations in PDGFRA/B were associated with myeloid diseases with eosinophilia, including AML [241].
FGF2	Systems containing VEGF and FGF2 have both successfully produced endothelial progenitors from cord blood progenitor cells [194, 195]. Conflicting results on proliferation of $CD34^+$ cells in culture [242-244].	FGF2 derived from stromal cell microparticles protected against toxicity from tyrosine kinase inhibitors [245]. Increased growth and migration in AML and protected against AraC-induced apoptosis [246, 247].
VEGF	Accelerated SDF-1-mediated endothelial transmigration [248]. Systems containing VEGF and FGF2 have successfully produced endothelial progenitors from cord blood progenitor cells [194, 195].	VEGFA mRNA was increased in AML $CD34^+$ cells, VEGFA overexpression increased viability of KG1 cell line and $CD34^+$ cells [249]. Overexpression was associated with poor prognosis [250].

TGF-β	Increased expression of CXCR4 [251]. Decreased mRNAs of cell cycle cyclins and increased SMAD7 levels [252]. Decreased proliferation dose-dependently, decreased relative frequency of cells in S phase, and increased frequency of $CD34^+$ cells in culture [253]. Anti-TGF-β increased relative frequency of S phase only in c-Kit-high, not c-Kit-low $CD34^+CD38^-$ [254].	Suppressed proliferation of AML cells [240]. Neutralization of TGF-β enhanced proliferation and cell cycle progression in an AML/MSC co-culture system [255]. Increased resistance to PARP inhibitors by induction of double-stand DNA break repair mechanisms [256].
IL1β	Increased short-term growth but suppressed clonogenic potential in $CD34^+$ cells have been observed [240]. Supported development of CD14 $cells^+$, $CD15^+$ cells and supported NK cell maturation [257].	Induced cell cycle progression, induced apoptosis, and potentiated AraC sensitivity in $CD34^+CD38^-$ AML cells [258]. Increased cell growth 15-fold in 2/3 of AML samples [240].

1.3 Acute myelogenous leukemia

1.3.1 *Development of AML*

Acute myelogenous leukemia (AML) is a hematological disease with a homogenous clinical presentation, usually limited to bone marrow failure, but with heterogeneous biological phenotypes, as evident in the original FAB classification of 1976 [259]. Risk of AML increases with

age and certain germline mutations, but other than exposure to exogenous factors such as radiation, chemotherapy, and benzene, there are few known risk factors [260-263]. The genomics of AML have been thoroughly examined, and the first paper with AML whole-genome sequencing was published in 2008 [264]. Since then, genomic data has been further utilized to intricately adjust treatment of the disease based on the presence of specific somatic mutations in cancer cells and their associated prognosis and risk of relapse [265]. Although the exact genesis of the disease is undoubtedly multifactorial and variable depending on subtype, the comprehensive use of sequencing technology and xenograft models has aided researchers in understanding the development of AML.

Hematopoietic stem cells are quiescent and long-lived cells that are tightly regulated to protect them from DNA damage. Non-coding and coding mutations, however, accumulate throughout life, though most are not drivers of leukemogenesis [266]. In some cases, these mutations may slightly alter normal hematopoietic stem cells, resulting in a selective growth advantage for a particular cell or "clone" without altering its phenotype to a noticeable degree. This is not a malignant disease *per se*, rather a normal progression of age and has been termed "clonal hematopoiesis" [267].

Clonal hematopoiesis is often the basis of AML development, at least in the elderly. Whole-exome sequencing of blood cells from healthy individuals reveals the incidence of somatic mutations associated with hematological cancer increases significantly with age, from 5.6% in persons 60 to 69 years of age to 18.4% in persons 90 years of age or older [268]. The most prevalent mutations are found in genes for the epigenetic modulators *DNMT3A*, *TET2*, and *ASXL1*, a finding that has also been corroborated by a different research group [266]. Using an error-corrected NGS technique with a detection limit of a variant allele frequency (VAF) of 0.03%, somatic mutations in *DNMT3A* and *TET2* were found in 95% (19/20) of 50 to 60-year-old healthy controls [269].

Clonal hematopoiesis is associated with an increased risk of hematological malignancies (approximately 1 % per year [268]); hence, the term "preleukemic state" has been coined. Data from the US Women's Health Initiative show that having a preleukemic mutation increases risk of AML with an odds ratio of 4.86 (CI95% 3.07–7.77) [270].

Consistent with the cancer stem cell theory [271], AML has a hierarchical structure similar to normal hematopoiesis, with quiescent, long-term, and short-term stem cells [272]. AML was initially believed to originate from primitive hematopoietic cells (identifiable today as MPPs or HSCs) because only $CD34^{+}CD38^{-}$ cells were able to initiate AML in pioneering studies [273, 274]. More recent research, however, has revealed that other cell types can also initiate leukemia in more immunocompromised versions of the SCID mouse strain [275]. These cells were initially named "SCID Leukemia Initiating cells" (SL-ICs), but are now referred to as leukemia-initiating cells (LICs) or leukemia/leukemic stem cells (LSCs). LSCs can be distinguished from pre-LSCs and normal HSCs using single-cell transcriptomics combined with nuclear and mitochondrial somatic gene mutational analysis [276]. Even though AML is generally associated with blocked differentiation in hematopoiesis, AML cells from single patients can express transcription programs associated with up to six different cell types from stem cells to more mature myeloid cells [277]. This discovery further supports the need to identify and adapt treatment for a common stem or progenitor cell.

Consequently, the clinical role of LSCs is starting to reveal itself as an increased expression of HSC-associated genes in AML is linked to worse outcomes [277, 278]. LSCs rely more on oxidative phosphorylation than their HSC counterparts [279]. Fatty acid oxidation (FAO) in LSCs is regulated by the fatty acid transporter CD36, though its expression is heterogeneous among LSCs, as is their metabolic state [280]. In addition

to LSC homeostasis, upregulation of FAO is also an important mechanism for drug resistance [280, 281], which will be elaborated later.

Leukemogenesis drives cells to a state more similar to HSCs, which can be replicated with viral transduction of single fusion proteins. The introduction of the MLL-AF9 fusion protein into committed progenitor cells yields LICs with a functional identity close to committed progenitor cells, but with the self-renewal capacity and gene expression profile of leukemic stem cells [282], suggesting that AML could arise in more mature progenitor cells with only a single genetic hit. The malignant transformation of progenitor cells through retroviral transduction of oncogenic transcription factors depends on the epigenetic reprogramming actions of the methyltransferase PRMT1 and the demethylase KDM4C, at least for certain transcription factors [283].

Many real-life observations support the importance of DNA methylation and epigenetics in AML development and kinetics. Early work identified 16 regions that are generally hypermethylated in AML compared to healthy controls [284]. Though enhancers tend to be aberrantly hypomethylated, i.e., they do not overlap with natural hypomethylation during granulopoiesis, an effect that is only partially explained by mutations in epigenetic regulators [55, 285]. Results from bisulfite sequencing confirm that enhancer regions are hypomethylated, but show that promoter regions are hypermethylated in AML cells as compared to $CD34^+$ control cells, though with great variability depending on the presence of individual or multiple somatic mutations [285]. Similar observations have been made for histone modifying proteins, where expression levels diverge depending on mutational status [286].

In general, AML has a low median mutation frequency compared with other cancers [287, 288] and, as a result, fewer different coding mutations per case (~13; defined as protein influencing SNVs and indels) [289, 290]. However, one can usually identify at least one driver mutation [289, 291]. Despite the low mutational burden, transcriptomic

and proteomic signatures differ markedly different between AML cells from different subclones from the same patient, and from diagnosis to relapse, even with minimal genetic alterations [292, 293]. When compared with normal hematopoietic stem and progenitor cell controls, *IDH1*/*DNMT3A*-mutant AML cells contained an average of 2,524 differentially expressed genes [285].

AML is prone to clonal evolution, which can lead to treatment-resistant subclones, often in response to the chemotherapy treatment itself [294]. Additional cytogenetic abnormalities at first relapse (as a sign of clonal evolution) are associated with a poorer prognosis [295]. Clonal evolution of AML can be described by two different models: linear (sequential acquisition of mutations) and branching (outgrowth of subclones) [296].

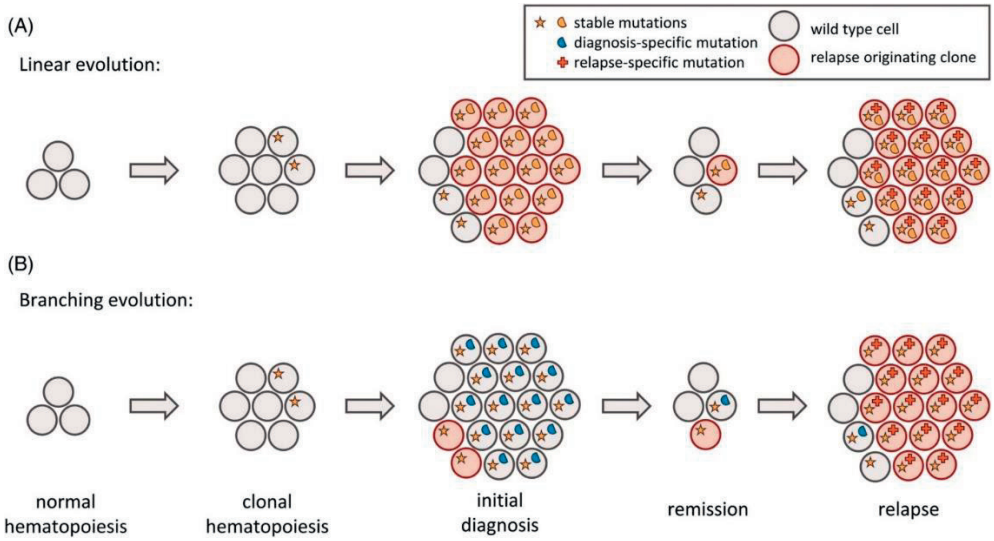

Figure 10. Linear and branching evolution of AML. In linear evolution (A) the relapse stems from the initial dominant clone gaining an additional mutation. In branching evolution (B) the relapse has evolved from a subclone or a pre-leukemic clone acquiring a new mutation. Reprint of Vosberg & Greif [296].

Although cytogenetic clonal heterogeneity assessed by karyotyping at the time of diagnosis does not correlate with poorer prognosis [297], clonal heterogeneity assessed with more sensitive single-cell sequencing

does [298]. Using a limited panel of 18 genes, clonal change (difference of ≥2x in VAF) was discovered in 57% of AML patients between diagnosis and relapse, mostly involving the *FLT3* and *NRAS* genes [299].

Patient-derived xenograft models (PDXs) provide increased sensitivity and reveal a level of clonal heterogeneity and clonal dynamics that conventional diagnostics are unable to discover at the time of diagnosis. This technique has revealed five different patterns of clonal dynamics: monoclonal, stable, loss, expansion, and burst (illustrated in Figure 11) [300]. Screening for 111 recurrent AML mutations with single-cell qPCR showed that the number of subclones at diagnosis ranged from one to six, and three out of seven where branching subclones [301]. In patients who had lost the *NPM1* mutation at relapse (9% of cases), all presumably preleukemic mutations persisted, and all presumably leukemia-driving mutations were lost [293]. However, in patients where the *NPM1* mutation persisted at relapse, at least three common leukemia driving mutations were found, and the preleukemic mutations persisted in only 6/10 patients. These results indicate that when the *NPM1* mutated clone is lost, AML relapse stems from a preleukemic clone (branching evolution), but when the *NPM1* mutation persists, AML relapse often stems from a surviving and evolving clone (linear evolution). Therefore, clearance of AML mutations after treatment, thus also clearing the preleukemic clone, probably leads to better relapse-free survival and overall survival [291].

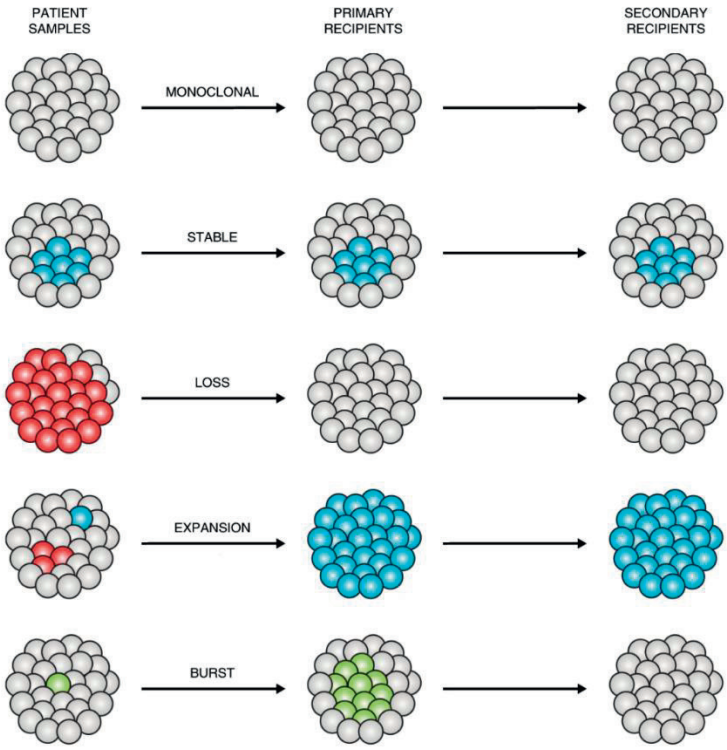

Figure 11. Clonal dynamics in AML. According to a patient derived xenograft model there are five patterns of clonal dynamics in AML: monoclonal (unaltered monoclonal composition in patient and xenografts), stable (unaltered clonal composition in patients and xenografts including subclones), loss (loss of major clone in xenografts), expansion (xenografts originate from subclones in patients), and burst (subclones from patients expand in primary xenograft but disappear in secondary recipient). This technique can reveal small subclones that are undetectable at diagnosis. Modified from Sandén et al. [300].

1.3.2 *AML and the microenvironment*

Bone marrow stromal cells show epigenetic and transcriptional discrepancies compared to healthy controls [302]. The effect of AML on the bone marrow microenvironment is even more evident in proteomic analysis, where 168 proteins are significantly differentially expressed compared to bone marrow plasma from healthy donors, including aberrant cytokine and chemokine networks [303]. In murine models, AML cells can, through exosome secretion, at least partially alter cellular

composition in the bone marrow, reducing their ability to sustain normal hematopoiesis [304]. Microparticles released by AML cells can transfer endoplasmic reticulum stress and induce an unfolded protein response (UPR) in bone marrow stromal cells, altering the fate of mesenchymal stem cells and osteoblastic progenitor cells [305]. Intravital microscopy of AML-infiltrated murine bone marrow revealed remodeling of the endosteal vascular niche, leading to depletion of HSCs and osteoblasts and decreased chemosensitivity, which can be reversed by inhibiting vascular remodeling [306]. Lymphocyte levels in AML patients are low due to blast proliferation in the bone marrow. In addition, remaining lymphocytes express less cytolytic markers and levels of anti-inflammatory immune cells are elevated, which blunts the immune response against malignant cells [307]. T cell function is suppressed by a $CD14^+$ monocyte-like fraction in AML [277] and stromal cells protect AML cells from $CD3^+$ T cells through interaction with the VLA4 antigen [308]. MSCs from AML have increased expression of cytokine mRNA and decreased *in vitro* T cell suppressive capabilities compared to healthy controls further underlining the dysregulated microenvironment in AML [309]. In addition, high expression of eight different microenvironment-related genes (*CD163*, *CYP27A1*, *KCNA5*, *PPM1J*, *FOLR1*, *IL1R2*, *MYOF*, and *VSIG2*) correlates with poorer prognosis [310]. Adipose tissue is a surprising reservoir for AML cells and LSCs, and the LSCs have a distinct transcriptome skewed towards pro-inflammatory cytokines and increased drug resistance [280].

It is clearly evident that AML cells and LSCs transform the bone marrow microenvironment with their altered immunomodulatory properties or through direct cellular communication. These characteristics have implications for the development of therapy resistance and thus prognosis, which will be elaborated later.

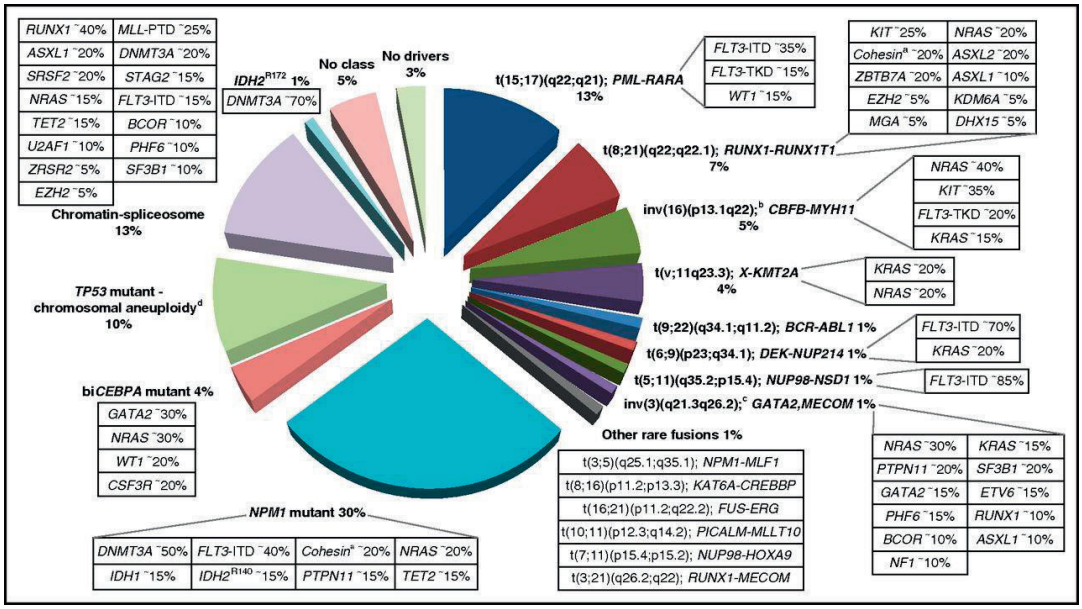

Figure 12. Recurrent chromosomal and genetic lesions in AML. The diagram illustrates the most common genetic aberrations in AML with frequent co-occuring mutations denoted in the boxes. Reprint of Döhner et al. [219].

1.3.3 *Diagnosis and treatment of AML*

The diagnostic approach to AML can be summarized by guidelines from the European Leukemia Network (ELN) [219]. With few exceptions, diagnosis requires a blast count of over 20% in a bone marrow smear. In addition, immunophenotyping, cytogenetic analysis, and molecular genetic testing are performed in order to categorize the disease further. Based on this extended evaluation, AML is classified according to the WHO classification [311]. Disease response has traditionally been monitored with bone marrow smears, but assessment of minimal residual disease (MRD) with multiparameter flow cytometry and/or PCR-based techniques or NGS is increasingly being implemented in clinical practice [312]. Classification of the disease and risk stratification are summarized in Table 3.

First line treatment of AML combines anthracyclines (daunorubicin, doxorubicin, idarubicin, or mitoxantrone) and AraC (cytarabine) in one or two induction treatments, followed by several intermediate-dose AraC (IDAC) or high-dose AraC (HDAC) cycles, and may also include autologous HSCT for consolidation therapy [219, 313-315]. High-dose cytarabine increases relapse-free survival compared to intermediate- or low-dose, but there seems to be no overall survival advantage [316].

Response to treatment is usually swift, and early blast clearance, defined as greater than a 1.50 log10 reduction of leukemic cells by day 4 in induction therapy, is a strong predictor of treatment response and overall survival [317]. Salvage treatment constitutes of intensive chemotherapy for fit patients with fludarabine, mitoxantrone or amsacrine based regimens combined with cytarabine, but allogenic HSCT is necessary for long-term survival because of dismal prognosis [219]. Moreover, recent years has seen a surge of new drugs which potentially could increase number of patients eligible for allogenic HSCT after relapsed or refractory disease [318].

Table 3. Risk stratification based on genetic aberrance from ELN. Reprint of Döhner et al. [219].

Risk category	**Genetic abnormality**
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*

Risk category	Genetic abnormality
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Wild-type *NPM1* and *FLT3*-ITD^{high}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

1.3.4 *Apoptosis in AML*

Targeting apoptosis in cancer is a biologically sound strategy that is currently evolving in clinical use [319]. Apoptosis can be divided into two separate pathways: the death receptor-initiated extrinsic pathway and the mitochondrial or intrinsic pathway. The extrinsic apoptotic pathway is initiated by the membrane-bound death receptors FAS/CD95, TNFR1, TRAIL-R1, TRAIL-R2, DR3, and DR6. Upon binding with their ligands, these receptors form a complex with FADD, c-FLIPP, and procaspase 8 and 10 to form the death-inducing signaling complex (DISC) [320]. In this complex, caspase-8 is activated via dimerization [321]. Caspase-8 activates caspase-3 and caspase-7, where the intrinsic and extrinsic pathways become interlinked, but it can also initiate intrinsic apoptosis through the cleavage of BID on the mitochondrial membrane, forming truncated BID (tBID) [322]. Caspase-3 and its related effector caspase-7 have multiple substrates [323], including the hallmark caspase-3 dependent DNase (CAD), leading to DNA degradation and cell death upon activation [324].

The intrinsic apoptotic pathway is initiated by several intracellular factors, such as DNA damage or cellular stress, and not by receptor-ligand binding. It is regulated by BCL2-family proteins, which includes both anti-apoptotic proteins, such as BCL2, MCL1, and BCL-XL, and pro-apoptotic proteins. Pro-apoptotic BCL2-family proteins are further

divided into two groups: the effector proteins BAX/BAK and BH3-only proteins (BAD, BID, NOXA, HRK, BMF, PUMA, and BIM) [325]. DNA damage or other cellular stresses activate p53, which upregulates the pro-apoptotic BH3-only proteins PUMA and NOXA [326, 327]. These proteins shift the equilibrium between anti- and pro-apoptotic proteins in the intrinsic apoptotic pathway, but PUMA can also directly activate the effector proteins BAK and BAX [325, 328]. Similar activities are seen for BIM and BID, and these BH3-only proteins are consequently labeled "activators" [329]. Upon activation, the mainly mitochondrial outer membrane (MOM)-bound BAK and mainly cytosolic BAX proteins oligomerize in the MOM, causing cytochrome c and SMAC leakage from the mitochondria [330, 331]. This is often erroneously seen as a "point of no return" in apoptosis, as it causes mitochondrial depolarization. However, select cells in several cell lines are resistant to mitochondrial outer membrane permeabilization (MOMP), as shown by Tait et al. [332]. In a minority of the mitochondria, levels of MOM-bound BCL2 are especially elevated, leading to a failure to induce MOMP as activation of BAX and BAK is inhibited. These mitochondria can then repopulate the cell by mitochondrial fission, rescuing the cell from apoptosis as apoptotic signaling downstream of permeabilization fades.

Albeit, the usual development is that generalized MOMP leads to cytochrome c release from the mitochondria, which then forms apoptosomes with APAF1. Apoptosomes activate pro-caspase 9 while mitochondrial SMAC inhibits XIAP, further promoting caspase-9 activation, which then activates caspase-3 [333]. Caspase-3 initiates cell death and contributes to further activation of caspase-8 and caspase-9 in a feedback loop, strengthening the apoptotic drive [334]. A schematic illustration of the extrinsic and intrinsic apoptosis is shown in Figure 13.

Dysregulation of the intrinsic apoptotic pathway is an important step in carcinogenesis. Regulatory and anti-apoptotic proteins in the BCL2-family are upregulated in hematological malignancies [335, 336], which

gain survival benefits by downregulating pro-apoptotic effector proteins and desensitizing mitochondria for BAX/BAK permeabilization. In fact, sensitivity of the intrinsic apoptotic pathway to activation is a good predictor of the clinical outcome of AML treatment, and BAX levels directly correlate with the effect of genotoxic treatment [337]. Thus, targeting the balance of BCL2-family proteins is a proposed strategy to overcome treatment resistance and increase the efficiency of standard AML cytotoxic chemotherapy protocols [338].

In addition to BCL2, MCL1 is also upregulated in AML and has independent prognostic value [335, 336]. Numerous drugs targeting BCL2 and MCL1 are under development or have been approved for the treatment of a variety of hematological malignancies, including AML [319]. The most prominent of these is the BCL2 inhibitor ABT-199, known as venetoclax, which is preferably added in low-intensity regimes, but are also evaluated for high-intensity regimes [339].

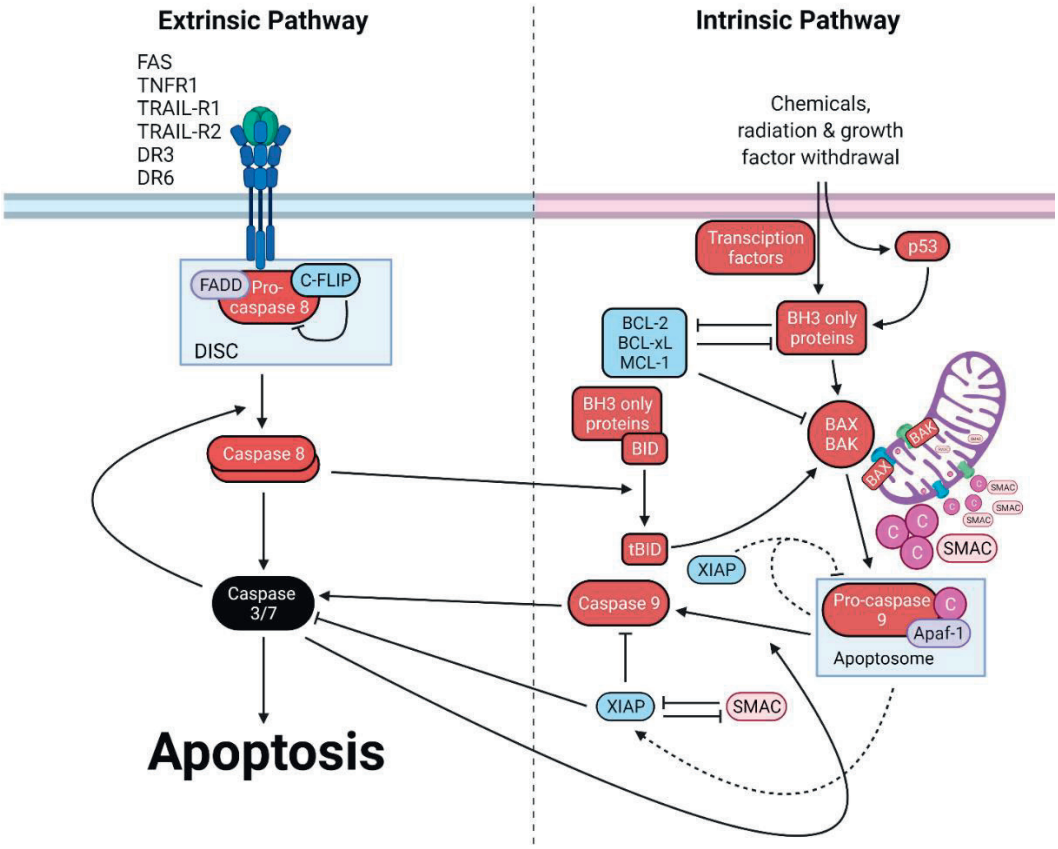

Figure 13. Apoptosis in AML. Apoptosis in AML can be divided in extrinsic and intrinsic apoptosis. Extrinsic apoptosis is initiated by transmembrane receptor-ligand binding, while intrinsic apoptosis is initiated by internal damage like DNA damage or ER stress. The pathways are interlinked at several stages, and ultimately converge in activating caspase-3 and -7, which initiates apoptosis through several substrates [323]. Created with BioRender.com.

1.3.5 *Development of treatment resistance in AML*

Treatment resistance in AML can develop through multiple mechanisms: genetic alterations that change the function of key proteins, overexpression of microRNAs that downregulate expression of pro-apoptotic proteins, protection by the AML microenvironment, and dysregulation of anti-apoptotic systems [340]. However, complete remission is usually achievable in younger patients with intensive

chemotherapy, even when assessed with sensitive techniques such as multi-color flow cytometry or qPCR. This suggests that treatment evasion by the AML population is an extremely rare event during high-intensity treatment.

Some mechanisms of treatment resistance can be dynamically induced by treatment. ABCB1 is a drug efflux pump and a member of the ATP-binding cassette transporter family. Anthracycline resistance induced in the K562 cell line by chemotherapy was due to increased expression of *ABCB1* as a result of an integrated stress response [341]. Contrary to these findings, even though increased ABCB1 activity is associated with worse patient outcomes and correlates with gene expression patterns associated with a poorer prognosis, it does not seem to correlate with *ex vivo* drug resistance in primary AML patient samples [342].

Autophagy, also linked to the integrated stress response, is another cellular mechanism for induced treatment resistance [343]. Autophagy is a cellular recycling process that degrades damaged organelles to increase the availability of nutrients and eliminate toxic waste and is indispensable for cellular survival. Thus, disruptions in normal autophagy are an important feature of drug resistance in LSCs [344]. Furthermore, treatment with chemotherapeutics increases autophagy, and knockdown of the regulating genes subsequently increases chemosensitivity [345, 346].

The unfolded protein response or UPR is a defense mechanism against ER stresses that cause misfolding of proteins [347]. If challenged above its capacity, UPR can also trigger apoptosis [348]. UPR reduces protein synthesis in general, increases synthesis of chaperone proteins, and increases ER size and ER-associated degradation proteins [348]. The transcription factor JUN, which upregulates UPR, is critical for the survival and expansion of several AML cell lines and could potentially induce chemotherapy resistance [349]. However, the importance of the UPR in treatment resistance is disputed. A review by Feral et al. [348]

summarized the effects of UPR in the treatment of leukemic cells and showed that in only 10 out of 91 chemical compounds tested did UPR increase resistance to therapy. In the remaining compounds, UPR caused cytotoxicity through activation of a terminal response. In addition, a meta-analysis of gene expression profiles revealed that *JUN* is generally downregulated in chemoresistant AML [350].

Wnt signaling constitutes several pathways important for cellular development and is linked to the initiation and progression of AML [351]. High expression of the Wnt-mediator β-catenin and the related γ-catenin are associated with a poor AML prognosis [352, 353]. Upregulation of the Wnt/β-catenin pathway in LSCs is associated with increased resistance to inhibitors of bromodomain and extra-terminal motif (BET) proteins [354]. More importantly, treatment with Wnt inhibitors decrease resistance to doxorubicin both *in vitro* in AML cell lines and *in vivo* in xenotransplant models [355].

One of the most convincing features of AML that eventually leads to therapy failure is the presence of leukemic stem cells, which do not necessarily share the same properties of the downstream cells in the AML hierarchy. Similar to HSCs, quiescent and therapy-refractory LSCs reside in hypoxic regions of the bone marrow [356], a milieu that protects cells against chemotherapy [306]. Although LSCs usually contain similar somatic mutations to the downstream cells, the phenotype of the quiescent LSCs is altered due to increased hypomethylation, especially in the regions of the HOXA cluster, an important regulator of dormancy [357]. Paradoxically, a potential weakness of these cells is that they may rely more on oxidative phosphorylation than their HSC counterparts, potentially increasing their susceptibility to apoptosis through BCL2 inhibition and MOMP [279]. However, the quiescent LSCs maintain low levels of ROS by high expression of the antioxidant glutathione peroxidase 3 [358], and can upregulate fatty acid oxidation further to overcome cell death induced by venetoclax/azacitidine [281]. The LSC-

associated mechanisms for evasion of the treatment are summarized in Figure 14.

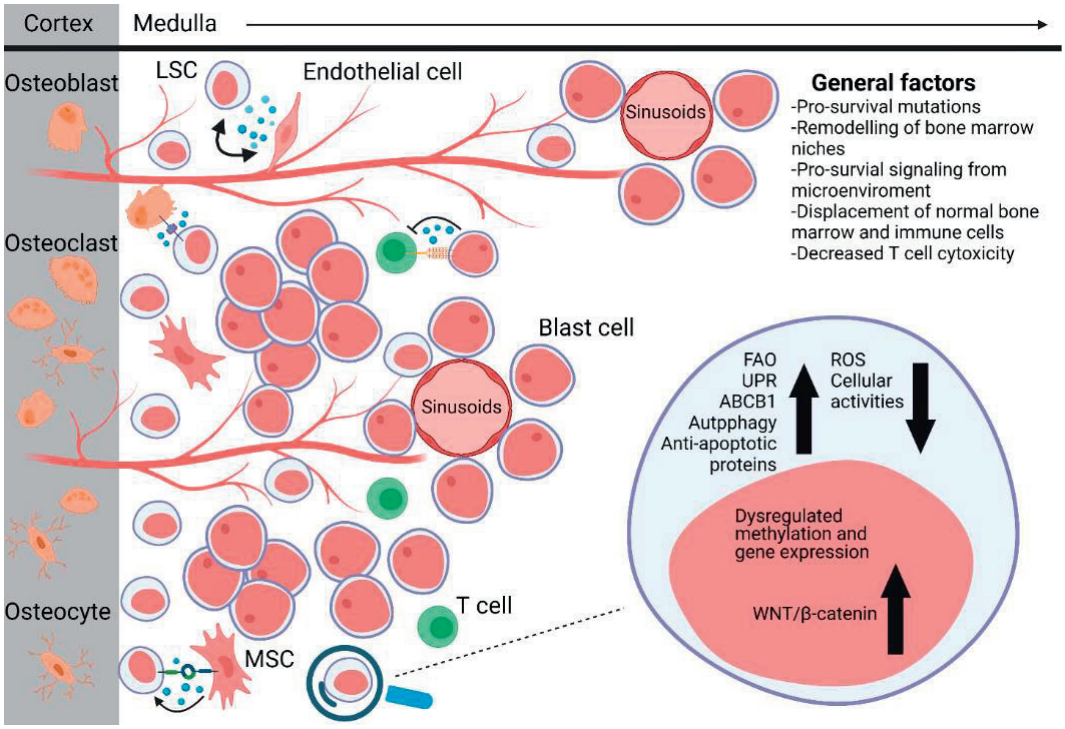

Figure 14. Therapy resistance in leukemia. Leukemic stem cells (LSCs) are important contributors to therapy resistance because of their dormant phenotype, which is regulated by genetic and epigenetic factors and the tumor microenvironment. Furthermore, therapy resistance is increased through internal factors like upregulation of anti-apoptotic compounds and increased fatty acid oxidation and autophagy. In addition to the factors associated with LSCs, several general alterations, such as remodeling of the microenvironment and immune response, can lead to treatment resistance. Created with BioRender.com

1.3.6 *New aspects on risk stratification in AML*

As noted in Table 3, the prognosis of AML is assessed by identifying recurrent genetic abnormalities, after which therapy is adjusted. However, MIR-omics, proteomics, transcriptomics, and maybe even a "multi-omics" combination could provide additional prognostic information. A recent study by Aasebø et al. used liquid chromatography

tandem mass spectrometry to show that 351 proteins were differentially expressed in peripheral blood AML cells at diagnosis between patients with and without relapse after five years of follow-up [359]. Proteins that were more abundant in relapse-free patients included those associated with ATPase activity, proton-exporting ATPase activity, phagosome acidification, and iron ion transport, whereas proteins involved in ribosomal processes had higher levels in patients with relapse. In addition, Hu et al. stratified AML into 154 different functional proteomic patterns using reverse phase protein lysate microarrays, yielding an independent prognostic value [360].

Lim et al. developed a model using the combined expression of 36 microRNAs to categorize pediatric AML into low, intermediate, and high risk of relapse [220]. In addition, Gao et al. discovered that upregulation of nine microRNAs, with limited overlap with the 36 microRNA model, was independently associated with poorer prognosis in adult AML [361].

Li et al. published a model based on the expression of 24 genes that provided additional prognostic value to the common ELN risk stratification [362]. A similar approach using a penalized Cox regression model on whole transcriptomic profiling by RNA-sequencing of 1,946 genes produced superior AUC for prognosis prediction compared to Li's model and the standard ELN model (0.73 vs 0.65) [363]. A transcription signature based on *GUSB*, *ALDH3B1*, *AMOT*, and *RAB32* has been developed to assess the prognosis of refractory AML [364]. AML with a positive signature had a median survival of 10 months, while a negative signature resulted in a median survival of 33 months.

CircularRNA is a recently discovered type of regulatory RNA. Papaioannou found that expression of specific circRNAs correlates well with distinct expression signatures related to the regulation of chromatin state and transcription, transcription factors, and signal transduction

mediators implicated in leukocyte activation and differentiation [365]. There was also an independent correlation with overall survival.

A methylation-based predictor model derived from MALDI-TOF mass spectrometry-based promoter methylation analysis was significantly associated with survival in patients with AML [284]. Similar findings have been obtained using whole-genome bisulfite sequencing [366]. High DNA methylation at polycomb targets correlated well with better prognosis, though this measure did not provide independent values as significance was lost when data were adjusted for age, WBC, and selective mutational analysis [367]. In contrast, genome-wide CpG methylation profiles at CGI, CGI shore, CGI shelf, and opensea gene contexts offered independent prognostic value [368]; however, the model only included *FLT3* mutational status, age, and sex in addition to the methylation profile. Methylation of CAGE-defined enhancers showed that hypo/hypermethylation correlated well with survival in AML in an enhancer specific matter, but it was not complemented with a multivariate analysis [55].

Thus, several biological characteristics of AML can provide independent prognostic value to aid clinicians in therapy choice and to aid researchers in therapy development. However, implementation will always depend on cost. The implementation of whole-genome sequencing, no longer a futuristic notion, may be the first step toward a more advanced diagnosis, improving outcome of treatment by identifying high-risk disease [369].

2 Aims and objectives

The main objective of this project is to characterize the interactions between platelets and hematopoietic cells that are central to hematopoiesis and hematologic diseases.

More specifically the project seeks to:

- Investigate the effects of platelet releasate on hematopoietic stem and progenitor cell function, differentiation, and proliferation.
- Investigate the effects of platelet microparticles on acute myelogenous leukemia cell activity and chemosensitivity.

3 Methodological considerations

3.1 Ethical considerations

All studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the regional ethics committee, *Regional Etisk Komite Vest* (reference number for Paper I: 2017/634, reference number for Paper II and III: 8144). The biobanks from which the biological materials were derived have the following reference numbers: Paper I: 2011/996. Paper II: REK III 060.02, REK III 059.02, REK Vest 2015/1759, REK Vest 2015.03, and REK Vest 2031.06.

3.2 Patient samples

3.2.1 Hematopoietic progenitor cells

$CD34^+$ cells were isolated from G-CSF stimulated leukapheresis harvest products of healthy donors for planned allogeneic HSCT after informed consent at the Department of Immunology and Transfusion Medicine, Haukeland University Hospital (Bergen, Norway). Donors received non-glycosylated G-CSF 10 g/kg/d for 4 days prior to harvesting. Apheresis was performed when the CD34 count exceeded 15–20 × 10^3/mL, as described by Melve et al. [370]. The samples were kind gifts from Doctor Guro Melve at Haukeland University Hospital and sent as cryopreserved pilot vials (1–5 ml).

3.2.2 AML cells

Primary AML cells were isolated by density gradient separation of peripheral blood from consenting patients at the Department of Medicine, Section of Hematology, Haukeland University Hospital (Bergen, Norway). The relative frequency of leukemic blasts to nucleated cells was usually greater than 80% to minimize the

contribution from other cell types. The cells were cryopreserved in liquid nitrogen until further use. All but one patient with polycythemia vera had no previous history of myeloid disease. See Paper II for further characteristics of patient samples. This material was a kind gift from Professor Håkon Reikvam at the University of Bergen (Bergen, Norway) and Haukeland University Hospital and was received as cryopreserved PBMCs.

3.2.3 Platelets

Platelet concentrates, obtained from consenting blood donors, were kindly donated by the Department of Immunology and Transfusion Medicine, Stavanger University Hospital (Stavanger, Norway). Platelet concentrates were produced using the automated TACSI® system (as routine at the department) and always composed of platelets from four donors. The plasma content was 35% and the remaining 65% was additive solution (PAS-III). Platelet count was generally greater than 0.90×10^9 /ml, though one concentrate with a platelet count of 0.88×10^9 was also included because the most important feature of the concentrates used in these experiments was the ability to produce a sufficient number of PMPs.

3.3 Isolation/separation of hematopoietic stem and progenitor cells

$CD34^+$ cells were isolated from the thawed leukapheresis harvest products using positive selection with immunomagnetic beads. Briefly, pilot tubes were thawed in a water bath, aliquoted in two 50 mL tubes and washed with StemSpan containing 10 mg/mL BSA and 20 µg/mL DNase. Cells were then isolated using the "UltraPure" variant of the $CD34^+$ microbead kit from Miltenyi Biotec (Bergisch Gladbach, Germany) following the manufacturer's instructions, although DNase was added at each step until final magnetic separation to avoid cell clumping. During protocol development, magnetically labeled cells were

first separated over one MACS column. However, this yielded an insufficient purity (approximately 50%). When separating over an additional column, the purity generally increased to greater than 90%.

Cryopreservation affects the purity, recovery, and viability. Viability of cultured cells was assessed by light scatter rather than with a specific assay. Although assessing viability with light scatter has its limitations compared to more advanced fluorescence based viability assays, it provides a good indication of the frequency of dead cells [371]. More sophisticated viability assays also have significant limitations and will always be outperformed by investigating *de facto* proliferation rates.

All experiments conducted in this study had sensible proliferation rates, therefore none were excluded other than the initial test runs to develop methods and determine expected cell growth.

3.4 Cell cultivation

3.4.1 HSCs

Hematopoietic stem cells were cultured in StemSpan SFEM medium (StemCell Technologies, Vancouver, BC, Canada) with the addition of stem cell factor (50 ng/mL), thrombopoietin (10 ng/mL), FLT3L (50 ng/mL), and IL3 (20 ng/mL). This mix has previously yielded good proliferation rates in our lab from $CD34^+$ cord blood cells (unpublished data and not included in this project). In addition, some versions of this mix are commonly used when expanding $CD34^+$ hematopoietic progenitor cells, though numerous combinations of growth factors can be used for this purpose [372]. In this project, the aspiration was to use commercially available $CD34^+$ cells. Initial experiments using cells from two renowned distributors, however, generated almost no viable cells after one and two weeks of cultivation. Thus, one could question the quality of the cells. This issue was eventually overcome by using donor cells from allogeneic donors for hematopoietic stem cell treatments as

described earlier. In a pilot experiment, expected proliferation rates were achieved with no indication of overcrowding of the cell cultures (data not shown). In addition, it was tested if co-culture with HUVECs increased proliferation extensively, which would indicate insufficient growth stimulus from the culture medium. However, co-culture only increased proliferation rates by approximately 50%–60% (Appendix A). Considering that cryopreserved and not fresh hematopoietic progenitor cells were used, the culture system yielded efficient proliferation rates compared to previously published work, further supported by the relatively low gain of proliferation in HUVEC co-cultures [56, 58].

3.4.2 AML cells

THP-1 cells (ATCC no. TIB-202) were cultured under standard conditions with Iscove's Modified Dulbecco's Medium (IMDM) + 10% FBS. The doubling time was approximately 48 h, and batches were never cultured for more than three months.

Primary AML cells were cultured under serum-free conditions with StemSpan SFEM medium with the addition of stem cell factor, G-CSF, and FLT3L, all at 20 ng/mL. Serum-free conditions allow for better reproducibility as it avoids the batch-to-batch variability often seen with FBS, and is a standard procedure in the research group of Håkon Reikvam, who kindly donated these cells.

3.5 Platelet releasate and isolation of microparticles

Platelet releasate was produced by adding human thrombin at a final concentration of 1 U/mL to the platelet concentrates and incubating them for one hour in a 37 °C water bath.

Microparticles were isolated as described in Paper II and III. Briefly, the releasate was thawed, centrifuged at 15,000 × g for 90 min, and the

supernatant was discarded. Because it was not possible to discard the supernatant completely due to its viscosity, manual pipetting was used to measure the volume of remnants. This was approximately 40 µL when using a 50 mL tube, the only tube size that fit available adapters for the centrifuge. The PMP-containing pellet and remnant releasate supernatant were diluted with culture medium and transferred to cell suspension in culture plates. Given the volume of supernatant carryover, the final platelet releasate content in the cell culture medium could be up to about 2%, with some variation. The effect of the centrifuged releasate supernatant (reduced in PMPs) at a 5% concentration (a rough overestimation of the upper limit) was investigated in Paper II. To avoid any carryover of releasate, platelets could be washed before activation, though platelets and PMPs are lost in the process of washing. Washing was performed during the development of the protocol, but unwashed concentrates were preferred because more platelets were retained and PMPs are spontaneously formed in the concentrates, which could reduce the number of concentrates needed to approximately half. Since platelet concentrates were kind gifts from the Department of Immunology and Transfusion Medicine at Stavanger University Hospital, the less resource-demanding approach was chosen. Data using washed platelet concentrates, albeit with lower PMP concentrations and a 16 h co-incubation before analysis or daunorubicin treatment was also obtained (Appendix B). Co-incubation times of 24 h were later chosen to simplify scheduling. These data coincide with the findings of co-culture of THP-1 cells with PMPs produced from unwashed platelet concentrates (as presented in Paper II). The PMP effects will be further elaborated in "Results" and "Discussion". However, there was little difference between the observed effects of using unwashed or washed platelet concentrates for production of PMPs; thus, the validity of the results, regardless of the potential minor contamination of platelet releasate are strengthened.

The aim of this PhD project was to investigate the interactions between platelets and hematopoietic cells. To examine this, platelet releasate was used as a culture supplement with hematopoietic progenitor cells and PMPs for co-culture with leukemic cells. Platelet releasate was chosen for hematopoietic progenitors because, at the time of initiation of the project, little evidence supported that hematopoietic progenitor cells could internalize PMPs, and surprisingly, some evidence to the contrary [134]. That platelet releasate, however, could affect the differentiation of $CD133^+$ hematopoietic progenitor cells had been proven but not extensively investigated [373]. However, the platelet secretome represents both humoral and paracrine factors, making it difficult to assess the relevant concentration of platelet releasate in cell cultures. On the other hand, *in vitro* studies with PMPs may be easier to translate into clinical relevance, as their effects should be less dependent on short transfer between close contact cells because of their abundance in plasma [212]. Thus, it is easier to construct models closer to real-life situations by mimicking the quantity of microparticles. However, the lack of standardization in microparticle quantitation makes comparison with earlier work difficult. Strategies for standardization have been proposed, and flow cytometric protocols show little inter-apparatus variability [374], however, there also needs to be an implementation of consensus, which for now is lacking.

3.6 Proliferation assay

Proliferation was assessed either through direct cell counting with flow cytometric beads or indirectly through assessment of the dilution of intracellular bound dyes (Cell Trace™; Thermo Fisher Scientific, Waltham, MA, USA). Assessment with count beads requires very little volume, often 25–50 µL depending on cell concentration, to obtain a representative sample. Thus, cultures can be analyzed with further assays, if desired. However, as the technique measures cell concentration, it is influenced by culture medium evaporation, which can

vary from well to well in cell culture plates and thus decrease the sensitivity of the method [375]. This effect was minimized by mirroring the plate.

Cell Trace™ is a brand of intracellular dyes that binds covalently to amines and are distributed evenly from mother to daughter cells. Thus, it is possible to track generations as the fluorescence intensity is halved by every cell division [376]. According to the manufacturer, they are associated with little cytotoxicity, which holds true for low concentrations in freshly isolated cells [377]. However, when used with cryopreserved cells, some cytotoxicity was noticed, indicating the need to slightly modify the manufacturer's protocol before implementing it in the final experiments as described in Paper I. In cell lines, Cell Trace™ dyes often fail to produce distinct peaks for generation distribution estimation as observed in Paper II, possibly due to synchronized cell cycles, but can still produce doubling time estimates that match those obtained by manual or automatic cell counting [376].

3.7 Flow cytometry

Flow cytometry was utilized for several analyses in experiments across all the papers. This technology is versatile and allows for highly sensitive readouts of data to a single cell level, with multiplexing capability. Briefly, the sample is stained with fluorochrome-labeled antibodies (or other compounds) and then spread out like beads on a string by a fluidic system followed. Fluorochromes are excited with lasers and emission from each fluorochrome is transferred to an electric current in specific detectors [378]. All analyses were performed on a CytoFlex flow cytometer (Beckman Coulter; Brea, CA, USA) with blue (488 nm) and red (638 nm) lasers and the following detectors: FITC, PE, PerCP Cy 5.5, and APC.

Most of the analyses are comprised of commercialized kits from renowned suppliers, and will not be elaborated, and I refer to the individual papers.

3.7.1 Immunophenotyping

Immunophenotyping, which identifies cell types by expression of certain antigens, was performed as a standardized procedure. Samples were washed, resuspended in a small volume of binding buffer containing 0.5% BSA, and then incubated at the time and temperature recommended by the manufacturer. As recommended by Miltenyi Biotec when using their REAffinity™ line of antibodies, which compromised most of the antibodies used, blocking reagents for surface antigen staining to avoid non-specific FCR binding of the monoclonal antibodies was not used. For background signal subtraction, unstained samples (direct labeling) or samples without primary antibody (indirect labeling) was chosen. For "no primary" controls, only the secondary antibodies were used in the case of indirect flow cytometry instead of isotype controls, as all experiments were run and compared pairwise. Gating was performed as described in the individual papers.

3.7.2 Selection of antibodies for immunophenotyping

One purpose of Paper I was to identify the effects of platelet releasate on the differentiation of hematopoietic progenitors.

As terminal maturation of blood cells often requires specific conditions, for example, EPO for erythrocytes and G/GM-CSF for neutrophils, in order to analyze different lineages in the same cultures, experiments were limited to antigens present at intermediate or early differentiation stages. Thus, it was important to identify lineage-restricted antigens for early commitment. The choice fell on CD14 (monocytes), CD15 (granulocytes), CD34 (progenitors), CD41 (megakaryocytes), CD71 (erythroid cells), and CD235a (erythroid cells). CD14 is a hallmark

antigen for monocytes and is expressed on day 5 of cell culture of hematopoietic progenitors on cells with monocyte progenitor morphology, albeit when using a slightly different mix of cytokines than ours [379]. The CD14 gene is also expressed in granulocytes, but at a level several orders of magnitude lower than on monocytes [94]. CD15 is expressed at low levels on the surface of monocytes [380], but it is mostly expressed on promyelocytes and more mature granulocyte stages [381], and is likely not expressed at the stage of the earliest commitment to granulopoiesis. CD41 was chosen as a megakaryocyte marker and is generally regarded as a lineage-specific antigen, though it has been found on basophils, possibly as the result of platelet binding [382]. CD71 and CD235a are expressed at different levels in erythropoiesis [85], but recent evidence suggests that CD71 is also expressed in neutrophil progenitors [381]. However, early neutrophil progenitors were likely rare in our experiments, as most cells were $CD34^-$.

3.7.3 Selection of fluorochromes and compensation

Ideally, when running multicolor flow cytometry experiments, dim fluorochromes should be chosen for frequent antigens and bright fluorochromes should be chosen for sparse antigens to increase sensitivity and decrease spillover. Pilot studies revealed that CD41 was relatively highly expressed; thus, FITC was chosen as the fluorochrome. CD14 and CD15 were prioritized for the bright fluorochromes PE and APC, as they were sparsely expressed using the cytokine mix in question. In addition, optimal sensitivity for measuring differences between the two groups (“platelet releasate” vs. “no platelet releasate”) for these antigens was consider more important than for CD34. Thus, dim PerCp Cy 5.5 was the only option for the less expressed CD34 antigen in cultured cells, which was not ideal. However, CD34 was only implemented to show the majority of cells had differentiated toward any one of the lineages, as would be indicated by loss of antigen expression. Erythroid immunophenotyping with CD71 and CD235a was performed

with FITC-and APC-labeled antibodies in separate experiments (from the same cultures because of limited possibility for multiparameter runs). As multiple fluorochromes have spillover effects on multiple detectors, compensation is necessary. This was performed using the CytExpert ver. 2.4 (Beckman Coulter; Brea, CA, USA) and was calculated using the difference in fluorescence in samples stained with single antibodies from the fluorescence of an unstained sample.

3.8 Selection of proteins for BCL2 family analyses

Analysis of BCL2 family proteins was investigated in Paper II and III with an adaptation of the protocol from Ludwig et al. [383]. Proteins were selected as potential targets for miR-125a/b based on the publicly available microRNA databases miRDB [384] and TargetScanHuman [385] or other previously published papers [232, 233]. BCL2 is not generally considered a target for these microRNAs but was chosen because it is the major regulator of mitochondrial outer membrane permeabilization.

3.9 Selection of microRNAs to analyze in Paper II and III

A few studies have investigated the extent of the microRNAome in platelets and PMPs [199, 200, 216, 230]. These data were compared with the levels of different microRNAs in THP-1 cells [386]. Generally, to detect a relative increase in microRNAs because of PMP internalization, one should select microRNA candidates with high levels in PMPs and low levels in THP-1 cells. However, microRNAs that were previously shown to have a biological effect in leukemia cells, especially the THP-1 cell line, are of great interest, for example, miR-125a/b [232, 233].

3.10 Limitations of the methodology

3.10.1 Paper I

This study had several limitations. First, because of the scarcity of materials and time-consuming setups, it was impossible to perform all assays in all donor samples. Hematopoietic stem and progenitor cells are highly heterogeneous and their composition, performance in transplantation, and response to growth factors vary greatly, which is not only explained by the source of origin [32, 387-389]. In other words, the frequency of differentiation stages or phenotypes on any given day of culture may differ between donors because of variable overall maturation, which is independent of the platelet releasate effect. This variability increases the need for donor samples when considering inter-donor variability in the analysis. Thus, in order to keep the scope of the paper manageable, data were compared intra-individually. I believe this choice is valid because there were very few contradictions in the data regarding qualitative effects, that is, differentiation toward specific lineages or proliferation inhibition. Furthermore, the main conclusion of the study is supported by consistent data from multiple donors.

Another limitation of this study is that the cells were not terminally differentiated. As previously described, attaining terminal differentiation would require specific culture conditions for specific lineages and thus greatly increase the scope of this work. In addition, there are very few publications investigating the effects of platelet-derived products on hematopoietic progenitors; thus, a broader approach was needed as a proof of concept. A further limitation is that the study was limited to one platelet releasate, although it was derived from four donors. Extending this project to compare different releasates would increase the scope by the same order of magnitude as the number of releasates. Furthermore, inter-donor variance, at least for a core set of the proteome, is not extensive [390].

3.10.2 Paper II and III

In these studies, only a single cell line (THP-1) was used to discover a potential relationship between PMPs, chemoresistance, and cell activity; thus the mechanistic findings only apply to this cell line. The findings regarding chemoresistance were replicated in AML samples, but the mechanistic findings were not replicated due to the lack of biological material. However, proliferation analysis using a Cell Trace™ assay was performed in AML samples, though proliferation dyes like Cell Trace™, as previously mentioned, can be cytotoxic. The protocol was adapted for cryopreserved $CD34^+$ cells, with little effect on cell growth but cytotoxicity was high for the more fragile AML cells. Though the effect of Cell Trace™ on AML cells was variable, viable cells were often limited to a couple of hundred events in the flow cytometry analysis. For that reason, these data were excluded, but they did indicate proliferation inhibition, like what was found in the THP-1 cell line (data not shown).

3.11 Statistical analyses

Data were generally analyzed using a paired-sample t-test. A paired sample t-test was best suited because data pair comparisons were always from the same batch of cells and were therefore dependent values. An independent t-test or one-sample t-test was chosen when appropriate. Data were always checked for normality, as described in the individual papers, and non-parametric tests were chosen for non-normal data. All calculations were performed using IBM SPSS software (IBM Corp., Armonk, NY, USA).

4 Results

4.1 Paper I

Cacic, D.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Releasate Augments *in vitro* Monocytopoiesis and Erythropoiesis. Manuscript.

Although the primary role of platelets is in hemostasis, other important platelet functions, especially in tissue regeneration, immunology, and cancer biology, are currently being discovered. Some of these properties are utilized when platelet-derived products, such as platelet lysate or platelet releasate, are used for the expansion of cells intended for cell therapy. However, few studies have included $CD34^+$ hematopoietic stem and progenitor cells; therefore, the potential effects of the platelet secretome on $CD34^+$ cells remain poorly understood. Our group investigated the *in vitro* effects of platelet releasate in combination with stem cell factor, thrombopoietin, FMS-like tyrosine kinase 3 ligand, and interleukin-3, on basic cellular functions, proliferation, and differentiation in mobilized peripheral $CD34^+$ cells. Although platelet releasate inhibited the growth of $CD34^+$ cells overall, cell tracking analysis with Cell Trace Far Red indicated that the effect may be variable for different $CD34^+$ subsets. Using mRNA expression analyses, flow cytometric immunophenotyping, and CFU assays, it was also demonstrated for the first time that platelet releasate can induce monocytopoiesis and the expansion of erythroid progenitors compared to cytokines alone, making it a suitable medium supplement for *ex vivo* differentiation of hematopoietic stem and progenitor cells into monocytes and erythroid cells.

4.2 Paper II

Cacic, D.; Reikvam, H.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Protect Acute Myelogenous Leukemia Cells against Daunorubicin-Induced Apoptosis. Cancers 2021, 13, 1870.

Platelets are now recognized to contribute to cancer development and progression, and several platelet-cancer interactions through cell-to-cell contact or signal molecule pathways have been identified. In addition, platelet microparticles (PMPs) can be shed by platelets and internalized by cancer cells, thus transferring a bulk of platelet contents. Proteins and small RNAs from the parental platelets are packed in PMPs and are known to have significant biological effects in a variety of cells from different tissues. However, the effects of PMP internalization on AML remain unknown. Thus, this study was conducted to investigate whether PMPs could transfer platelet contents to the THP-1 cell line and whether this transfer could change the biological behavior of the cells. Using fluorescence microscopy, acridine orange-stained PMPs were shown to be internalized by THP-1 cells, and the findings were corroborated by flow cytometry analysis. This internalization consequently increased the levels of miR-125a, miR-125b, and miR-199. More importantly, PMP co-incubation with THP-1 or primary AML protected the cells against daunorubicin-induced cell death. Furthermore, flow cytometry-based analyses revealed that PMP-co-incubation also impaired THP-1 cell growth, partially inhibited cell cycle progression, decreased mitochondrial membrane potential, and induced differentiation toward macrophages. The results from this study suggest that a PMP-associated change in phenotype and decrease in cell activity may explain the observed resistance to daunorubicin-induced apoptosis, as similar results were produced with serum starvation of THP-1 cells. Platelet inhibition with a reduction in plasma PMP level thus emerges as a possible treatment for AML, but further research is needed.

4.3 Paper III

Cacic, D.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Decrease Daunorubicin-Induced DNA Damage and Modulate Intrinsic Apoptosis in THP-1 Cells. Int. J. Mol. Sci. 2021, 22, 7264.

Platelets can modulate cancer through the budding of platelet microparticles (PMPs), which can transfer a variety of proteins and RNA molecules to cancer cells through the internalization of these microparticles. In AML, PMP internalization can increase resistance to chemotherapy, partially through a decrease in cell activity, as shown in Paper II.

This study was conducted to investigate whether the internalization of PMPs protected the monocytic AML cell line THP-1 from apoptosis solely via a decrease in cell activity, or if PMP co-incubation could decrease daunorubicin-associated DNA damage independently of the cell cycle effect or potentially also modulate apoptosis directly. Furthermore, we examined whether PMPs have general anti-apoptotic properties, protecting against apoptosis after treatment with different apoptotic inducers, primarily associated with either the intrinsic or the extrinsic apoptotic pathway. The results of this study showed that the anti-apoptotic properties of PMPs were restricted to agents targeting intrinsic apoptosis. By assessing the levels of phosphorylated H2AX (gH2AX), a measure of the DNA damage response, it was evident that DNA damage was reduced in both 2N and 4N cells after PMP co-incubation. The levels of BCL2-family proteins before and after treatment with daunorubicin revealed that PMP co-incubation led to a daunorubicin-independent decrease in the levels of the pro-apoptotic PUMA protein. The levels of other BCL2-family proteins (BAK1, BMF, BCL2, and MCL1) were also altered; however, these changes only followed the expected trends in apoptosis and could not be categorically connected to downregulation of the proteins *per se*. Interestingly, PUMA is a predicted target of miR-125a/b, whose levels were shown to be

elevated after PMP co-incubation in Paper II. The findings of this study indicate that PMPs may protect AML cells against apoptosis by reducing DNA damage both dependently and independently of the cell cycle phase, and via direct modulation of the intrinsic apoptotic pathway by leading to a downregulation of PUMA. Thus, the study of the effects of platelet inhibition to reduce PMP levels in AML patients is further strengthened; however, the results need to be corroborated in more AML cell lines and patient samples, and by using more advanced models.

5 Discussion

5.1 The role of platelet-derived products in cellular therapy and regenerative medicine

Platelet-rich plasma has been tested for applications in tissue regeneration with little or only moderate effect [391-393]. However, there are reoccurring issues in this research as evident in the cited systematic reviews as production of platelet-rich plasma lack standardization and treatment is often limited to a single injection. On the other hand, platelet lysate and releasate outperform FBS as culture supplements for proliferation induction that do not bias the differentiation potential of MSCs [394-397]. Given the pluripotent nature of MSCs, these cells are paramount to the field of regenerative medicine and the role of platelet lysate as a culture supplement has cemented itself as commercialized products now are readily available. Data are scarce, though, on the use of platelet-derived products as supplements in the culture of hematopoietic stem and progenitor cells. Our results indicate that platelet-derived products may serve a purpose in the cultivation of these cell types for *ex vivo* production of erythroid cells and monocytes. The results from Paper I indicate that culturing mobilized peripheral $CD34^+$ progenitor cells with platelet releasate increase CD14 antigen expression and elevate levels of monocyte-associated mRNAs. In addition, the platelet releasate conserved erythroid phenotype in hematopoietic progenitors and increased the number of CFU-Es. Thus, platelet releasate may support both monocytopoiesis and erythropoiesis.

Ex vivo production of functional erythrocytes is a long-awaited game changer in transfusion medicine. However, several obstacles must be overcome, including the limited availability of progenitor cell sources, such as cord blood or human embryonic stem cells. In addition, the lack of proper functionality of the cultivated cells remains concerning and

probably results from incorrect or absent stimuli from the artificial extracellular environment [398]. The introduction of induced pluripotent stem cells has revitalized research in regenerative medicine and could potentially solve the general constraints of availability, as evident in a recent study using a single line of iPSC-derived MSCs to treat steroid refractory GVHD [399]. Hemoglobinized and enucleated erythrocytes have been produced from iPSCs, however, there still are technical difficulties to overcome as the switch from fetal to adult hemoglobin was only achieved post transfusion [400]. Moreover, the ultimate challenge in cellular therapy, regardless of the source of stem cells, will always be cost. Thus, the protocols need to be optimized for maximum output, which could be a potential role for platelet-derived products in the case of *ex vivo* production of erythrocytes, but this use needs to be verified both in more advanced *in vitro* models and animal transfusion models before final conclusions are drawn.

Ex vivo expansion of monocytes is currently exploited for the production of dendritic cell vaccines, a growing field of advanced immune therapy. DCs are usually generated either from $CD34^+$ cord blood progenitor cells through an intermediate monocyte stage or from peripheral blood monocytes directly [401]. Platelet releasate is known to alter phenotypes in mature monocytes [402, 403]. However, the results in Paper I indicate its potential use as a growth supplement for producing monocytes from hematopoietic progenitors. We provide little evidence of the effects of platelet releasate on the functionality of the generated monocytes, other than a general increase in the phagocytic capacity in cells cultivated with platelet releasate. Thus, our findings, although promising, should be interpreted with caution regarding the role of platelet releasate as a growth supplement in this specific setting, but further research is warranted.

Paper I, which addresses the interactions between platelets and hematopoietic progenitors, does not provide many mechanistic findings. The platelet granule proteome, or platelet secretome, is vast [160, 390],

and the observed effects likely stem from specific combinations of growth factors and other substances. As noted in Table 2, several platelet-associated growth factors are known to affect hematopoietic progenitor cells. Considering that the releasate contains additional compounds, including RNAs, providing mechanistic findings is a major undertaking, especially when the biological effects in the first place are largely unknown. Noroozi-Aghideh et al. conducted a similar study at a smaller scale and found that platelet releasate induced differentiation towards megakaryocytes from $CD133^+$ cord blood cells, but other lineages were not investigated [373]. We could not be reproduce these results, perhaps because of differences in sources of progenitor cells, selection of immunophenotype of progenitors, or choice of cytokines, which is discussed further in Paper I. Moreover, to the best of my knowledge, no other publications support platelet releasate as an inducer of megakaryocytopoiesis in isolated hematopoietic progenitor cells.

One needs to be careful when interpreting the *in vivo* relevance of the findings in Paper I or other *in vitro* studies addressing the role of platelets in hematopoiesis. Platelets communicate with cells in multiple ways, including by cell-to-cell contact, by production of exosomes/microparticles, and through release of soluble chemical compounds as humoral or paracrine factors. The addition of thrombin-stimulated platelet releasate to static cell cultures is therefore a crude model for investigating these interactions, with many shortcomings. Using platelet releasate in this setup is, however, a natural starting point, as it is simple and can reveal new hypotheses regarding basal interactions of platelets with different cells while simultaneously providing evidence for its use as a culture supplement in emerging cellular therapies. Platelet lysate, which contains the total platelet proteome, may prove to be a more viable culture supplement than platelet releasate, at least for MSC cultivation, because lysate could potentially be obtained from expired blood bank products [404]. However, releasate more closely resembles the *in vivo* platelet secretome and is better suited as a model for

potentially relevant interactions. An obvious obstacle to any *in vitro* platelet study is that platelet function is dependent on mechanistic forces such as shear stress and interactions with endothelial cells [164, 405, 406]. Thus, it is very challenging to develop a realistic *in vitro* model to study the close-contact interactions between blood cells and platelets in addition to the effects of platelet-secreted soluble compounds and microparticles. However, results from *in vitro* studies can be corroborated using knockout or knockdown animal models to evaluate the effects of deleting or inhibiting individual genes that are important for specific platelet functions such as alpha granule exocytosis or PMP shedding. Alternatively, one can study patients with specific genetic disorders that affect platelet function. For example, investigating hematopoiesis, coagulation, and the occurrence of cancers in patients with Scott's syndrome, which causes defective PMP release, could reveal key information about the roles of these cell fragments.

5.2 The role of platelets and platelet inhibition in cancer

Monocytes and monocyte-derived dendritic cells play an important role in cancer immunology [407]. This also includes AML, where a monocyte-like subfraction is known to modulate the immune response [277]. Thus, the monocyte-cancer-platelet axis could be significant for the development and progression of cancer. In addition, monocyte-platelet interactions are well supported [155]. Platelets are believed to bind to monocytes via P-selectin and P-selectin glycoprotein ligand-1 (PSGL), which then upregulates other adhesion molecules on the monocyte cell membrane [408]. Other binding mechanisms likely contribute, which is especially evident in children, where the levels of monocyte-platelet aggregates are elevated in the absence of increased expression of P-selectin [409]. Han et al. showed that platelets can induce dendritic cell differentiation in mature monocytes via this P-selectin/PSGL binding [410]. Furthermore, these platelet-induced

dendritic cells stimulated T cell proliferation better than cytokine-stimulated bone marrow-derived dendritic cells.

We provide evidence that the platelet secretome may be an important regulator for monocytopoiesis. This may represent an indirect effect in cancer, however, it is difficult to deduce whether this is an anti- or pro-cancer effect based on our results. On the other hand, in some hematological malignancies where the monocytes themselves represent the malignant cells, it is easier to assume that the observed effects of platelet releasate on monocytopoiesis may be disease driving. One such disease is chronic myelomonocytic leukemia (CMML), which is dominated by monocytes with pro-inflammatory phenotype [411], a trait also seen in platelet-activated monocytes [403, 412].

It is now commonly accepted that frequent use of platelet inhibitors is associated with a decreased risk of a variety of cancers and an increased cancer-specific survival rate for some cancer types [167-170]. However, a number of issues and unanswered questions remain regarding the effect of platelet inhibition in cancer. Data supporting these conclusions are usually retrospective, and findings from prospective studies are less convincing [413-415], perhaps due in part to the short follow-up time in some of those studies [413]. “Frequent” or “regular” use of platelet inhibitors is also poorly defined, with great heterogeneity between studies [167]. Presumably, one would suspect a clearer effect with daily use as compared to occasional use. The major risk of using platelet inhibitors is bleeding, but the U.S. Preventive Services Task Force has nonetheless concluded a beneficial risk-reward ratio for their use in patients aged 50–69 years for the prevention of colorectal cancer and cardiovascular disease [416]. This policy is not implemented globally and would be strengthened by further data from randomized studies with sufficient follow-up time, also including other cancers. The mechanism of these cancer-specific effects is not yet known and a deep understanding may be necessary to fully utilize the therapeutic potential. As platelet inhibition decreases the generation of PMPs [417], the

findings from Paper II and III that PMPs have anti-apoptotic properties suggest one possible mechanism, but platelet inhibition has never been investigated as a treatment for AML. In addition, PMPs have anti-tumoral properties in some cancer types, such as lung and colorectal cancer [139]. Although some studies show that frequent use of aspirin may reduce the risk of AML or leukemia in general [418, 419], this has not been confirmed in larger meta-analyses [167]. Platelet inhibition as an adjuvant treatment in AML is intriguing, but it faces one obvious challenge: the prolonged bone marrow aplasia after intensive chemotherapy. During this period, however, interactions between platelets and cancer cells are very limited as platelet production is absent or very low and blood levels are maintained at a minute level only through prophylactic transfusions.

The findings from Paper II and III suggest that PMPs can be internalized by AML cells, resulting in the transfer of small molecules, including microRNAs. The levels of miR-125a and miR-125b were elevated after a short co-incubation of THP-1 cells with PMPs. These microRNAs are known to downregulate the pro-apoptotic PUMA protein [232, 233]. We also provide evidence for the downregulation of PUMA protein in PMP co-incubated THP-1 cells, which therefore could be a result of the transfer of these microRNAs. PMPs inhibited apoptosis in leukemic cells, though these effects may be specific to AML, as PMP-associated compounds could vary in importance in different cancers, especially across different tissues and germ layers. Thus, it is premature to conclude that the anti-tumoral effect of aspirin is due to it reducing the transfer of anti-apoptotic microRNAs in PMPs to host cells, as this mechanism might be cancer-type-specific. Plenty of evidence shows that miR-125a and miR-125b have anti-apoptotic properties in multiple leukemic cell lines [232, 233, 420, 421], but their effect on solid cancer cell lines is more controversial, with mounting evidence for the opposite. Ectopic expression of miR-125a is pro-apoptotic in colorectal cancer [422]; however, chemoresistance increased with overexpression of miR-125b

[423]. In addition, miR-125a induced chemosensitivity in cell lines from breast cancer [424], esophageal cancer [425], and cervical cancer [426]. Moreover, opposing biological effects were seen when different miR-125a isoforms (-3p vs. -5p) were overexpressed in lung cancer cell lines [427], further complicating our understanding of their roles in solid cancer. To the best of my knowledge, Paper II is the first study to show that PMPs have anti-apoptotic properties in primary AML cells. Although Vasina et al. have previously shown that PMPs can inhibit apoptosis in THP-1 cells [207], this knowledge was expanded with multiple compounds with different apoptosis-inducing properties in Paper III. More importantly, Paper II showed a significant increase in chemoresistance to a clinically relevant concentration of daunorubicin, a standard chemotherapy for AML treatment. Thus, these findings have additional clinical relevance.

PMPs significantly inhibited the activity of AML cells, which partially explained the increase in chemoresistance. A minor reduction in growth rate in a very aggressive cancer may not mean much for survival *per se*, when excluding the effect of apoptosis. However, this inhibition could be a result of the maturation of leukemic cells, as reported in Paper II, where PMPs induced differentiation towards macrophages. This could be of importance for disease stabilizing of more indolent myeloid diseases such as the myeloproliferative diseases or MDS, where one often adapts a form of "watchful waiting" strategy or symptomatic treatment, but the effect needs to be verified in a wider array of cell lines and representative patient samples.

AML can have different maturation characteristics according to the FAB classification [259]; however, all types of AML are defined by a considerable proportion of immature cells in the bone marrow. A potentially clinically beneficial effect of PMPs through the maturation of these cells will likely depend on multiple mechanisms and pathways, but when deciphered it may inspire development of therapies with similar

effects as seen for all-trans retinoic acid (ATRA) in patients with AML M3 or acute promyelocytic leukemia.

Based on the findings from Paper II and III, there is a relationship between cell activity, chemoresistance, and PMPs. However, the results were derived from *in vitro* studies and should be confirmed in more sophisticated translational animal models. For example, this could be done by adapting the approach of Michael et al. [139] where NSG mice were transplanted with Lewis lung carcinoma cell line and reduced in PMP levels by genetic knockout. Another possible model could be to treat AML-transplanted mice with or without PMPs and chemotherapy.

Our findings introduce another aspect in the debate of using therapeutic contra prophylactic platelet transfusions in AML patients undergoing intensive chemotherapy. For a long time it has been common place for all cancer patients under active treatment to receive prophylactic platelet transfusion with platelet count < 10 x 10^9/L as a threshold to limit risk of bleeding. Although data are scarce, the 2017 guidelines from American Society of Clinical Oncology (ASCO) opens for limiting platelet transfusions to sign of bleeding, although only for patients receiving autologous HSCT in experienced centers, which predominantly apply to patients with multiple myeloma and lymphoma [428]. There is also recent real life observational data supporting the safety of this practice [429]. As platelet concentrates are rich in PMPs, it is possible that transfusion can induce chemoresistance in the cancer cells, but our data only supports this notion in the context of AML. However, based on randomized data, prophylactic transfusions seem necessary to limit severe bleedings when limiting analysis to AML patients receiving intensive chemotherapy [430, 431]. Thus, there is an increased need for identifying bleeding risk in these patients other than platelet count alone. Only by abandoning the practice of prophylactic platelet transfusion in the setting of clinical studies can the potential harmful effects of platelet transfusions in AML be thoroughly investigated.

The scope of the biological effects of PMPs in leukemic cells is likely only partially revealed by our experiments. To fully understand these mechanisms requires combined whole-transcriptome and whole-proteome analyses at different time points. One can then use advanced bioinformatics to better identify which pathways are affected, and to differentiate "immediate" effects, due to the transfer of chemical compounds from PMPs, and effects that result from *de facto* genetic reprogramming.

6 Concluding remarks

The broader role of platelets outside coagulation is increasingly appreciated and represents a potential therapeutic target in multiple diseases, especially cancer. In this project, potential interactions between normal and leukemic hematopoietic cells and platelets are identified, which can inspire further research that will improve our understanding of the interplay between these cells and develop potential interventions with significant clinical relevance. Platelet releasate possibly induces monocytopoiesis and erythropoiesis, which supports the use of platelet-derived products as cell culture supplements for *ex vivo* production of monocytes and erythrocytes. In addition, it potentially provides important insights into the basal mechanisms for differentiation of hematopoietic progenitors. Moreover, PMPs have been identified as a potential therapeutic target in AML as they seemingly increase resistance to daunorubicin and show general anti-apoptotic properties.

7 Future perspectives

Recent years have seen a surge in advanced and costly cancer treatments derived from our increasingly profound understanding of cancer biology down to the level of epigenetics. However, these treatments are not available to most people in low-income countries or even richer countries without universal health care. Thus, it is important to fully utilize this profound knowledge of cancer biology to revisit older and cheaper drugs with proven safety profiles that may have therapeutic effects for other indications. Prophylactic aspirin for colorectal cancer is one example of this, but aspirin likely has a plethora of other undiscovered indications, as do the numerous other platelet inhibitors that exert their effects through different mechanisms. In addition, when fully understanding the basal mechanisms by which platelets affects different diseases, one can likely develop targeted therapies with greater potency than the platelet inhibitors available today. The availability of platelets and PMPs and their great cell adhesion properties also make them suited for utilization as drug delivery systems to increase intracellular concentration, a research field that still is in its mere infancy.

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Appendix A – supporting information for HSC experiments

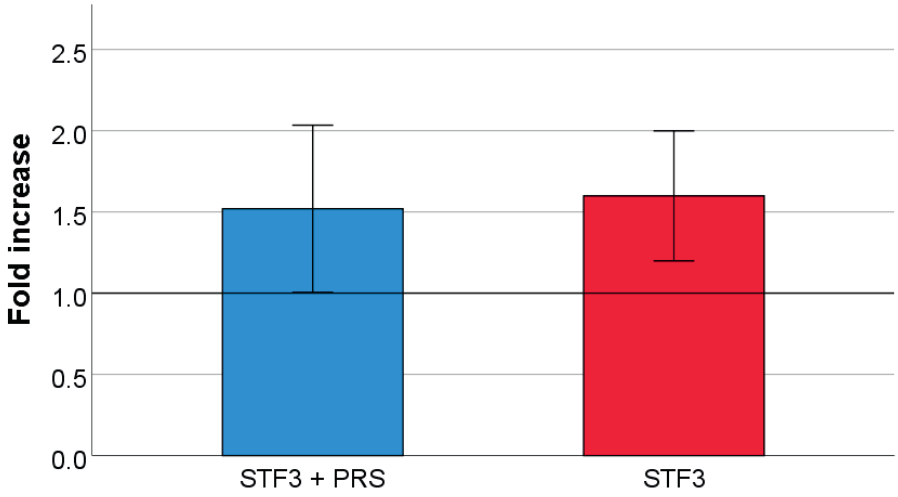

Figure 1. Relative increase in cell count of mobilized peripheral $CD34^+$ cells after HUVEC co-culture. Mobilized peripheral $CD34^+$ cells were cultured in either STF3 medium (see Paper I for detailed explanation of culture conditions) with or without the addition of 10% platelet releasate on a confluent lining of HUVEC cells. Mean relative increase in cell count of HUVEC co-culture versus standard suspension culture were calculated after one week of cultivation. Error bars represent 95% CIs. n (technical replicates)=10.

Appendix B – supporting information for AML experiments

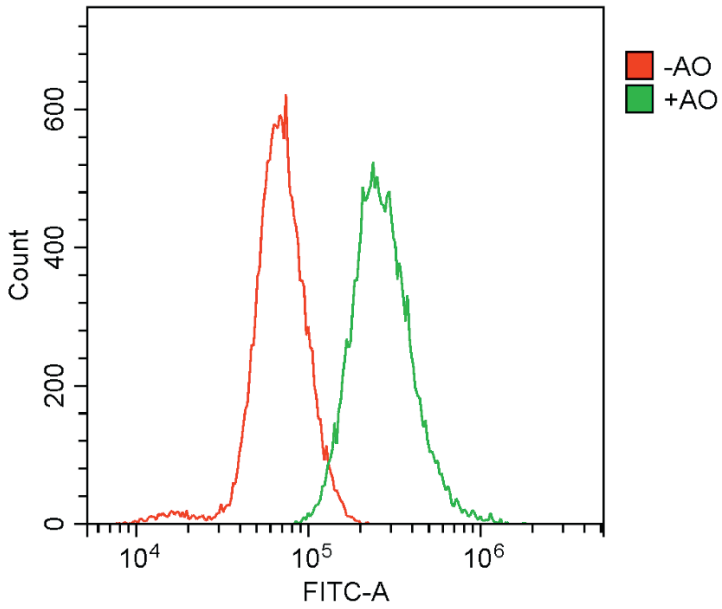

Figure 1. Internalization of platelet microparticles by THP-1 cells, flow cytometry analysis. THP-1 cells, at a concentration of 5 x 10^5/mL, were co-incubated with 5 x 10^6 PMPs/mL (stained with acridine orange (AO) as described in Paper II) for 16 hours before analysis. Platelet concentrate was washed with IMDM and centrifuged at 400 g for 10 minutes before resuspension in IMDM. Platelet releasate was then produced and PMPs were isolated according to the standard procedure described in Paper II and III.

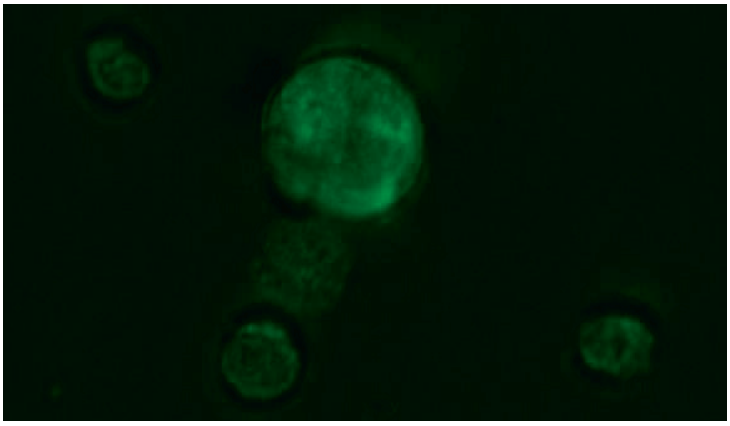

Figure 2. Internalization of platelet microparticles by THP-1 cells, fluorescence microscopy. Picture of representative cells from the samples from Figure 1 at 400 x magnification using SpGreen filter.

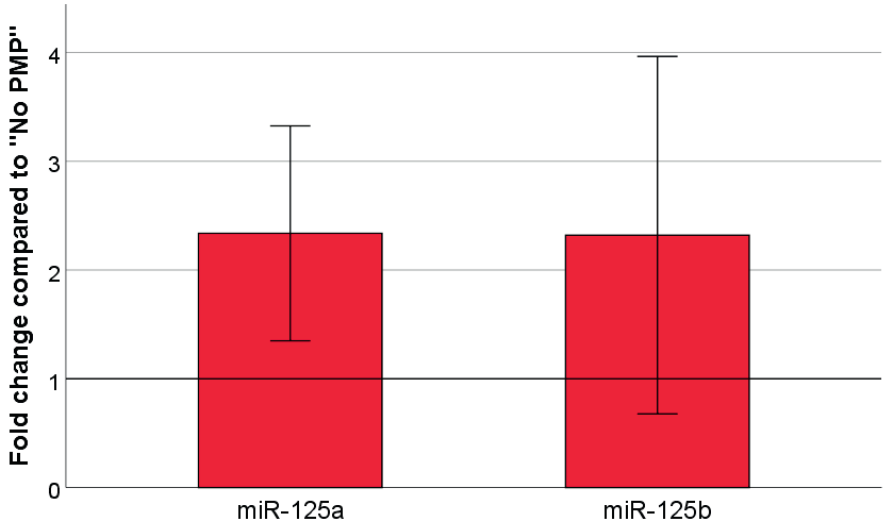

Figure 3. Levels of miR-125a/b after PMP co-incubation. THP-1 cells were co-incubated with PMPs as described in Figure 1. Levels of microRNAs were then analyzed as described in Paper II. The bars represent the fold increase in microRNA levels of cells with PMP co-

incubation compared to cells in standard conditions. Error bars represent 95% CIs. n=3.

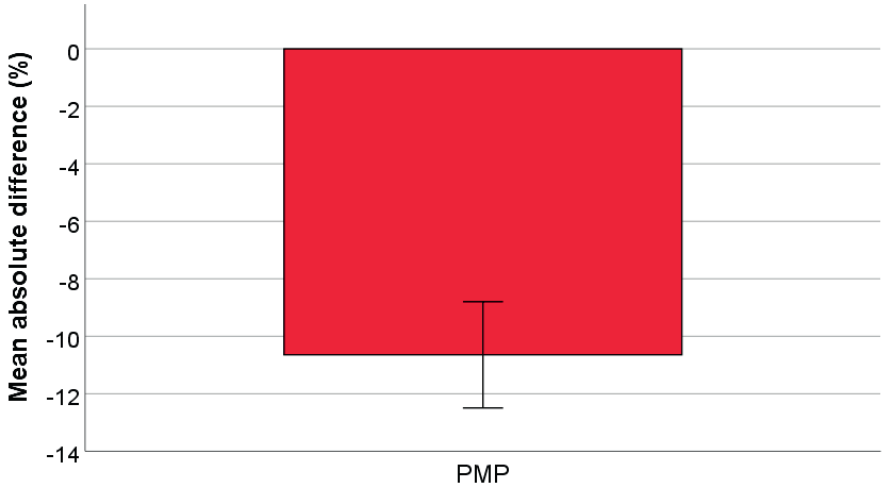

Figure 4. Effects of PMP co-incubation on apoptosis. THP-1 cells with or without co-incubation with PMPs from a washed platelet concentrate were cultured as described in Figure 1. Then cells were treated with daunorubicin at 0.5 μM and analyzed for apoptosis as described in Paper II and III. The bar represents the mean difference of relative frequency of dead and apoptotic cells with or without PMP co-incubation. Error bar represents 95% CIs. n=4.

Appendix C – papers

Paper I

Cacic, D.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Releasate Augments *in vitro* Monocytopoiesis and Erythropoiesis. Manuscript.

Platelet Releasate Augments *in vitro* Monocytopoiesis and Erythropoiesis

Running head of title: Platelets increase monocytes and erythrocytes

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Home institution for study: Stavanger University Hospital

Mesh terms/keywords: Hematopoietic stem cells, platelets, erythroid precursor cells, monocytes

Abstract

Background

Although the primary role of platelets is in hemostasis, other important functions, especially in tissue regeneration, immunology, and cancer biology, are currently being discovered. Some of these properties are utilized when platelet-derived products, such as platelet lysate or platelet releasate, are used for the expansion of cells intended for cell therapy. However, few studies have included CD34+ hematopoietic stem and progenitor cells; therefore, the potential effects of the platelet secretome on CD34+ cells remains poorly understood.

Methods

We investigated the *in vitro* effects of platelet releasate in combination with stem cell factor, thrombopoietin, FMS-like tyrosine kinase 3 ligand, and interleukin-3 on basic cellular functions, proliferation, and differentiation in mobilized peripheral CD34+ cells. Basic cellular function was assessed with measurement of glucose uptake, cell cycle distribution, mitochondrial membrane potential, and reactive oxygen species, all by flow cytometry. Proliferation analyses was performed with flow cytometric count beads and Cell Trace generation tracking. Differentiation analyses was performed using reverse transcription PCR and flow cytometric immunophenotyping of lineage specific mRNAs and antigens.

Results

Platelet releasate inhibited growth of CD34+ cells overall, but cell generation tracking implied that the effect may be variable for different CD34+ subsets. Frequency of CD14+ cells and the levels of CD14 and VCAN mRNA were increased in the presence of platelet releasate. In addition, erythropoiesis was expanded compared to stimulation with

cytokines alone through conservation of an erythroid immunophenotype and increased CFUe number.

Conclusions

We show for the first time that platelet releasate can stimulate monocytopoiesis and erythropoiesis. This suggests possible new roles for platelets in normal and pathological hematopoiesis and should encourage the use of platelet releasate for ex *vivo* production of monocytes and erythroid cells.

Background

Platelets were first recognized as an important contributor to hemostasis in the late 19th century [1]. Later, it became clear that platelets exhibit many other functions, with important roles in tissue regeneration, immunology, and cancer biology [2-7]. Retrospective and observational data have even linked inhibition of platelets with a reduced risk of cancer and improved cancer-specific survival [8-11], underpinning the broader functionality of platelets.

For the last few decades, mobilized peripheral blood stem cells (mPBSCs) have been preferred over bone marrow transplantation for autologous and allogenic stem cell treatment. This has resulted in shorter time to engraftment, but also more chronic graft versus host disease [12-14]. A notable difference between the two methods is that mobilized cells are more exposed to activated platelets than bone marrow cells because platelets are activated during the harvesting procedure [15].

When platelets become activated, they release their alpha granule contents, which consist of several hundred different proteins [16, 17]. Apheresis grafts of patients receiving autologous stem cell transplantation contain a higher level of platelet-associated growth factors, such as platelet factor 4, ß-thromboglobulin, and platelet-derived growth factor (PDGF), compared to the peripheral blood of the patients and healthy controls [18]. Some of the platelet-associated growth factors are known to affect proliferation or basic cellular functions of CD34+ cells [19-23]. Platelets also contain a large selection of microRNAs and have processing capacity for microRNAs and pre-microRNAs [24, 25]. These non-coding RNAs regulate fundamental functions in normal and malignant hematopoiesis [26-29], and there is evidence of the transmission of microRNAs from platelets through microparticle fusion or internalization in different cell types, changing the biological behavior of these cells [30-32].

Even before the significant findings in the area of platelet proteomics and transcriptomics, researchers were encouraged to use platelet-rich plasma in clinical experiments to enhance cell growth for accelerated bone and wound healing, with mixed results [33-35]. However, not until enriched products such as platelet lysate or releasate were used as cell culture supplements could one see a potential use. This was especially evident for the expansion of mesenchymal stem cells (MSCs) because it does not seem to interfere with multipotency or lineage differentiation [36-39]. For expansion of cells for clinical use, platelet-derived products, which are now commercially available in the form of platelet lysates, are replacing fetal calf serum, which theoretically carries the risk of xenogenic immune reaction and infection.

Platelet microparticles (PMPs) are also produced, or released, during platelet activation. According to early work by Janowska-Wieczorek et al., the reduced time to engraftment for transplantation with mPBSCs could be explained by the binding of PMPs to the hematopoietic stem and progenitor cells, thereby increasing the expression of platelet-endothelium attachment receptors or "anchor proteins", such as CD41, CD42, CD61, and P-selectin [40]. The same study showed that incubation of human cord blood CD34+ cells with PMPs led to shorter time to engraftment after transplantation in NOD/SCID mice. After a short incubation with PMPs (<2 hours), the expression of CD41 increased in the cord blood cells to a level similar to mPBSCs. The assumption that increased expression of "anchor proteins" accelerates bone marrow reconstitution has been tested in a small clinical trial in which fucosylation of CD34+ cells resulted in a shorter time to engraftment in allogenic cord blood transplantation [41].

Even though there is evidence that platelets can influence the adhesion capacity of hematopoietic stem and progenitor cells, knowledge regarding the potential interactions of platelets with the developing hematopoietic progenitor cell, or hematopoiesis, is largely incomplete. Therefore, we conducted this study to examine the effects of platelet

releasate on the proliferation, differentiation, and basic cellular functions of hematopoietic stem and progenitor cells.

Methods

Cell isolation

We used cryopreserved samples prepared after routine allogenic stem cell apheresis donations from donors who had provided informed consent (Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway). Cryovials were quickly thawed in a 37°C water bath and washed with Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) + 1% BSA (Thermo Fisher Scientific). CD34+ cells were isolated using the Ultra Pure CD34 MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. DNase I (Merck KGaA, Darmstadt, Germany) was added at multiple steps throughout the process, from thawing to final magnetic separation, to avoid cell clumping and ensure optimal recovery. Purity was assessed by staining with monoclonal anti-CD34 PerCP Cy 5.5 and analyzed on a CytoFLEX flow cytometer using CytExpert software ver. 2.4 (Beckman Coulter, Brea, Ca, USA) and was generally >95%. List of donors with the associated analyzes performed can be found in Table S1.

Cell culture

Isolated CD34+ cells were seeded at 5 x 10^4 cells per well in 0.5 ml of StemSpan SFEM medium (StemCell Technologies, Vancouver, Canada) supplemented with the following recombinant cytokines: 50 ng/ml stem cell factor, 10 ng/ml thrombopoietin, 50 ng/ml FMS-like tyrosine kinase 3 ligand, and 20 ng/ml interleukin-3 on day 0. All cytokines were purchased from PeproTech EC (London, UK). Cell cultures with StemSpan SFEM + recombinant cytokines are hereafter abbreviated STF3. In select experiments, 10% of StemSpan SFEM in STF3 was

exchanged for platelet releasate. These cultures are abbreviated STF3 + PRS.

Medium was added on days 3, 7, and 10. Cells were grown in 24-well culture plates (Corning Co., Corning, NY). For experiments with higher cell demand on day 5, 1.0 x 10^5–1.6 x 10^5 cells were seeded in 2 ml of complete medium in 6-well plates on day 0 (Thermo Fisher Scientific).

Platelet concentrates

A single prepared platelet concentrate pooled from four donors after written consent was provided by the Department of Immunology and Transfusion Medicine, Stavanger University Hospital (Stavanger, Norway). The platelet concentrate was produced using the TACSI system (Terumo BCT, Lakewood, Co, USA). The platelet concentration was 1.02 x10^9/ml. The concentrate was leukofiltrated to a residual LPK absolute value of <1.00 x 10^6. In the final concentrate, the storage medium contained approximately 65% additive solution (PAS-III, Baxter, Lake Zurich, IL, USA) and 35% plasma.

Platelet releasate

Platelet releasate was produced as described in [42]. The platelet concentrate was transferred from the blood bag to 50 ml tubes and human thrombin (Sigma Aldrich, St. Louis, MO, USA) was added to a concentration of 1 U/ml. The tubes were manually inverted every five minutes and incubated for 60 minutes in a 37°C water bath. Both clot formation and clot retraction was observed and the mixture was centrifuged at 900 g for 10 minutes after incubation. The supernatant was transferred to new tubes and stored at -80°C. The characteristics of the platelet releasate are summarized in Tables S2 and S3.

Cell counting

Cell counting was performed by the CountBright count bead assay (Thermo Fisher Scientific). Beads were identified using the APC channel

on a CytoFLEX flow cytometer, and at least 2000 bead events were collected. The fold change from day 0 was calculated until cells were harvested for other analyses, except for the colony forming unit (CFU) assay, in which only 1 x 10^4 cells were harvested (<1% of total cell number for all cultures).

Flow cytometric proliferation analysis

Proliferation analyses with an assessment of the distribution of generations were performed using the Cell Trace Far Red Proliferation Kit (Thermo Fisher Scientific). On day 0, CD34+ cells were washed, stained with 5 µM of Far Red reagent in Dulbecco's phosphate-buffered saline (DPBS; Sigma Aldrich), incubated briefly for 5 minutes in a 37°C water bath to avoid excessive cell toxicity, and then washed with StemSpan SFEM. The cells where then cultured as described previously. A sample of the cells was analyzed on day 0 to identify the 1st generation and on day 5 with the APC channel on the CytoFLEX flow cytometer. Approximately 40,000 gated cells were acquired. Generations were identified as discrete peaks in the flow histograms. For this and all other flow cytometric analyses, technical replicates of STF3 and STF3 + PRS were analyzed interchangeably to minimize any effects of prolonged incubation or time decay after sample preparation.

Glucose uptake

On day 5, 2.5 x 10^5 cells from the expansion culture were washed, resuspended in StemSpan SFEM, and incubated with 100 µM 2-NBDG (2-[N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino]-2-deoxyglucose;
Thermo Fisher Scientific) for 30 minutes. The cells were washed and analyzed using the FITC channel on the CytoFLEX flow cytometer. Approximately 10,000 gated cells were acquired. Experiments were compared according to the mean fluorescence intensity (MFI) of stained cells after subtracting the MFI of unstained cells specific for both culture conditions.

Cell cycle analysis

For cell cycle analysis on day 5, 5 x 10^5 cells were washed and resuspended in StemSpan SFEM. The cells were then incubated with 10 µM Vybrant Dye Cycle Green Stain (Thermo Fisher Scientific) for 30 minutes and analyzed using the FITC channel on the CytoFLEX flow cytometer immediately after incubation. Approximately 20,000 gated cells were acquired.

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed using the MitoProbe DiIC1(5) Assay Kit (Thermo Fisher Scientific). On day 5, 5 x 10^5 cells in 1 ml IMDM were stained with DiIC1(5) using carbonyl cyanide 3-chlorophenylhydrazone as a control for background signal and incubated for 30 minutes following the manufacturer's instructions. Approximately 30,000 gated cells were acquired.

Reactive oxygen species

Reactive oxygen species (ROS) were measured using the Cell Rox Green Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. On day 5, 5 x 10^5 cells were stained and incubated with 1000 nM Cell Rox Reagent for 30 minutes. Approximately 20,000 gated cells were acquired.

Colony forming units assay

After 1 week of culturing in STF3 or STF3 + PRS, the cells were seeded (900 cells/ml) in 1.1 ml MethoCult Classic medium (StemCell Technologies) in 35 mm dishes (StemCell Technologies) in duplicate and incubated for another 12 days. The colonies were scored manually under an inverted light microscope. For the first CFU experiment, colonies were also harvested for immunophenotyping. Culture dishes were kept for 2 hours at 4°C before adding 1 ml of IMDM and transferring the mixture to a 15 ml tube. Another 1 ml of IMDM was

used to thoroughly wash any residual colonies from the dishes. The cells were then washed and stained with monoclonal anti-CD71 FITC for further flow cytometric analysis.

mRNA analysis

Total RNA was isolated using the RNeasy kit (Qiagen GmbH, Hilden, Germany) and the RNA concentration measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) including "NO RT" experiments. Real-time PCR was performed on the Mx3005P qPCR system (Agilent Technologies, Palo Alto, CA, USA) using TaqMan gene expression assays (Thermo Fisher Scientific) and the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) following the manufacturer's instructions. β-Actin was used as a reference gene, and the relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method for paired samples. For a comprehensive list of the TaqMan gene expression assays see Table S4.

Immunophenotypic analysis

For flow cytometric immunophenotyping, the cultured cells were counted and 2.5 x 10^5 (day 5 and 7) or 5.0 x 10^5 (day 12) cells washed in DPBS (Thermo Fisher Scientific) and incubated with conjugated antibodies in DPBS containing 0.5% BSA. The cells were then washed and resuspended in DPBS and kept on ice until analysis. For a comprehensive list of the antibodies see Table S5. An unstained sample from each experiment was used as a control. At least 20,000 cell gated events were recorded depending on cell concentration but always matched for the two culture conditions. Debris and non-viable cells were gated out based on light scatter properties as described by Reardon et al. [43]. Spectral overlap was corrected when necessary using the compensation module of CytExpert software and cells from the same

experiment as analyzed (i.e., we performed separate calculations for cells from STF3 and STF3 + PRS). For gating strategy see Fig. S1 and S2.

Phagocytosis assay

Phagocytosis was assessed on day 12 with FITC-conjugated *Escherichia coli* (K-12 strain) BioParticles (Thermo Fisher Scientific). Briefly, the BioParticles were reconstituted and incubated with opsonizing reagent for 1 hour before washing. Then, 5 x 10^5 cells were washed, resuspended in IMDM, and incubated with 5 x 10^7 BioParticles for another hour in a CO_2 incubator. Finally, the cells were washed and kept on ice before flow cytometric analysis using the FITC channel. Approximately 20,000 gated cells were acquired. Trypan Blue was used to quench the signal from bound, but not phagocytized material, at a final concentration of 1.2 mg/ml.

Statistical analysis

Statistical analyses were performed using SPSS 26 software (IBM Corp, Armonk, NY, USA). Comparisons were made between STF3 and STF3 + PRS only in experiments within each individual donor using the paired sample t test or the Wilcoxon signed-rank test unless otherwise specified. The data were checked for normality using P-P plots, Shapiro-Wilks test, and Kolmogorov-Smirnov test. $p<0.05$ was considered significant. All mean values are reported with 95% confidence intervals; "n" denotes the number of technical replicates.

Results

Platelet releasate inhibits proliferation of CD34+ progenitor cells

To study the effects of platelet releasate on the proliferation of hematopoietic stem and progenitor cells, isolated CD34+ cells from mPBSC grafts were cultured in STF3 or STF3 + PRS and counted on days 5, 7, and 12 for select donors. Cell counting was performed in experiments from seven different donors for up to three time points,

yielding a total of 13 comparable data pairs. We observed significantly lower expansion for STF3 + PRS compared to STF3 in 6/13 experiments, and a trend towards lower expansion in additional 6/13 experiments (Fig. 1A).

Although cell counting using flow cytometry and count beads is a highly automated procedure, the technique does have sources of errors. The variance is also naturally falsely elevated due to uneven evaporation of the medium [44]. To minimize this effect, culture dishes were always mirrored. In addition, to avoid this issue and further evaluate the inhibition of proliferation induced by platelet releasate, we used the Cell Trace Far Red Proliferation Kit that tracks cell generations. Analyzing cells from Donor 6, we successfully identified eight generations on day 5, and all gated cells were generation 3 or above. Cell counting with count beads resulted in a mean 34.2% higher cell concentration for STF3 ($p=0.012$). Thus, we expected a right-shift of the generation distribution curve for STF3 + PRS. Surprisingly, STF3 + PRS yielded a relative increase in not only low-proliferating generation 3 and 4 cells, but also high-proliferating generation 9 and 10 cells (Fig. 1B and S3). This shows that, even though overall proliferation is inhibited as evaluated with cell counting – proliferation may be increased in subsets of CD34+ cells on day 5 when adding platelet releasate.

Platelet releasate does not affect basic cellular functions

To further examine the biological effects of stimulating CD34+ cells with platelet releasate, we analyzed basic cellular functions, such as cell cycle distribution, glucose uptake, mitochondrial membrane potential, ROS, and mRNA levels of stemness and differentiation markers. We found no significant difference in the frequency of cells in G0/G1 cell phase or in the uptake of 2-NBDG (Fig. 2A-B). We also did not find a clear trend in the expression of self-renewal or quiescence-associated genes, as HLF and GATA3 were downregulated but HOXB6 was upregulated (Fig. 2E). We had conflicting results for the mitochondria

membrane potential and ROS production for two different donors (Fig. 2C-D and S4). The MFI was higher for Donor 5 (the only donor where PRS increased proliferation) and lower for Donor 6 in STF3 + PRS for both mitochondrial membrane potential and ROS. Thus, these functions appear to depend more on the actual proliferation effect, rather than the culture conditions per se.

Platelet releasate does not drive hematopoietic progenitor cells to the megakaryocytic linage

Flow cytometric immunophenotyping and mRNA analyses were performed on day 12 to evaluate the differentiation of CD34+ progenitor cells. As an earlier study indicated that platelet releasate can stimulate megakaryocytopoiesis [45], we analyzed the cells for CD41 antigen (Fig. 3A and S5A) and NFE2, GPIBA, and ITGA2B mRNA expression (Fig. S5C). We found no or marginal differences in the frequency of CD41hi cells between culture conditions in 3/3 donors, and no trend in megakaryocytopoiesis-specific mRNA analysis in a single donor, indicating that platelet releasate does not stimulate megakaryocytopoiesis in mobilized peripheral CD34+ cells.

STF3 and STF3 + PRS do not induce granulocytopoiesis

In three different donors, we found almost no CD15+ cells and did not detect CD15hi cells on day 12 (Fig. 3A and S5A). This indicated that granulocytopoiesis does not progress above the promyelocyte level [46], probably because of a lack of G-CSF or GM-CSF. However, we observed a striking trend towards a decreased level of myeloperoxidase (MPO) mRNA for STF3 + PRS at all time points for all experiments (Fig. 2F, 3C, and Table S6). The relative expression of MPO has been reported to be higher in progenitor cells than in mature blood cells and to be upregulated in early neutrophil progenitors [47, 48]. The results were similar for CLEC12A mRNA levels (Fig. 2F and 3C), which are also known to be upregulated in neutrophil progenitors [47]. This could mean that platelet releasate inhibits granulocytopoiesis at the early

progenitor cell level, but further research is needed, including a stimulus that will terminally differentiate the progenitor cells.

Platelet releasate increases the expansion of erythroid progenitors from CD34+ cells

On day 12, we observed a trend towards a reduced fraction of CD71hi cells and lower mRNA levels for KLF1, EPOr, and GYPA in the presence of platelet releasate (Fig. 3C and S5A). However, there was also evidence of conservation of a small CD71lo population that was not seen when the platelet releasate was not added (Fig. 3B). Our culture systems lack EPO, which is generally thought to be indispensable for terminal differentiation of the erythroid lineage. Previous studies of erythropoiesis also imply that most CD71hi and CD235+ cells are downstream of EPO dependency (i.e., the CFUe level), and that early erythroid progenitors are CD71lo/mid [49]. Thus, analyses using our culture systems should be focused on the effects of platelet releasate on erythroid progenitors, as we did not expect any significant effect on terminal maturation. To explore this further, we performed additional flow cytometric immunophenotyping with anti-CD71 FITC and anti-CD235a APC on days 5, 7, and day 12. Even though we identified the same decrease in the relative distribution of CD71hi cells for STF3 + PRS on day 12, there was a clear trend towards conservation of a CD71lo/mid population (Fig. 4). In addition, on day 5, the frequency of CD235+ cells was higher for STF3 + PRS before the expression decreased significantly (Fig. S5B). The trend that platelet releasate conserve an erythroid cell phenotype on days 5 and 7 was confirmed by analyzing mRNA from these time points (Fig. 2F and Table S6). On day 12, we measured reduced KLF1 mRNA levels in 3/4 donors for STF3 + PRS. However, on day 7, we measured a slight increase in 2/2 donors and more than twice as high levels on day 5 for all erythroid-associated mRNAs in a single donor. To finally confirm the theory that platelet releasate conserves erythroid potential, we measured CFUe on day 7. As expected, the number of erythroid colonies was higher in STF3 + PRS

for all three donors analyzed, and the MFI of CD71 was significantly higher when all colonies were harvested (Fig. 5).

Platelet releasate stimulates differentiation into CD14+ and phagocytizing cells

Addition of platelet releasate led to a uniform increase in expression of monocyte differentiation antigen CD14 on day 12 (Fig. 3A and S5A). We also found an increase in the levels of CD14 and VCAN mRNA from day 5 onwards, whereas FCGR1B mRNA was elevated only in day 12 cells (Fig. 2F, 3C, and Table S6). Surprisingly, we could not find elevated levels of chemokine receptor CX3CR1 and CCR2 mRNA, which should increase upon monocytic maturation [50]. Although CD14 antigen expression is not restricted to monocytes, we know that the cells could not have been of granulocytic origin because CD15 antigen expression was either negligible or absent. To evaluate whether the CD14+ cells could be of dendritic cell lineage, we included ZBTB46 mRNA, which is a transcription factor specific for classical dendritic cells [51]. In our experiments, ZBTB46 mRNA levels were generally decreased with the addition of platelet releasate (Fig. 3C). CD14 is also expressed on macrophages. However, the CD14+ cells could easily be discriminated from THP-1 derived macrophages based on the light scatter properties (Fig. S6). These findings led us to believe that platelet releasate stimulates the differentiation of CD34+ stem and progenitor cells towards CD14+ cells of the monocytic linage.

In a single experiment, we cultured cells in STF3 for 1 week before washing and either continuing the culture with fresh STF3 or changing to STF3 + PRS (Fig. S5D). The cells were analyzed on day 15 for relative frequency of CD14+ cells, which was increased, proving that platelet releasate also could induce monocytopoiesis in more mature, cultivated progenitor cells.

One hallmark of mature monocytes is their phagocytic ability. To evaluate the differentiation into phagocytes in our culture systems, we

included the *E. coli* BioParticles assay (Fig. 6). We measured increased MFI in STF3 + PRS, indicating that platelet releasate induces differentiation of CD34+ cells towards phagocytes.

Discussion

There is increasing interest in studying platelet interactions with cells from a variety of origins. Platelet-derived products (e.g., platelet lysate or platelet releasate) are often added as a supplement for *in vitro* culture in expansion protocols intended for cell therapy [52]. However, this sort of experiments will also give valuable knowledge for understanding the variety of interactions that could have *in vivo* relevance for platelets in tissue regeneration, inflammation, and cancer biology [53-56].

Our results show a clear biological effect of platelet releasate on mobilized peripheral CD34+ cells. Platelet releasate at a concentration of 10% inhibited proliferation, conserved erythroid progenitors, and stimulated monocytopoiesis (Fig. 7). These findings imply that, although platelet-derived products may not play a role in non-linage driving *ex vivo* expansion of CD34+ progenitors as seen in mesenchymal stem cells [38], they seem applicable as a differentiation supplement in the cultivation of erythroid cells and monocytes.

Scarce evidence is available on the effects of platelet-derived products on hematopoietic progenitor cells and mostly limited to cells of cord blood origin. Noroozi Aghideh et al. [45] showed that platelet releasate inhibited the growth of CD133+ cord blood cells. In contrast to our findings, they observed a significant increase in the relative CD41+ cell frequency. However, there is evidence of some heterogeneity between CD34+ hematopoietic progenitor cells from different sources in the distribution of progenitor subsets and the *in vitro* response to growth factors, and between the CD133+ and CD34+ populations [57-59]. Whether the conflicting findings in our study regarding CD41+ cell frequency is de facto or stems from this heterogeneity is unknown. The distribution of generations on day 5 suggests that platelet releasate can

affect proliferation differently for different subsets of CD34+ progenitor cells. Therefore, one could speculate that the discrepancies stem from a potential skew of lineage-restricted progenitors between sources – that our samples had too few progenitors with megakaryocytic potential to yield a meaningful difference. However, this is unlikely, as the relative frequency of CD41+ cells was similar in both studies for an approximately similar "control" medium.

Platelet releasate is rich in both PDGF and TGF-β1 [45, 53, 60], which could influence the proliferation of CD34+ cells. PDGF has been shown to increase *ex vivo* proliferation of CD34+ cells of cord blood origin [19, 61], whereas TGF-β1 maintains quiescence in CD34+ hematopoietic stem and progenitor cells [62]. However, the platelet secretome extends vastly beyond these two cytokines and the effects of platelet releasate likely stem from a combination of different growth factors and other molecules.

Platelets are important actors in inflammation through interactions with several immune cells [63]. The interaction with mature monocytes is evident through multiple mechanisms: formation of monocyte-platelet complexes is increased during acute myocardial infarction [64], platelets modulate monocyte/macrophage responses in infection [65], platelet-derived β2-microglobulin, TGF-β1, and CXCL12 regulate monocyte survival and differentiation [56, 66], and platelet releasate has been found to polarize monocytes into the pro-angiogenic CD14++CD16+ phenotype [67]. We found that stimulation with platelet releasate yielded a significant increase in monocytes, or at least monocytic cells, as the relative frequency of CD14+ cells and expression of monocyte-associated mRNAs were increased. To the best of our knowledge, this is the first time a platelet-derived product has been shown to induce monocytopoiesis.

The generation of monocytes is generally thought to be regulated by M-CSF, but type 1 interferons also contribute, especially in emergency

monocytopoiesis [68]. However, none of these cytokines are found in platelets. TGF-β1 can effectively differentiate HL-60 cells into monocytes [69], but HL-60 cells are not the preferred model for studying monocytopoiesis. Lentiviral transfection of miR-142-3p, which is also present in platelet releasate [70], augments monocytic differentiation in both THP-1 cells and CD34+ cord blood cells, and to some extent in bone marrow-derived CD34+ cells from acute myelogenous leukemia (AML) patients [71]. This is relevant as the transfection yielded only a 1.9-fold increase in miR-142-3p expression, which is below what we have observed for several microRNAs in experiments with microRNA transfer from PMPs to THP-1 cells [72].

In summary, although we have not characterized the CD14+ cells with extensive immunophenotyping, we are confident that these cells represent the monocytic linage and think our findings should encourage further research on platelet releasate as a supplement for *ex vivo* monocyte production. This could also have relevance for the emerging field of dendritic cell-based cancer vaccines, as dendritic cells are often generated from either CD34+ progenitor cells through an intermediate monocyte stage or peripheral blood monocytes directly [73]. As for platelets, the central role of monocytes in cancer biology is becoming evident with both pro- and anti-tumoral properties [74]. Some hematological malignancies are also closely associated with or influenced by monocytic cells, such as AML and chronic myelomonocytic leukemia (CMML). For example, a monocyte-like CD14+ fraction of AML cells has been found to suppress T-cell function, blunting the immune response [75]. On the other hand, CMML is characterized by an accumulation of abnormal classical monocytes [76]. Therefore, we suggest that our results provide a basis for further research on the use of platelet inhibition in models of these diseases, as blocking a developmental stimulus for monocytes theoretically could be beneficial.

Here, we have also provided evidence that priming CD34+ cells for 7 days with platelet releasate and cytokines could conserve the erythroid potential compared to cytokines alone. This could be explained by conservation of a CD71lo/mid population in which the CFUe subset is localized [77]. However, cultivation without platelet releasate preserved a more easily recognizable CD71hi population and generally resulted in higher relative expression of erythroid-related mRNAs on day 12. Nevertheless, lower expression of erythroid genes does not necessarily mean that cells are in a less-differentiated stage, as the copy number per cell of important transcription factors (e.g., KLF1 and GFI1B) decreases throughout erythroid differentiation [49]. In addition, we consider these findings to have less significance at that time point because our culture conditions probably did not effectively stimulate erythropoiesis beyond the progenitor level. Even though EPO-independent erythroid maturation of hematopoietic stem and progenitor cells has been described [78], it is unknown whether either of our culture conditions provided sufficient stimulus for terminal maturation of erythroid progenitors. Stem cell factor or IL-3 alone or a combination of stem cell factor, IL-3, and IL-6 is at least not sufficient to drive *in vitro* erythropoiesis to the erythroblast stage within the timeframe of our study [49, 78]. Stimulation with TPO alone has, at the minimum, preserved cells with erythroid colony formation capacity [79], but the effect on terminal maturation is unknown. Juutistenaho et al. reported that a culture system with TPO, IL-3, and IL-6 produced both anucleated and benzidine-positive cells [80], but they used volume-reduced cord blood, not isolated CD34+ cells. Thus, the antigen expression of CD71 and CD235a on day 12 may not represent mature and functional erythroid cells. Cell culture expansion from day 5 to day 7 did not match the relative decrease in the relative frequency of CD71+ and CD235a+ cells, meaning that these cell subsets were expanding, but that is not equal to terminal erythroid differentiation.

Therefore, we chose to focus on the data from day 5 and day 7 cells, analyzing the conservation of early erythroid progenitors, as this seemed to be more relevant than erythroid maturation per se in the absence of EPO. Both CD71 and CD235a antigen expression categorically decreased with time, strengthening our argument. We observed an increase in the CFUe and CD71 MFI for all colonies on day 7 in the presence of STF3 + PRS, which corroborated the day 5 and 7 immunophenotyping findings, underlining our conclusion that the addition of platelet releasate increased erythroid progenitors compared to STF3 alone.

VEGF, which is found in platelet releasate [60], is known to increase commitment to early hematopoiesis and erythropoiesis in embryonic stem cell models [81]. However, the augmentation of erythroid development was only observed when the cytokine was added prior to hematopoietic commitment [82]; thus, it is unlikely that the presence of VEGF in the platelet releasate explains the observed difference in erythroid potential in our study. miR-451, which we identified in our platelet releasate batch (Table S3), is known to be an important regulator of erythropoiesis [83, 84]. Forced expression of miR-451 also promoted erythroid differentiation of the mouse erythroleukemia cell line MEL [85]. This could be a possible explanation for our findings, but we did not evaluate the transmission of this or other microRNAs after stimulation with platelet releasate.

Conclusion

We have shown that platelet releasate can inhibit the overall growth of CD34+ stem and progenitor cells, conserve erythroid progenitors, and stimulate monocytopoiesis. Our results warrant further research regarding the use of platelet releasate to supplement medium for erythroid and monocytic development in cell cultivation. This insight should also inspire the involvement of platelet interactions in models studying diseases involving erythropoiesis and monocytopoiesis.

Abbreviations

2-NBDG 2-[N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino]-2-deoxyglucose. **AML** Acute myelogenous leukemia. **APC** Allophycocyanin. **CFU** Colony forming unit. **CMML** Chronic myelogenous leukemia. **EPO** Erythropoietin. **FITC** Fluorescein isothiocyanate. **MEL** Mouse erythroleukemia cell line. **MFI** Mean fluorescence intensity. **mPBSC** Mobilized peripheral blood stem cells. **MPO** Myeloperoxidase. **MSCs** Mesenchymal stem cells. **PDGF** Platelet-derived growth factor. **PMP** Platelet microparticles. **PRS** Platelet releasate. **ROS** Reactive oxygen species. **STF3** Stem cell factor, thrombopoietin, FMS-like tyrosine kinase 3 ligand, interleukin-3. **TGF-ß1** Transforming growth factor beta 1. **TPO** Thrombopoietin

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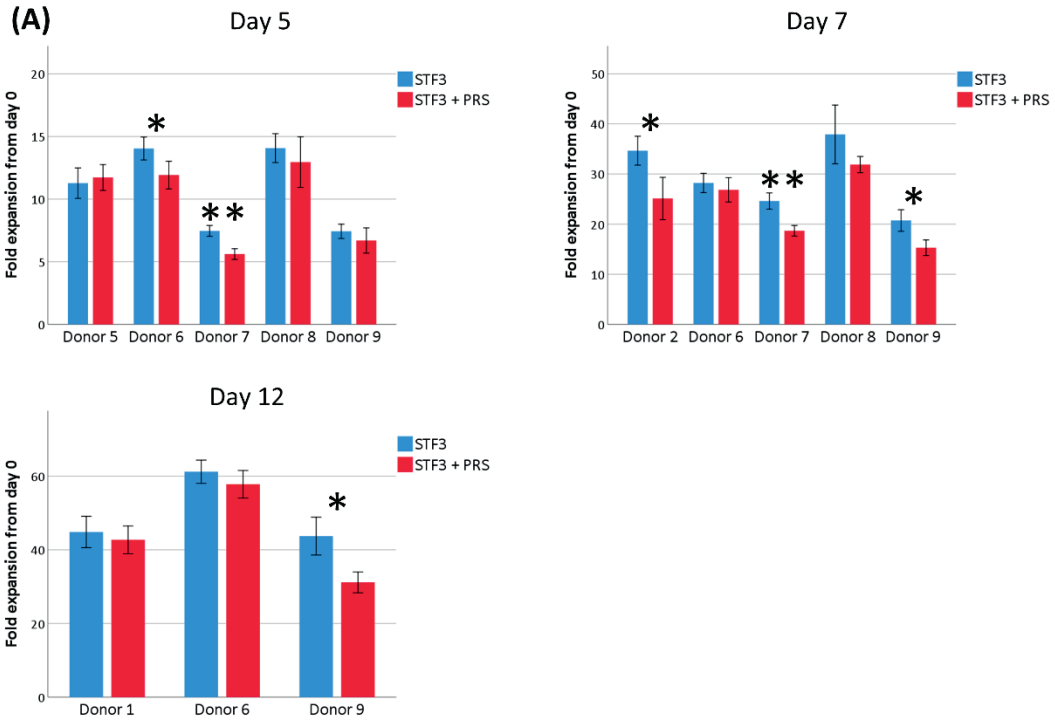
Figures and legends



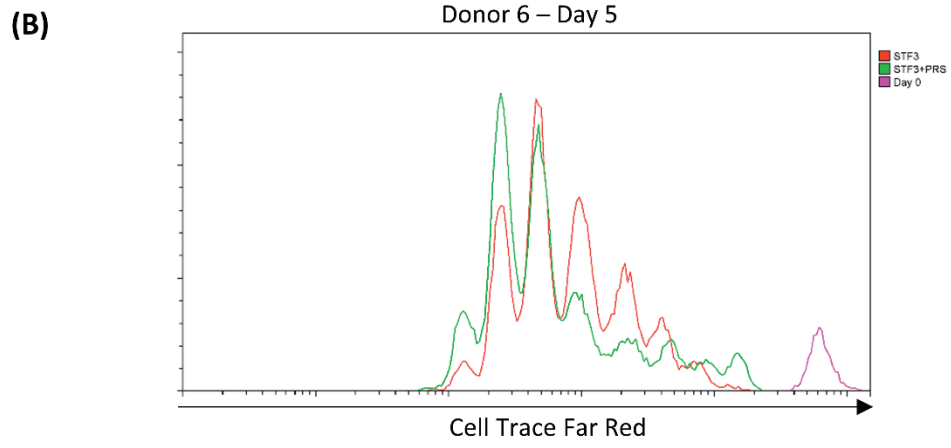


Figure 1

Proliferation analysis of cultured CD34+ cells. **A**, CD34+ cells were cultured with STF3 or STF3 + PRS and counted on days 5, 7, and 12 using flow cytometric count beads (n = 6 to 12). Data are presented as the mean fold expansion from day 0 with 95% CIs and analyzed by the paired sample t test. *$p<0.05$, **$p<0.001$. **B**, CD34+ cells were stained with Cell Trace Far Red and cultured for 5 days before analysis. Different cell generations were identified as distinct peaks in the flow histograms (n=6).

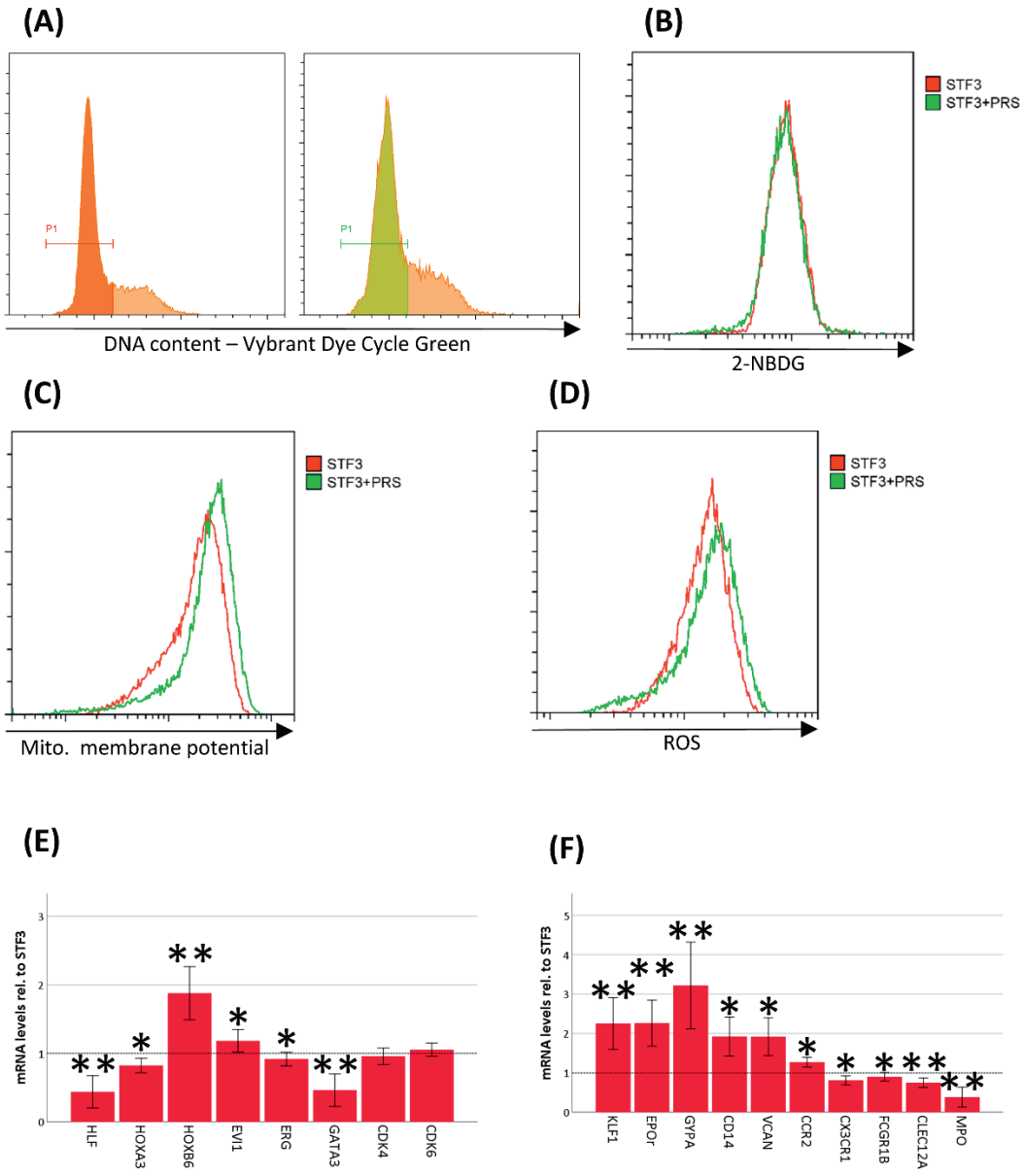


Figure 2

Effects of platelet releasate on basic cellular functions on day 5. **A**, Cell cycle analysis. The P1 gate represents G0/G1 cell phase (n=5, p=0.500).

B, 2-NBDG uptake (n=5, p=0.500). **C**, Mitochondrial membrane potential (n=4, p=0.068). **D**, Reactive oxygen species (n=5, p=0.043). Mean MFI values were compared by the Wilcoxon signed-rank test. **E**, Stemness or self-renewal mRNA markers (n=6). **F**, Differentiation mRNA markers (n=6). The mRNA data are presented as mean relative expression with 95% CIs compared to STF3 and normalized for β-actin and analyzed with the one-sample t test. A-D are derived from Donor 5, and E-F are derived from Donor 4. *p<0.05, **p<0.001.

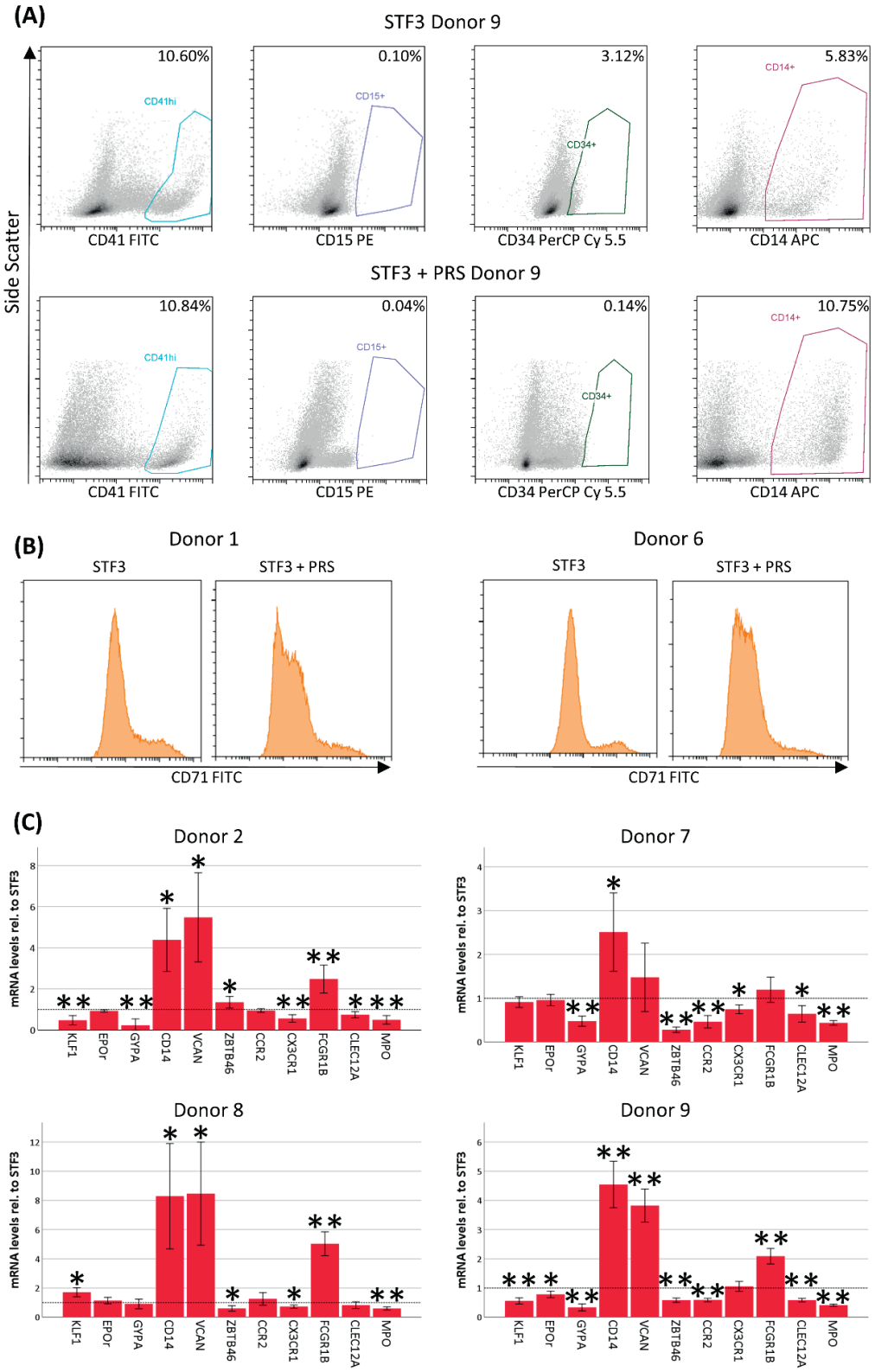

(A)
STF3 Donor 9
10.60%
CD41hi
CD41 FITC
0.10%
CD15+
CD15 PE
3.12%
CD34+
CD34 PerCP Cy 5.5
5.83%
CD14+
CD14 APC
Side Scatter
STF3 + PRS Donor 9
10.84%
CD41hi
CD41 FITC
0.04%
CD15+
CD15 PE
0.14%
CD34+
CD34 PerCP Cy 5.5
10.75%
CD14+
CD14 APC
(B)
Donor 1
STF3
STF3 + PRS
CD71 FITC
Donor 6
STF3
STF3 + PRS
CD71 FITC
(C)
Donor 2
Donor 7
Donor 8
Donor 9
mRNA levels rel. to STF3
KLF1
EPOr
GYPA
CD14
VCAN
ZBTB46
CCR2
CX3CR1
FCGR1B
CLEC12A
MPO

Figure 3

Differentiation analysis of cultured CD34+ cells on day 12. **A**, Flow cytometric immunophenotyping (n=5). "Cell gate" is set using FSC-A versus SSC-A plots. Antigen gates are set based on unstained samples using fluorescence versus SSC-A plots and annotated with the mean frequency relative to the parent population. See Fig. S1 and S2 for complete gating strategy. **B**, Flow histogram of anti-CD71 stained cells (n=9 for both donors). **C**, mRNA levels of differentiation markers (n = 5 to 6). The mRNA data are presented as mean relative expression with 95% CIs compared to STF3 and normalized for β-actin and analyzed with the one-sample t test. *p<0.05, **p<0.001.

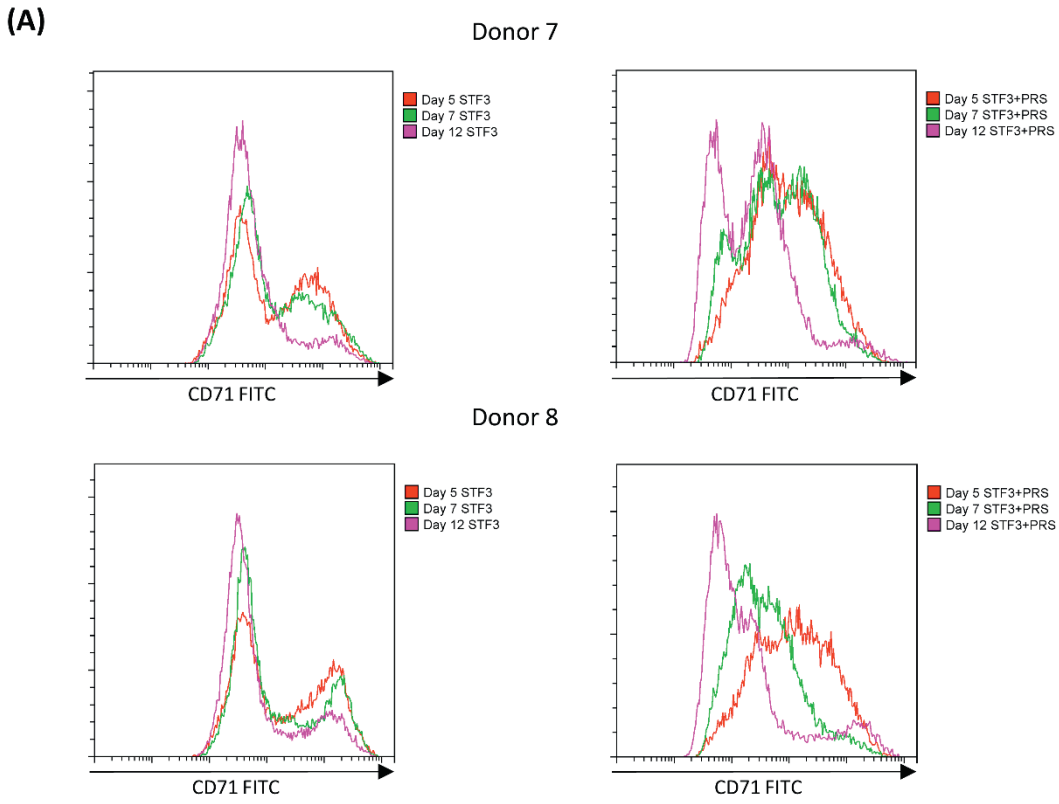
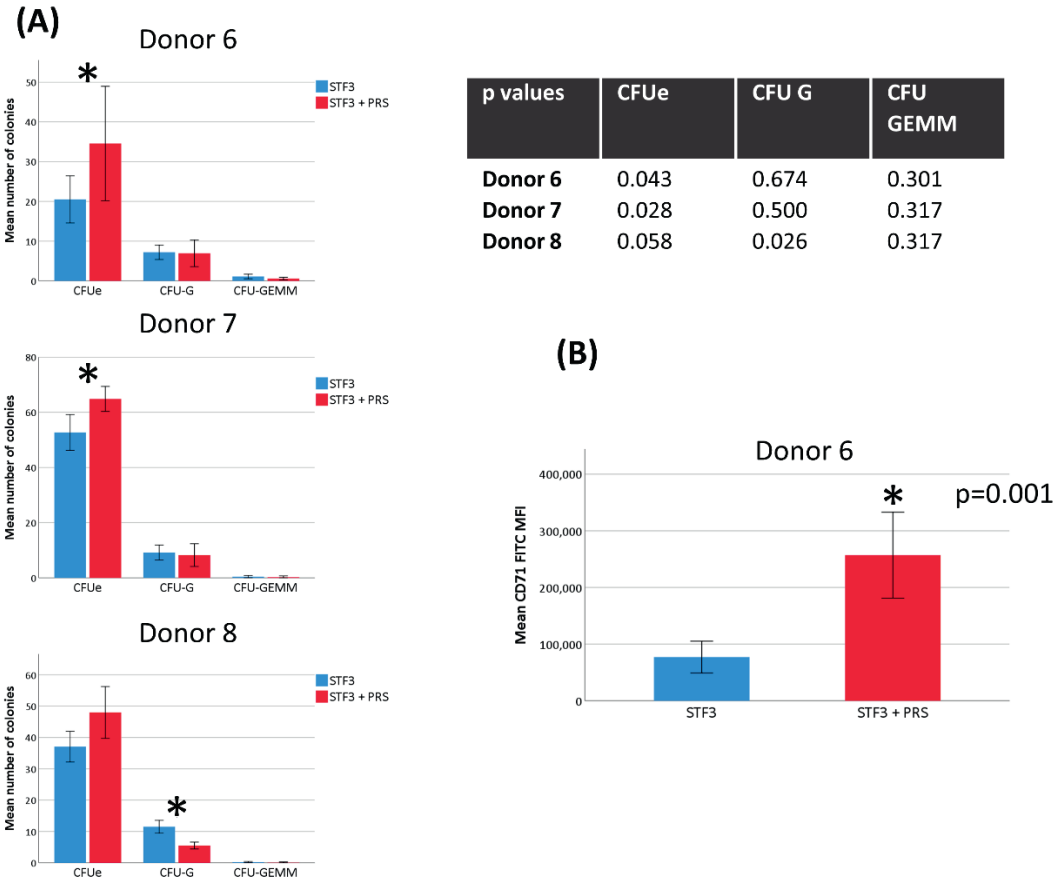


Figure 4

Flow cytometric analysis of CD71 at different time points. CD34+ cells were cultured under the described conditions and analyzed with anit-CD71 FITC on days 5, 7, and 12 (n=5).



p values	CFUe	CFU G	CFU GEMM
Donor 6	0.043	0.674	0.301
Donor 7	0.028	0.500	0.317
Donor 8	0.058	0.026	0.317

Figure 5

CFU analysis on day 7. Cells from expansion cultures were harvested and seeded in a methylcellulose-based medium at 900 cells/ml. **A**, Distribution of CFUs scored as CFUe, CFU-G, and CFU-GEMM (n = 6

to 7). **B**, CD71 MFI for harvested colonies. MFI was measured and background signal (unstained sample) subtracted. Data are presented as mean values with 95% CIs and analyzed by Wilcoxon signed-rank test for colony scores and paired sample t test for MFI data. *p<0.05.

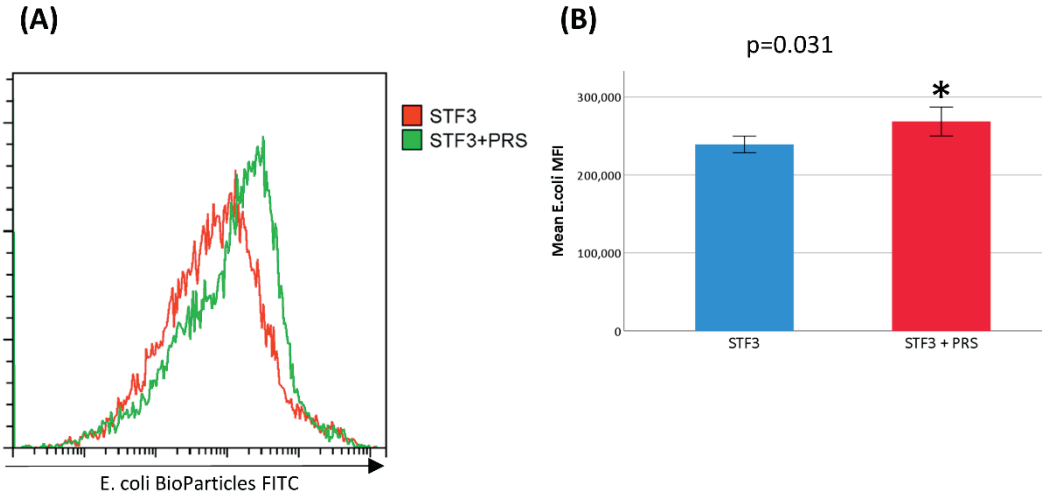


Figure 6

E. coli phagocytosis of cultured cells on day 12. **A, B**, Cultured CD34+ cells were analyzed for *E. coli* BioParticle uptake. MFI was measured and background signal (unstained sample) subtracted. The signal from bound, but not phagocytized, material was quenched with Trypan Blue (n=4). Data are presented as mean values with 95% CIs and analyzed by the paired sample t test. *p<0.05.

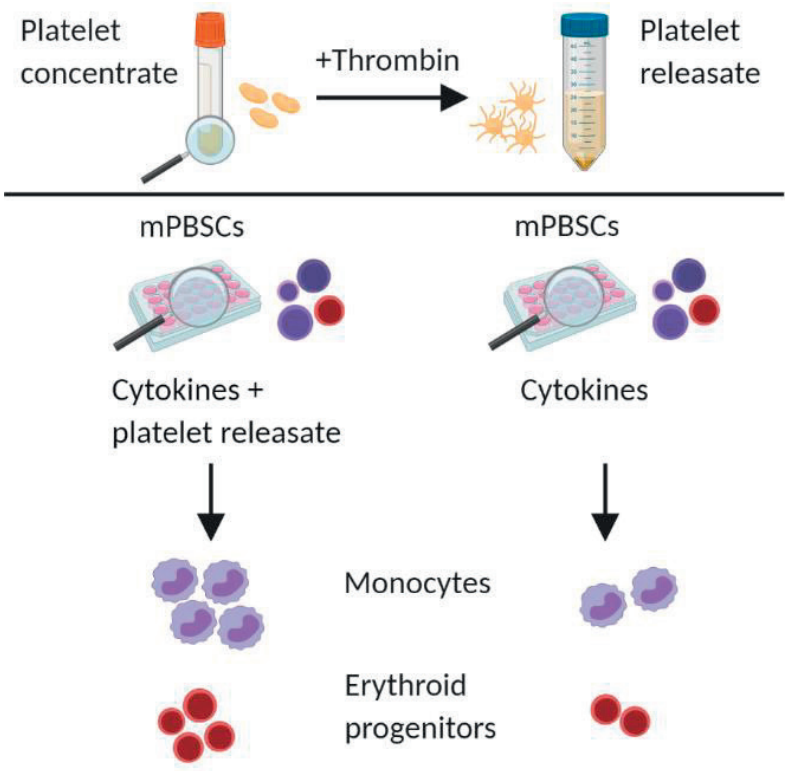
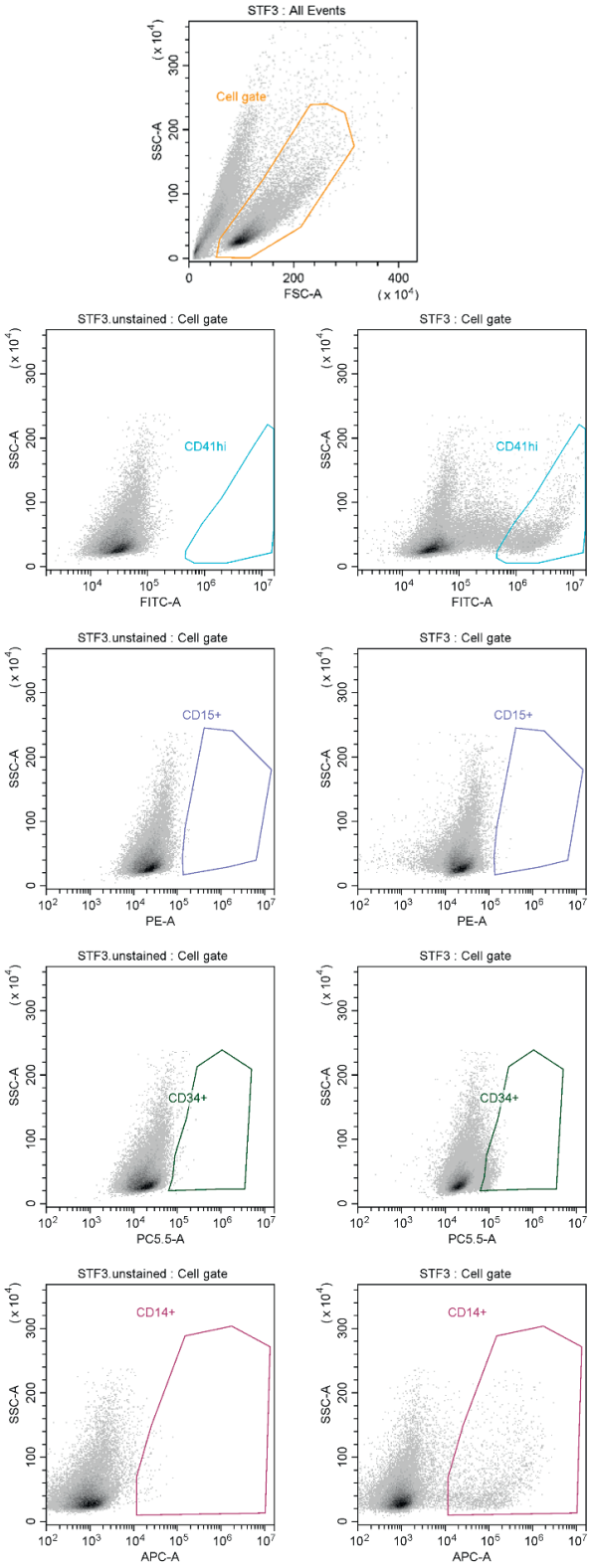
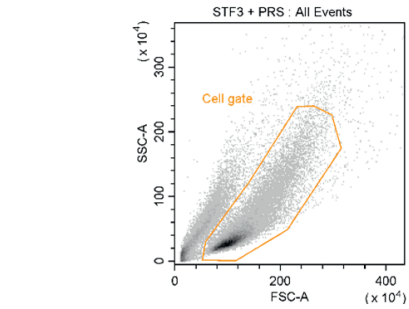
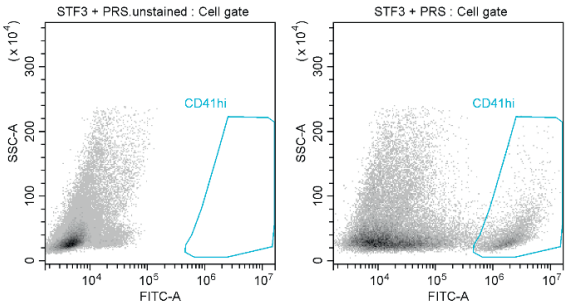


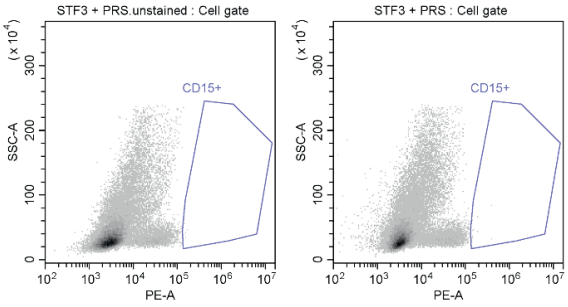
Figure 7

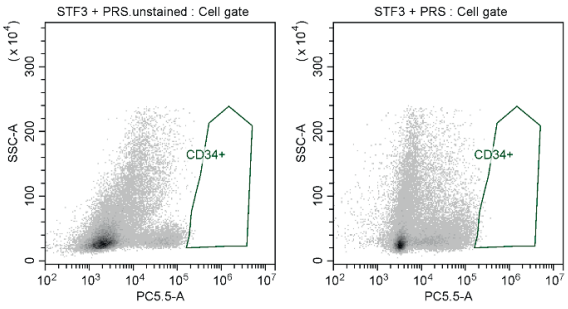
Schematic summary of the main effects of platelet releasate on mobilized peripheral CD34+ cells. Created with BioRender.com.


STF3 : All Events
Cell gate
SSC-A
FSC-A
STF3.unstained : Cell gate
CD41hi
FITC-A
STF3 : Cell gate
CD41hi
FITC-A
STF3.unstained : Cell gate
CD15+
PE-A
STF3 : Cell gate
CD15+
PE-A
STF3.unstained : Cell gate
CD34+
PC5.5-A
STF3 : Cell gate
CD34+
PC5.5-A
STF3.unstained : Cell gate
CD14+
APC-A
STF3 : Cell gate
CD14+
APC-A


STF3 + PRS : All Events
Cell gate
SSC-A
FSC-A
(x 10⁴)


STF3 + PRS.unstained : Cell gate
CD41hi
STF3 + PRS : Cell gate
CD41hi
SSC-A
FITC-A


STF3 + PRS.unstained : Cell gate
CD15+
STF3 + PRS : Cell gate
CD15+
SSC-A
PE-A


STF3 + PRS.unstained : Cell gate
CD34+
STF3 + PRS : Cell gate
CD34+
SSC-A
PC5.5-A

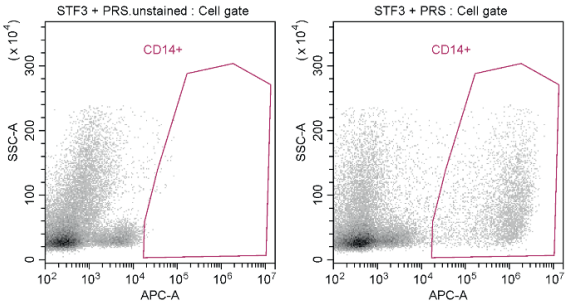

STF3 + PRS.unstained : Cell gate
CD14+
STF3 + PRS : Cell gate
CD14+
SSC-A
APC-A

Figure S1 and S2

Gating strategy for flow cytometric immunophenotyping for cells from Donor 9. Antigen gates for experiments with STF3 (Fig. S1) or STF3 + PRS (Fig. S2) were set either as "+" or "hi" populations as shown in the plots. This strategy was also representative for experiments with other donors or antigens not shown in the figure.

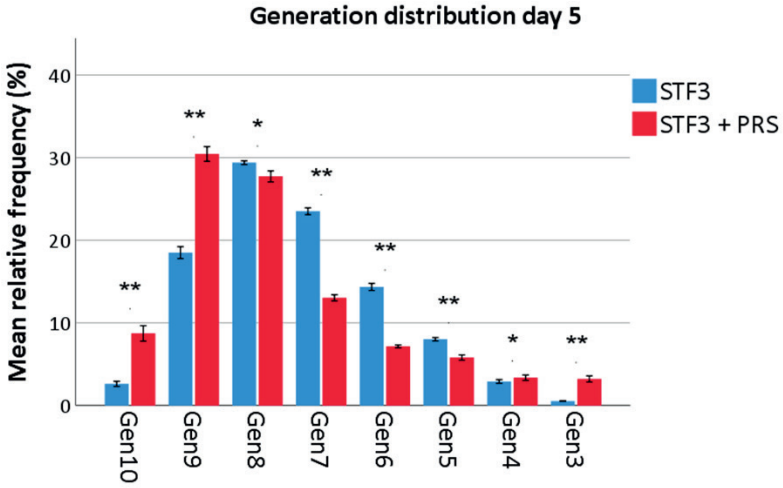


Figure S3

Generation distribution of cultured CD34+ cells on day 5. CD34+ cells were stained with Cell Trace Far Red on day 0 and analyzed for generation distribution after 5 days of culturing with STF3 or STF3 + PRS (Donor 6; n=6). Data are presented as the mean relative frequency of different cell generations with 95% CIs. *p<0.05, **p<0.001 using the paired sample t test.

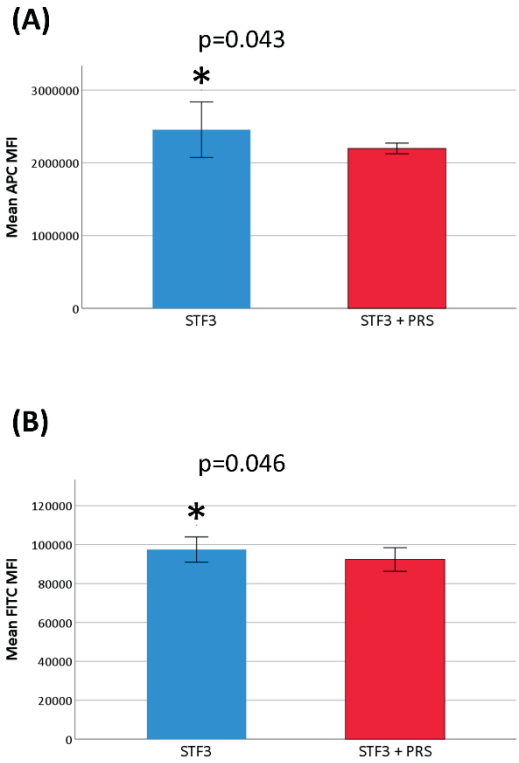


Figure S4

Effects of platelet releasate on basic cellular functions on day 5 in Donor 6. **A**, Mitochondrial membrane potential (n=5). **B**, Reactive oxygen species (n=5). Note the opposite trend from Donor 5 (Fig. 2C-D) and the difference in the proliferation effect of the platelet releasate for the two donors (Fig. 1). Data are presented as mean MFI values with 95% CIs. *p<0.05 using the Wilcoxon signed-rank test.

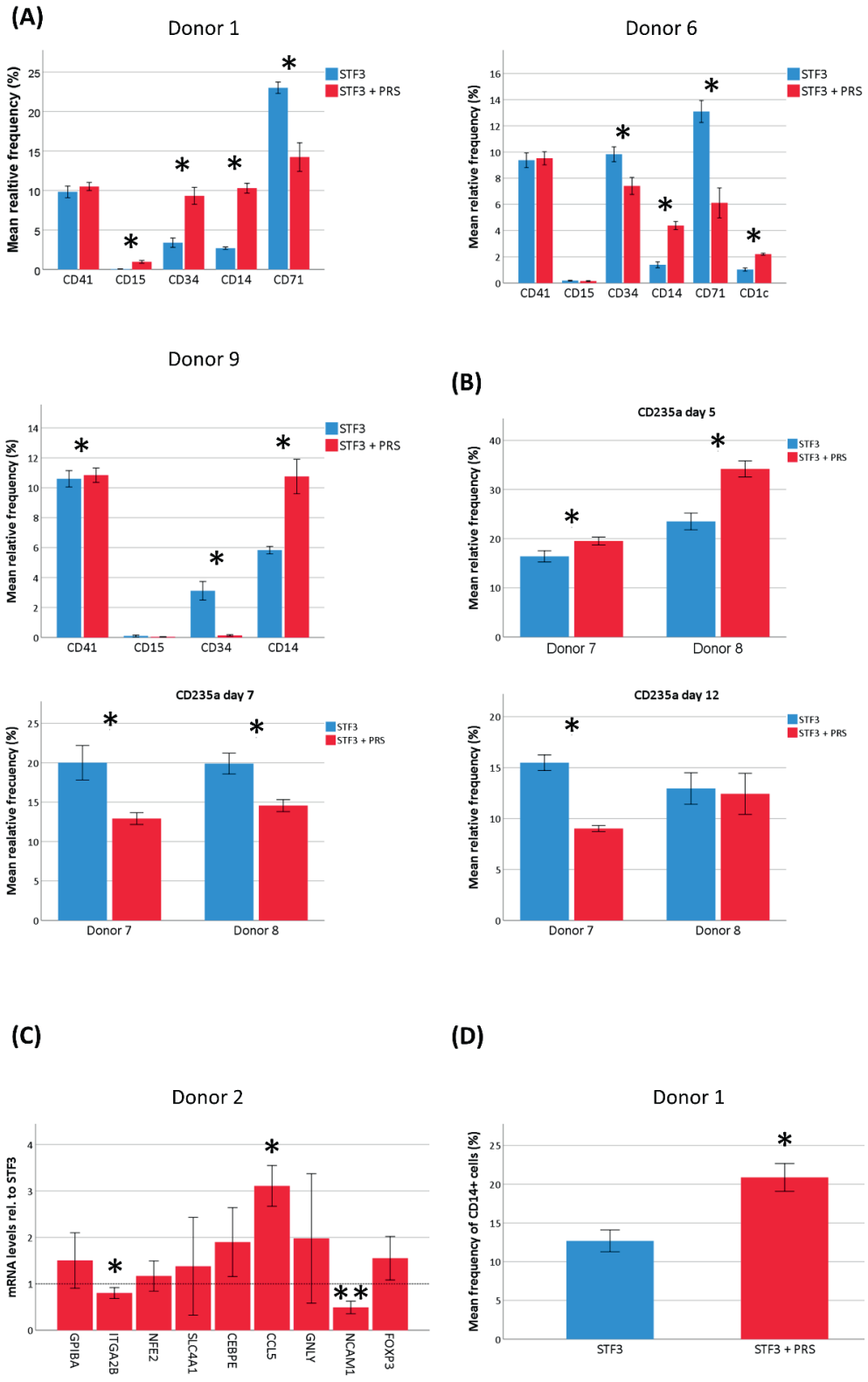

(A)
Donor 1
Mean realtive frequency (%)
STF3
STF3 + PRS
CD41
CD15
CD34
CD14
CD71
Donor 6
Mean relative frequency (%)
CD1c
Donor 9
(B)
CD235a day 5
Donor 7
Donor 8
CD235a day 7
Mean realative frecuency (%)
CD235a day 12
(C)
Donor 2
mRNA levels rel. to STF3
GPIBA
ITGA2B
NFE2
SLC4A1
CEBPE
CCL5
GNLY
NCAM1
FOXP3
(D)
Donor 1
Mean frequency of CD14+ cells (%)

Figure S5

Differentiation effects of the platelet releasate. **A-C**, CD34+ cells were cultured with STF3 or STF3 + PRS. **A**, Immunophenotyping on day 12 (n = 5 to 9). CD41 and CD71 gates were restricted to high fluorescence intensity events. Data are presented as the mean relative frequency with 95% CIs. **B**, Frequency of CD235a-positive cells on days 5, 7, and 12 (n=5). **C**, mRNA levels of markers only analyzed in donor 2 on day 12 (n=6). Data are presented as the mean relative expression with 95% CIs compared to STF3 and normalized to β-actin. **D**, Frequency of CD14+ cells on day 15 in cells cultured for 7 days with STF3 and then for 8 days with STF3 or STF3 + PRS (n=3). *$p<0.05$ using the Wilcoxon signed-rank test (**A-B**), the one-sample t test (**C**), or the paired sample t test (**D**).

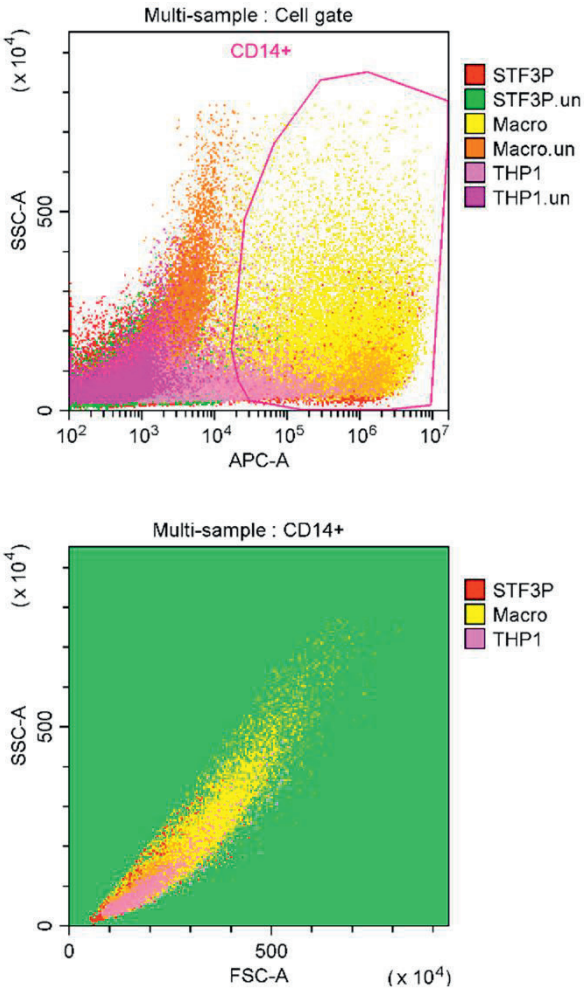


Figure S6

Light scatter properties of STF3 + PRS (STF3P) cultured CD34+ cells on day 12. FSC-A and SSC-A were compared between CD14+ THP-1 cells, CD14+ THP-1 derived macrophages, and CD14+ cells derived from STF3P stimulated mobilized peripheral CD34+ cells. CD14+ cells from STF3P align well with THP-1 cells, but have a significantly lower signal than macrophages (Macro) in both channels.

Table S1

Donor	
1	CountBright, immunophenotyping
2	CountBright, mRNA
3	No results included in the paper
4	mRNA
5	CountBright, cell cycle, 2-NBDG, mito. membrane potential, ROS
6	CountBright, Cell Trace, mito. membrane potential , ROS, E. coli, immunophenotyping, CFU
7	CountBright, longitudinal CD71 and CD235a, CFU, mRNA
8	CountBright, longitudinal CD71 and CD235a, CFU, mRNA
9	CountBright, immunophenotyping, mRNA

Overview of analyses in respective donors

Table S2

Protein content	30.29 mg/ml PRS
PMP quantification	4.58 x 106/ml PRS
RNA content	10.62 ng/ml PRS

Basic characteristics of the platelet releasate used in this study. Protein level is measured with the BCA assay. The value represents the average of three different dilutions of the platelet releasate in duplicate.

PMP quantification was performed with flow cytometric assessment of CD61+AnnV+ events relative to count beads in triplicate.

RNA content was measured using the Qubit RNA HS kit. The value represents an average of two separate isolations.

Table S3

MicroRNA	Cq value
miR-15a-5p	23.48
miR-26a-5p	18.51
miR-125a-5p	22.98
miR-125b-5p	25.52
miR-199a-5p	25.47
miR-223-3p	15.90
miR-451a	21.19

MicroRNA analysis of platelet microparticles. MicroRNA was isolated from isolated platelet microparticles. The input for PCR reactions was 443 pg of cDNA template. Data are presented as Cq values from a single run.

Table S4

Gene	**Assay ID**		**miR**	**Assay ID**
ACTB	Hs99999903_m1		**hsa-miR-15a-5p**	000389
CCL5	HS00982282_m1		**hsa-miR-26a-5p**	000404
CCR2	HS01560352_m1		**hsa-miR-125a-5p**	002198
CD14	Hs00169122_g1		**hsa-miR-125b-5p**	000449
CDK4	Hs00364847_m1		**hsa-miR-199a-5p**	000498
CDK6	Hs01026371_m1		**hsa-miR-223-3p**	002295
CEBPE	Hs00357657_m1		**mmu-miR-451**	001141
CLEC12A	Hs00370621_m1			
CX3CR1	Hs00365842_m1			
EPOr	Hs00959427_m1			
ERG	Hs01554629_m1			
EVI1	Hs00602795_m1			
FCGR1B	Hs00417598_m1			
FOXP3	Hs01085834_m1			
GATA3	Hs00231122_m1			
GNLY	HS01120727_m1			

GP1BA	Hs00173947_m1			
GYPA	HS01068072_m1			
HLF	Hs00171406_m1			
HOXA3	Hs00601076_m1			
HOXB6	Hs00980016_m1			
IRF8	Hs00175238_m1			
ITGA2b	Hs01116228_m1			
KLF1	Hs00610592_m1			
MPO	Hs00165162_m1			
NCAM1	HS00941830_m1			
NFE2	Hs00232351_m1			
SLC4A1	HS00978603_m1			
VCAN	Hs00171642_m1			
ZBTB46	HS00982282_m1			

Overview of mRNA and miR assays.

Table S5

Antibody	**Supplier**	**Clone**	**Catalogue number**
CD41 FITC	Miltenyi Biotec	REA386	130-120-719
CD71 FITC	Miltenyi Biotec	REA902	130-115-028
CD15 PE	Miltenyi Biotec	VIMC6	130-113-485
CD34 PerCP-Cy 5.5	BD	8G12	347222
CD1c APC	Miltenyi Biotec	REA694	130-110-537
CD14 APC	Miltenyi Biotec	REA599	130-110-520
CD61 APC	Miltenyi Biotec	Y2/51	130-117-369
CD235a APC	Miltenyi Biotec	REA175	130-118-356

Overview of conjugated antibodies in flow cytometry analyses.

Table S6

mRNA **Donor 7**	**Mean relative expression**	**CI95%**
KLF1	1.24	0.91–1.57
EPOr	1.24	0.80–1.68
GYPA	0.58	0.29–0.87
CD14	5.20	1.96–8.44
VCAN	5.51	0.13–10.89
MPO	0.41	0.18–0.64
mRNA **Donor 8**	**Mean relative expression**	**CI95%**
KLF1	1.27	0.53–2.02

EPOr	1.22	0.36–2.07
GYPA	1.11	0.98–1.23
CD14	3.12	-2.61–8.84
VCAN	1.74	-0.01–3.50
MPO	0.62	-0.48–1.71

mRNA levels in cultured CD34+ cells on day 7. In select experiments, day 7 cells from the expansion culture were harvested and analyzed for mRNAs associated with hematopoietic differentiation (n=3). Data are presented as mean relative expression of STF3 + PRS with 95% CIs compared to STF3 and normalized to β-actin.

Declarations

Ethics approval

The study was approved by the regional ethics committee, *Regional Etisk Komite Vest* (ref 2017/634).

Consent for publication

Not applicable.

Availability of data and material

Data are available upon reasonable request.

Conflict of interest

The authors declared no potential conflicts of interest.

Funding

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Authorship statement:

D.C: Literature review, conception and design, financial support, data collection and analysis, manuscript writing, and final approval of manuscript. O.N: Administrative support, manuscript writing, assisted with training in RT-PCR technique. P.M: Manuscript writing. T.H: Conception and design, financial support, administrative support, provision of study material, manuscript writing, and final approval of manuscript.

Acknowledgments

We would like to thank the Department of Immunology and Transfusion Medicine, Stavanger University Hospital, for providing the platelet concentrates used in this work.

Supplementary Methods

BCA protein assay

Total protein level was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). Absorbance of platelet releasate diluted in NaCl was compared to a standard curve of nine standards from 0 to 2 mg/ml bovine serum albumin.

Platelet microparticles isolation and measurement

Platelet microparticles were isolated as previously described [42]. Briefly, platelet releasate was centrifuged at 15,000 g for 90 minutes at room temperature and the supernatant discarded. The microparticles were resuspended in 400 µl of 0.22 µm filtered Annexin V Binding Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). Then 200 µl of the microparticle solution was transferred to a second tube and stained with 20 µl of Annexin V FITC (Milteny Biotec) and 2 µl of anti-CD61 APC (Miltenyi Biotec) and incubated for 15 minutes at room temperature. For an unstained control, the stains were swapped with 22 µl of filtered Annexin V Binding Buffer. After incubation, 278 µl of filtered Annexin V Binding Buffer and 50 µl CountBright beads (Thermo Fisher Scientific) were added before analysis. Microparticle gates were set using Megamix-PLUS FSC beads (BioCytex, Marseille, France) using the side scatter channel and gated according to our previous publication [42].

MicroRNA analysis in platelet microparticles

Microparticles were isolated as described above. Total RNA was isolated using the miRNeasy Kit (Qiagen GmbH, Hilden, Germany) and measured on a Qubit 2.0 (Thermo Fisher Scientific) with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific). Reverse transcription was performed with the TaqMan MicroRNA Reverse Transcription Kit

(Thermo Fisher Scientific). Finally, Real Time PCR was performed using the TaqMan MicroRNA Assays (Thermo Fisher Scientific) and the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) on the MX305P qPCR system. The input of total RNA in the reverse transcription reaction was 5 ng. The input of cDNA in the Real-time PCR reaction was 443 pg.

Comparison of light scatter properties

THP-1 cell line was purchased from ATCC (Manassas, VA, USA). Cells from an established culture in exponential growth phase, at concentration of 5 x 10^5 cells in IMDM + 10% fetal bovine serum (Sigma Adrich, St. Louis, MO), were stimulated with 100 ng/ml Phorbol 12-myristate 13-acetate (Sigma Aldrich) for two days. The medium was then changed and the cells rested for three additional days before harvest of adherent cells using the Non-Enzymatic Cell Dissociation Solution (ATCC). Macrophages and unstimulated THP-1 cells where then stained with anti-CD14 APC for flow cytometric analysis as previously described.

Paper II

Cacic, D.; Reikvam, H.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Protect Acute Myelogenous Leukemia Cells against Daunorubicin-Induced Apoptosis. Cancers 2021, 13, 1870.

cancers

MDPI

Article

Platelet Microparticles Protect Acute Myelogenous Leukemia Cells against Daunorubicin-Induced Apoptosis

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Simple Summary: Activated or apoptotic platelets both shed platelet microparticles that are proven to be internalized by many different cell types, including cancer cells. Here, we have investigated whether platelet microparticles can transfer their contents to the monocytic leukemia cell line THP-1 and if this could change cell activity and resistance to chemotherapy. We show that platelet microparticles were internalized by THP-1 cells and that platelet-associated microRNAs were elevated after a brief co-incubation. Furthermore, differentiation toward macrophages was induced and cell cycle progression, proliferation, and mitochondrial activity were decreased. Co-incubation with platelet microparticles increased chemotherapy resistance, which also was evident in acute myelogenous leukemia cells from patient samples, and it could be explained by the decrease in cell activity. Thus, platelet microparticles may have a role in the evolution of acute myelogenous leukemia and contribute to development of chemotherapy resistance, making them an interesting target for treatment.

Abstract: The role of platelets in cancer development and progression is increasingly evident, and several platelet–cancer interactions have been discovered, including the uptake of platelet microparticles (PMPs) by cancer cells. PMPs inherit a myriad of proteins and small RNAs from the parental platelets, which in turn can be transferred to cancer cells following internalization. However, the exact effect this may have in acute myelogenous leukemia (AML) is unknown. In this study, we sought to investigate whether PMPs could transfer their contents to the THP-1 cell line and if this could change the biological behavior of the recipient cells. Using acridine orange stained PMPs, we demonstrated that PMPs were internalized by THP-1 cells, which resulted in increased levels of miR-125a, miR-125b, and miR-199. In addition, co-incubation with PMPs protected THP-1 and primary AML cells against daunorubicin-induced cell death. We also showed that PMPs impaired cell growth, partially inhibited cell cycle progression, decreased mitochondrial membrane potential, and induced differentiation toward macrophages in THP-1 cells. Our results suggest that this altering of cell phenotype, in combination with decrease in cell activity may offer resistance to daunorubicin-induced apoptosis, as serum starvation also yielded a lower frequency of dead and apoptotic cells when treated with daunorubicin.

Keywords: acute myelogenous leukemia; platelets; microparticles; apoptosis

Citation: Cacic, D.; Reikvam, H.; Nordgård, O.; Meyer, P.; Hervig, T. Platelet Microparticles Protect Acute Myelogenous Leukemia Cells against Daunorubicin-Induced Apoptosis. *Cancers* **2021**, *13*, 1870. https://doi.org/10.3390/cancers13081870

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1. Introduction

Acute myelogenous leukemia (AML) is a bone marrow malignancy originating in hematopoietic stem and progenitor cells [1–3]. The average 5-year survival rate for de novo

disease is approximately 50% in younger patients [4], but this may vary widely depending on the occurrence of a selection of genetic aberrances. According to the 2017 European LeukemiaNet genetic risk stratification of AML, survival varies from 20% to over 60% [5]. Curative treatment involves intensive chemotherapy and, for select high-risk patient groups, the addition of consolidating treatment with allogenic stem cell transplantation, which carries the risk of a fatal outcome [6]. Thus, there is a need for a better understanding of tumorigenesis and evolution of the disease to improve treatment strategies.

Platelet–cancer interactions are becoming increasingly evident, and there is proof of cancer disease fundamentally altering the platelet transcriptome [7]. In aggregates with cancer cells, platelet function is hijacked to evade the NK cell response [8,9] and induce cancer cell epithelial–mesenchymal transition to facilitate metastasis [10,11]. Platelets are also important mediators for the development and maintenance of the cancer cell microenvironment [12,13].

Platelet microparticles (PMPs) are small membranous platelet particles (<1000 nm), which either bud off as a result of platelet activation [14] or as apoptotic bodies [15,16]. These microparticles are internalized by a variety of cell types, transferring their contents during this process [16–19]. PMPs contain a selection of the myriad of parent platelet alpha granule proteins [20,21] and platelet-associated microRNAs [22,23], which may potentially affect the biological behavior of the cells that have internalized them. This transfer of microRNAs has been demonstrated in a number of cancer models [24–26], where, although the effects are dependent on the cancer type and model, the PMPs appear to have both pro and anti-tumoral properties.

Targeting anti-apoptotic proteins is a novel strategy in the treatment of AML [27]. BCL2 is an important regulator of the intrinsic or mitochondrial apoptosis pathway, inhibiting BCL2 Antagonist/Killer (BAK) and BCL2 Associated X, Apoptosis Regulator (BAX) oligomerization, thus preventing pore formation in the outer mitochondrial membrane and subsequently leading to the leakage of cytochrome c and activation of caspase-9 [28]. Both platelet releasate and lysate seem to counter the effects of agents that specifically target this pathway, revealing an anti-apoptotic potential of platelets in AML [29]. There is also evidence that intrinsic apoptosis can be affected by the transfection of certain microRNAs, which are also found to be overexpressed in AML and present in platelets, indicating the potential relevance of these regulatory RNA molecules in an interaction between AML cells and platelets [30,31].

The role of microRNAs in AML is further supported by several studies showing an association of microRNA expression with mortality and chemotherapy resistance in patients, in whom several of the microRNAs are known to be present in high concentrations in platelets and platelet microparticles [32–34]. In this study, we aimed to assess whether PMPs could be taken up by AML cells and if this would change the AML cells' microRNA levels and in vitro chemotherapy resistance.

2. Results

2.1. Platelet-Associated microRNAs Are Increased in THP-1 Cells after PMP Co-Incubation

To examine whether platelet microparticles could be internalized by AML cells, we cultured cells from the monocytic AML cell line THP-1, with acridine orange (AO)-stained PMPs for 18 h. There was a PMP concentration-dependent increase of fluorescence in the co-incubated cells, and fluorescence microscopy revealed that that the stain was indeed dispersed within the cell nucleus and not located to bound microparticles (Figure 1A,B). To further investigate whether this PMP internalization could increase microRNA levels, we analyzed a selection of platelet-associated microRNAs in THP-1 cells after 18 h of co-incubation with PMPs. miR-125a-5p, miR-125b-5p, and miR-199-5p levels were all markedly increased (Figure 1C). This was particularly true for miR-199-5p, where levels were undetectable without PMP co-incubation in 2/3 samples (range 37.55–not detected), versus an average Cq value of 33.20 (range 33.13–33.24) with PMP co-incubation. These

findings give indirect proof that microRNAs can be transferred from platelets to THP-1 cells through PMP internalization.

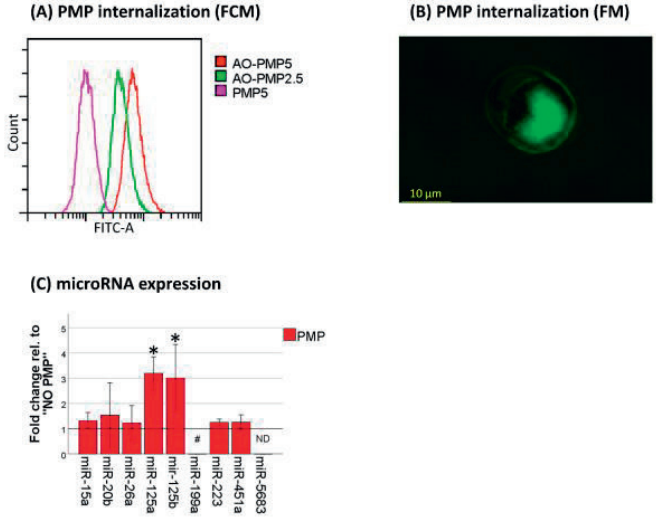

Figure 1. Internalization of platelet microparticles (PMPs) in THP-1 cells after 18 h of co-incubation. (**A**) Transfer of acridine orange from stained PMPs analyzed by flow cytometry (FCM). Histogram plot from a representative experiment ($n = 2$). Number following different PMP groups denotes the final concentration in million PMPs per mL medium. AO, acridine orange. (**B**) Transfer of acridine orange from stained PMPs analyzed by fluorescence microscopy (FM) at 400× magnification. (**C**) Changes in levels of microRNAs ($n = 3$). microRNA data were calculated as fold change from THP-1 without PMP co-incubation, normalized for *BCR*. p values were calculated using the one-sample t test. * $p < 0.05$. #, fold change was not calculated as levels were undetectable in 2/3 replicates for THP-1 without PMP co-incubation. ND, not detected.

2.2. PMPs Lead to Increased Resistance of THP-1 Cells to DNR

Both miR-125a-5p and miR-125b-5p have been associated with resistance to chemotherapy in retroviral transduction studies [30,31]. Therefore, we examined whether the cytotoxic effect of daunorubicin (DNR), a common front-line chemotherapeutic in AML, could be influenced by PMP internalization. Co-incubation of THP-1 cells with PMPs decreased the relative frequency of dead and apoptotic cells in a concentration-dependent manner following treatment with DNR (Figure 2A). Thus, for all other analyses, PMPs were co-incubated at a concentration of 1.5×10^7 per mL medium, unless otherwise specified, as this generated the highest chemoprotective effect. Vector control experiments, where the supernatant of isolated PMPs was added to Iscove's Modified Dulbecco's Medium (IMDM) + 10% FBS medium at a concentration of 5%, only had a small and non-significant effect on resistance to DNR ($p = 0.109$).

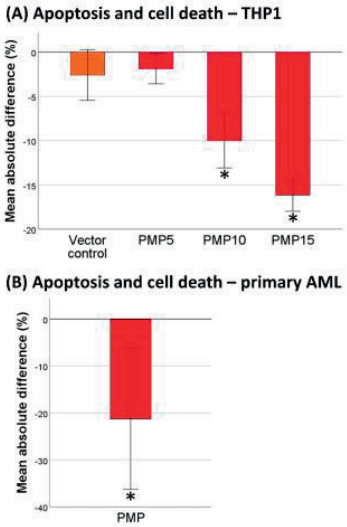

Figure 2. Apoptosis and cell death in THP-1 and primary acute myelogenous leukemia (AML) cells treated with 0.5 μM daunorubicin. (**A**) Difference in frequency of dead and apoptotic cells in THP-1 cells with or without co-incubation with platelet microparticles (PMPs) ($n = 3$). Number following different PMP groups denotes final concentration in million PMPs per mL medium. (**B**) Evaluation of the effect of PMPs on the frequency of dead and apoptotic cells in primary AML cells (nine patient samples, $n = 4$). p values were calculated using the one-sample t test (test value = 0). * $p < 0.05$.

2.3. Co-Incubation of Primary AML Cells with PMPs Also Increased Resistance to DNR

As there are limitations for the clinical relevance of cell line AML models [35], we examined whether the chemoprotective effect of PMPs could be observed in cells derived from AML patients. Using a similar approach, albeit with serum-free conditions, our results showed an identical effect on primary AML cells where we identified a significantly lower frequency of dead and apoptotic cells when PMPs were added (Figure 2B). The average absolute reduction in dead and apoptotic cells in individual patient samples ranged from 0.2 to 55.9% and was significant in 8/9 patients (Figure S1). One-way ANOVA analysis of the effects on THP-1 cells of PMPs from different releasates (when used in the primary AML experiments as quality controls) revealed no significant inter-releasate batch difference ($p = 0.823$).

2.4. THP-1 Cells Co-Incubated with PMPs Had Lower Caspase-9 Activity Following DNR-Treatment

Using microRNA databases (miRDB [36] and TargetScanHuman [37]), we found several predicted target mRNAs for miR-125a-5p and miR-125b-5p with important roles in the intrinsic apoptotic pathway, such as the pro-apoptotic BCL2 family proteins, BCL2 Modifying Factor (BMF) and BAK1 [38]. To investigate whether PMP internalization could influence the intrinsic apoptotic pathway, THP-1 cells were co-incubated with PMPs and treated with DNR using the established approach. Then, the cells were analyzed

for caspase-9 activation, which is a downstream effect of mitochondrial outer membrane permeabilization (MOMP). There was a lower frequency of caspase-9 positive cells in the THP-1 cell cultures co-incubated with PMPs (Figure 3), suggesting that the chemoprotective effect of PMPs could be the result of the effects on the intrinsic apoptotic pathway upstream of caspase-9 activation.

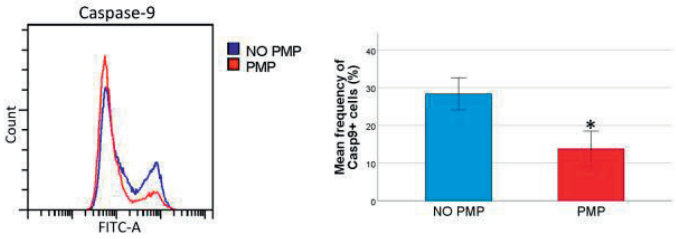

Figure 3. Caspase-9 activation in daunorubicin (DNR)-induced cell death. DNR-treated THP-1 cells were analyzed for caspase-9 activation ($n = 3$). Caspase-9 positive cells were identified as the distinct second peak in the flow histogram. p values were calculated using the paired-sample t test. * $p < 0.05$.

2.5. Decreased Cell Activity Protected THP-1 Cells against DNR

Cytotoxic chemotherapy is believed to be most potent in highly proliferating cancer cells. Inducing cellular dormancy to decrease DNA replication should theoretically offer a chemoprotective effect, as it would prevent DNR-triggered DNA damage. Thus, we investigated whether serum starvation would decrease cell activity and subsequently protect THP-1 cells against DNR. We analyzed proliferation, cell cycle distribution, and mitochondrial membrane potential, and we observed that 48 h of serum starvation in THP-1 cells induced a significant growth arrest (Figure 4A–C). To evaluate whether serum starvation affected DNR-resistance, we compared apoptosis and cell death in THP-1 cells, with or without serum starvation, 24 h after treatment with 0.5 µM DNR. We showed a marked reduction in the frequency of dead and apoptotic cells (Figure 4D).

To investigate whether the apparent chemoprotective effect of PMP co-incubation may be the result of a similar decrease in cell activity, we measured the effects of PMPs on cell proliferation, cell cycle distribution, and mitochondrial membrane potential. Our results showed that co-incubation with PMPs increased the frequency of cells in the G0/G1 cell phase, reduced mitochondrial membrane potential, and inhibited cell proliferation (Figure 5A–C). These findings lead us to believe that PMPs may protect THP-1 cells from DNR-induced cell death by partially inhibiting cell cycle progression and proliferation. Co-incubation with PMPs did not alter mRNA or protein levels of CDK4 (Figure 5D,E), which is fundamental for THP-1 viability and normal cell cycle progression [39,40].

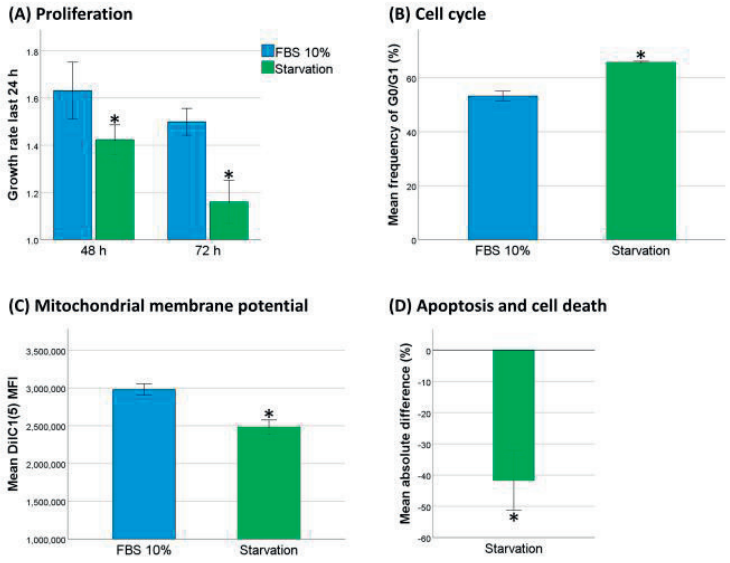

Figure 4. Effects of serum starvation on cell activity and daunorubicin (DNR)-resistance in THP-1 cells. (**A**) Daily proliferation rate analyzed by flow cytometric counting ($n = 4$). (**B**) Cell cycle analysis after 48 h of serum starvation ($n = 3$). Cells in the G0/G1 cell phase were gated. (**C**) Mitochondrial membrane potential after 48 h of serum starvation ($n = 3$), mean fluorescence intensity (MFI) data. (**D**) Difference in DNR-induced cell death and apoptosis after 48 h of serum starvation compared to standard conditions. (**A**–**C**) were compared using the paired-sample t test. (**D**) was compared using the one-sample t test (test value = 0). * $p < 0.05$.

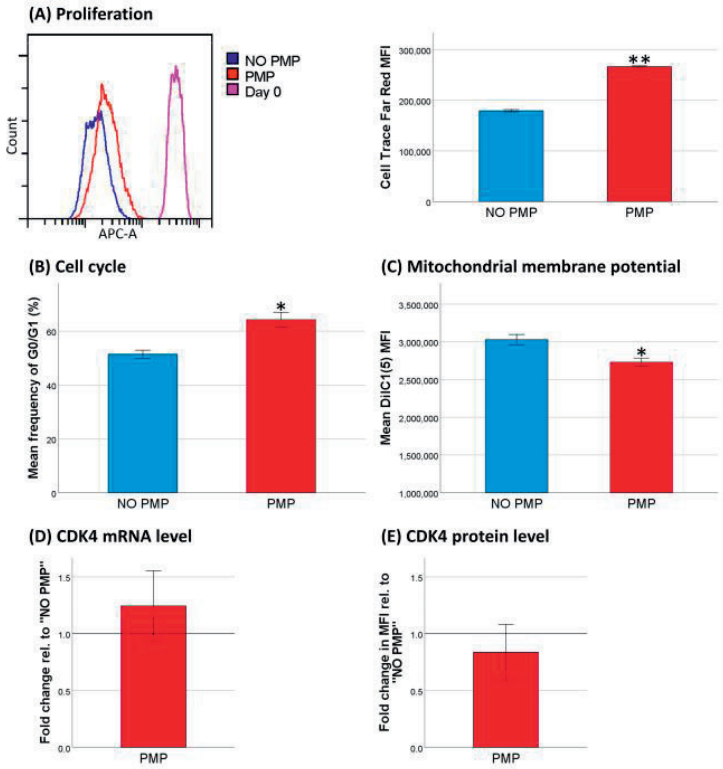

Figure 5. Effects of platelet microparticles (PMPs) on cell activity in THP-1 cells. (**A**) Proliferation analysis by Cell Trace Figure 5. (n = 5). (**B**) Cell cycle analysis (n = 3). Cells in G0/G1 cell phase were gated. (**C**) Mitochondrial membrane potential (n = 4), mean fluorescence intensity (MFI) data. (**D**) CDK4 mRNA levels (n = 3). (**E**) CDK4 protein levels (n = 5). (**B**–**E**) were analyzed after 24 h of PMP co-incubation. mRNA data are calculated as fold change from THP-1 without PMP co-incubation, normalized for *ACTB*. Protein data are calculated as fold change in MFI from THP-1 without PMP co-incubation. Data were compared using the paired-sample t test for data pairs or the one-sample Wilcoxon signed-rank test for ratios. * $p < 0.05$. ** $p < 0.001$.

2.6. PMP Co-Incubation Increased Differentiation of THP-1 Cells toward Macrophages

THP-1 cells are capable of macrophage differentiation, leading to cell growth arrest. Thus, we wanted to examine if increased differentiation of the cells could contribute to the observed decrease in cell cycle progression. PMP co-incubation increased both forward scatter and side scatter (Figure 6A), indicating increased cell size and granularity, which are two hallmarks of macrophage differentiation [41]. Surprisingly, we could not

corroborate the forward scatter findings with measurement of cell cross-sectional area using the particle analysis function in the ImageJ software with pictures taken under an inverted phase-contrast microscope (Figure 6B). However, co-incubation with PMPs led to a significant increase in CD14 antigen expression (Figure 6C). Thus, the decrease in cell cycle progression, and therefore part of the chemoprotective effect, could stem from differentiation of the cells toward macrophages.

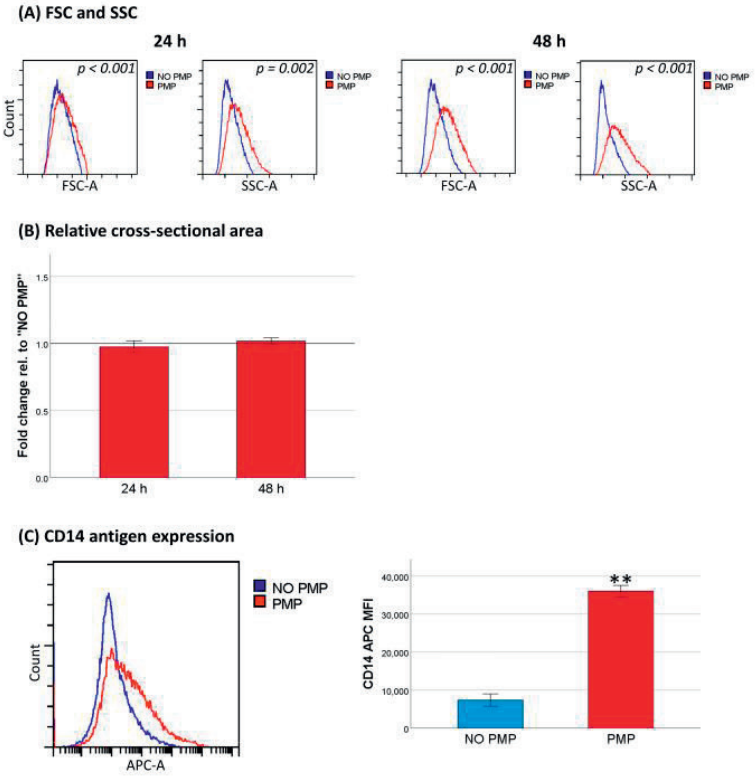

Figure 6. Phenotypical changes induced by platelet microparticles (PMPs) co-incubation. (**A**) Forward scatter (FSC) and side scatter (SSC; $n = 4$). (**B**) Ratio of cell cross-sectional area ($n = 4$). (**C**) CD14 antigen expression after 48 h of culture ($n = 5$). Data were compared using the paired-sample t test for data pairs or the one-sample t test for ratios. ** $p < 0.001$.

3. Discussion

The important role of platelets in cancer development, progression, and metastasis is becoming increasingly clear, and there are several known mechanisms in this interplay, including the transfer and uptake of platelet microparticles. We showed that co-incubating primary AML cells and THP-1 cells with PMPs increased their DNR resistance. In addition we demonstrated the inhibition of THP-1 cell proliferation, cell cycle progression, mitochondrial membrane potential, and induction of differentiation toward macrophages.

We observed internalization of PMPs by THP-1 cells and a subsequent increase of platelet-associated microRNAs. The internalization was observed using AO-stained PMPs. AO will stain DNA, RNA, and acidic vesicles. Platelets do not contain DNA, other than a small amount of mtDNA. However, platelets and PMPs both contain RNA and lysozomes, making AO staining suitable for our purpose.

Co-incubation of THP-1 or primary AML cells with PMPs increased their resistance to DNR-treatment at a concentration of 0.5 μM, which is close to the peak plasma concentration measured in patients receiving 60 mg/m^2 DNR [42]. In AML, DNR is routinely administrated at dosages of 45–90 mg/m^2 [43]. This observed chemoprotective effect could be the result of microRNA transfer from PMPs. The overexpression of both miR-125a and miR-125b have been associated with DNR resistance in AML cell lines, including THP-1 [30,31]. Using the transduction of THP-1 cells with murine stem cell virus (MSCV), these studies obtained a 4 and 4.9-fold increase of miR-125a and miR-125b. In our study, the respective levels were increased 3.0 and 3.2-fold. The transduction of miR-125a and miR-125b resulted in downregulation of the apoptotic proteins Grk2 and Puma [30,31]. The latter is a member of the pro-apoptotic BH3-only proteins of the intrinsic apoptosis pathway [38], where we show a decrease in activation with PMP co-incubation. These proteins are known to be crucial participants in apoptosis, as genetic knockout models are protected against several apoptotic stimuli [44]. However, the cited studies with the transduction of miR-125a and miR-125b did not include an analysis of cell activity in the THP-1 cells associated with microRNA overexpression, which may be decisive for the actual DNA-damage induced by DNR. Other studies have linked the ectopic expression of miR-125a with proliferation inhibition, although in solid tumor cell lines [45,46].

PMPs contain several hundred different proteins and small RNAs, meaning the underlying mechanism for chemoprotection are likely more complex than that reflected in the single microRNA transduction studies. The anti-cancer effect of DNR and other anthracyclines are believed to mainly be a result of interference with topoisomerase II (Top2) enzyme activity [47]; however, other mechanisms have been identified [48]. Top2 introduces double-strand DNA breaks during replication [47]; thus, an inhibition of proliferation should decrease the efficiency of Top2 poisons. We showed that decrease of cell activity through serum starvation protects THP-1 cells against DNR-triggered apoptosis and cell death, and we suggest that PMPs could offer chemoprotection through this mechanism.

Vasina and colleagues have previously shown that microparticles from apoptotic platelets can induce macrophage differentiation in THP-1 cells after 7 days of co-incubation [16]. Here, we show prominent upregulation of CD14 antigen already after 48 h using platelet microparticles from activated platelets from platelet concentrates containing a mixture of PMPs generated by activation and apoptosis, better resembling the in vivo milieu. Seemingly, there are conflicting results regarding cell size analyses, as forward scatter and side scatter were increased, but the measured cross-sectional cell area was unchanged. However, we believe the increase in light scatter was affected by morphological changes with more vacuolization in the cells treated with PMPs.

The observed differentiation effect can at least partially explain the decrease in cell cycle progression, as THP-1 cells treated with phorbol myristate acetate for macrophage differentiation only exit G1 phase to a little extent [49]. CDK4 mRNA and protein levels were unchanged, and the decrease in cell cycle progression would appear to be the result of a downstream target. However, notable downregulation of the CDK4 gene is known to be a later event in macrophage differentiation of THP-1 cells [49]. We have not identified the

exact substances that initiate differentiation or lead to the inhibition of cell cycle progression. The latter could partially be an independent process, because contrary to our findings, mitochondrial activity is increased with macrophage differentiation [41]. Transforming growth factor beta (TGF-β) is a potent cell cycle regulator known to be present in platelets. TGF-β induces dormancy or quiescence through several mechanisms, but it cannot be entirely responsible for the observed chemoprotective effect of PMPs, as it is known to be abundant in the platelet secretome [50,51], and we did not observe any significant effects on the frequency of dead and apoptotic cells in our vector control experiments.

Several research groups have reported that PMPs affect cancer development. Michael and colleagues showed that PMPs could infiltrate solid tumors and inhibit the growth of lung and colon cancer [26]. Others have linked PMPs to increased epithelial–mesenchymal transition and metastatic capacity in ovarian cancer [25] and lung cancer invasion [24]. Recent evidence has shown that platelets can have a bimodal effect in colorectal cancer where they inhibit growth but promote metastasis [52]. An extensive review of the role of PMPs in cancer progression can be found elsewhere [53].

Our protocol for the quantitation of microparticles has some limitations, as the PMP number per mL releasate varied on average by 10.0%, but it ranged from 0.4 to 34.9% between technical replicates. Thus, the final concentration of PMPs in the culture media may have varied extensively in some experiments. Accordingly, the concentration of microRNAs and proteins will vary from batch to batch of platelet concentrates. We accounted for the latter when we chose to use pooled platelet concentrates derived from four different donors. Furthermore, we found no inter-batch differences with respect to chemoprotective effect.

The induction of resistance to DNR by PMPs could have significant clinical relevance. Inhibiting the production of PMPs may present a potential therapeutic approach in AML to increase chemosensitivity. This can easily be achieved with platelet inhibition [54]. Platelet inhibition has also previously been linked to both lower cancer incidence and improved cancer-specific survival [55–58], although the exact mechanism is unknown. On the flip side, the differentiation of AML cells by PMPs might be beneficial to inhibit evolution of the disease.

4. Materials and Methods

4.1. Cell Line

The THP-1 cell line was purchased from ATCC (American Type Culture Collection; Manassas, VA, USA) and maintained in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) + 10% FBS (Sigma Aldrich, St. Louis, MO, USA). Only cells in the exponential growth phase were used, and cultures were kept for less than three months.

4.2. Primary AML Cells

Primary AML cells were isolated by density gradient separation of peripheral blood from consenting patients at the Department of Medicine, Section of Hematology, Haukeland University Hospital (Bergen, Norway). The cells were cryopreserved in liquid nitrogen until use. The cryopreservation solution consisted of insulin-free RPMI 1640 (Sigma Aldrich), supplemented with 10% dimethylsulfoxide and 20% FBS. Primary AML cells were cultured in StemSpan Serum-Free Expansion Medium (Stem Cell Technologies, Vancouver, BC, Canada) with the addition of the following recombinant cytokines in a final concentration of 20 ng/mL: stem cell factor (Peprotech EC, London, UK), G-CSF (Peprotech), and FMS-like tyrosine kinase 3 ligand (Peprotech). Charateristics of the AML patients can be found in Table 1.

Table 1. Characteristics of primary AML patients.

#	Sex	Age	Prev. Myeloid Disease	FAB	Cytogenetics	FLT3	NPM1	CEBPA	CD11b	CD14	CD33	CD34	CD45	CD64	CD117	HLA-DR	L-MPO
1	M	22	No	M5	del(9)	ITD (low ratio)	wt		neg	neg	pos	pos	dim		pos	pos	dim
2	F	60	PV		del(7)	wt	wt	wt	neg	neg		pos	dim		pos	pos	
3	F	56	No	M4	inv(16)	wt	wt		neg	neg	pos	pos	dim	neg	pos	pos	pos
4	M	44	No	M4	inv(16)	wt	wt		neg	neg	neg	neg	dim	dim	neg	pos	
5	F	92	No	M1					neg	neg	dim	neg	dim	dim	pos	pos	pos
6	M	49	No	M4	45, XY	wt	ins	wt	neg	neg		neg	dim	dim	pos	pos	
7	M	76	No	M5	Normal	wt	ins	wt	hetero	hetero		neg	hetero	pos	hetero	pos	
8	F	95	No	M4	Normal	wt	wt	wt				dim	dim		dim		
9	M	29	No	M4	Normal	ITD (high ratio)	wt	wt	neg	neg	neg	pos	dim	neg	neg	pos	pos

patient number.

4.3. Platelet Concentrate

Routinely prepared platelet concentrates pooled from four donors (Tacsi system; Terumo BCT, Lakewood, CO, USA) were provided by the Department of Immunology and Transfusion Medicine, Stavanger University Hospital (Stavanger, Norway), after written consent from the donors. The platelet concentrations were 0.88–1.08 $\times$ 10^9 per mL. Leukocytes were removed by filtration to a residual level of <1.00 $\times$ 10^6. In the final concentrate, the storage medium contained approximately 65% additive solution (PAS-III, Baxter, Lake Zurich, IL, USA) and 35% plasma.

4.4. Platelet Releasate

The platelet concentrate was transferred from the blood bag to separate 50 mL tubes and incubated with a final concentration of 1 U/mL human thrombin (Sigma Aldrich) for one hour in a 37 °C water bath. The tubes were gently agitated every 5 min. The platelet releasate was centrifuged for 10 min at 900$\times$ g, and the supernatant was transferred to new 50 mL tubes. The samples were stored at −80 °C. Fibrin clots that appeared after thawing were plucked using a 10 mL serological pipette.

4.5. Platelet Microparticles Isolation, Co-Culture, and Measurement

Platelet releasate was centrifuged at 15,000$\times$ g for 90 min at room temperature and the supernatant was carefully poured off. To examine the biological effects of platelet microparticles, a mastermix of StemSpan + cytokines (for primary cells), or IMDM + 10% FBS (for THP-1 cells), was used to resuspend PMPs before transfer to cell culture and thoroughly mixed with the cells by pipetting. Two hours after the PMPs were added to the cell cultures, the wells were mixed again by pipetting. For quantitation, the microparticles were resuspended in 400 µL of 0.22 µm filtered Annexin V Binding Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), before 200 µL of the solution was transferred to a second tube. Twenty µL of Annexin V FITC (Milteny Biotec), and 2 µL of anti-CD61 APC (clone Y2/51; Miltenyi Biotec), or 22 µL of 0.22 µm filtered Annexin V Binding Buffer for an unstained control, were added and incubated for 15 min at room temperature. Finally, 278 µL of 0.22 µm filtered Annexin V Binding Buffer and 50 µL CountBright beads (Thermo Fisher Scientific) were added before analysis. Microparticle gates were set with Megamix-PLUS FSC beads (size range of beads: 0.3 to 0.9 µm; BioCytex, Marseille, France) using the side scatter channel, according to Poncelet and colleagues [59]. At least 2500 bead events were collected. This as well as all other flow cytometric analyses were performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) using CytExpert ver. 2.4 acquisition and analysis software (Beckman Coulter).

4.6. Acridine Orange Staining of Platelet Microparticles

Platelet releasate was stained with 100 µg/mL of acridine orange (Thermo Fisher Scientific) and incubated for 30 min at room temperature. Then, the solution was washed and centrifuged two times at 15,000× *g* for 90 min. Tubes were changed after the first wash step to avoid any contamination of acridine orange that may have adhered to the plastic. Finally, the PMPs were resuspended in IMDM + 10% FBS and co-cultured with THP-1 cells for 18 h at a concentration of 5×10^6 PMPs per mL. The cells were harvested and washed twice in Dulbecco's phosphate-buffered saline (DPBS; Sigma Aldrich) before analysis with flow cytometry using the FITC channel, and with a Zeiss Axioplan 2ie MOT fluorescence microscope (Carl Zeiss, Göttingen, Germany) using an SpGreen filter. At least 25,000 gated cells were collected for flow cytometric analysis.

4.7. mRNA and microRNA Analysis

Total RNA was isolated using the miRNeasy kit (QIAGEN GmbH, Hilden, Germany), and RNA concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), and the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-Time PCR was done on the Mx3005P qPCR system (Agilent Technologies, Palo Alto, CA, USA) using TaqMan MicroRNA Assays (Thermo Fisher Scientific) with the TaqMan Universal Master Mix for microRNA analyses (Thermo Fisher Scientific), and the TaqMan gene expression assays (Thermo Fisher Scientific) with the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) for mRNA analyses, following the manufacturer's instructions. *BCR* or *ACTB* were used as reference genes, and the relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method. ΔΔCq was calculated as ΔCq value (target gene minus reference gene) for cells without PMP co-incubation minus ΔCq value for PMP co-incubated cells. For a comprehensive list of the microRNA and mRNA assays used in this study, see Table S1.

4.8. Daunorubicin Apoptosis Assay

Approximately 5×10^5 cells per mL of resuscitated primary AML cells, or THP-1 cells in exponential growth phase, were cultured under aforementioned conditions with or without PMPs for 24 h. The cells were then treated with 0.5 µM daunorubicin hydrochloride (Sigma Aldrich) for another 24 h before further analysis. THP-1 cells were also used as a quality control for the experiments with primary AML cells. Then they were kept in the same batch of StemSpan + cytokines to detect any false negative results in case of issues with the PMP isolation. Cell viability was analyzed with the Annexin V-FITC kit (Miltenyi Biotec), strictly following the manufacturer's instructions. Dead and apoptotic cells were analyzed using flow cytometry and gated out in a single gate using a pseudo color plot of FITC-A versus PerCP Cy 5.5-A after doublet discrimination. For analysis with primary AML cells, contaminating cells were gated out based on light scatter properties. See Figure S2 for gating strategies. At least 25,000 gated cells were collected.

4.9. Caspase-9 Activity

Caspase-9 activity in daunorubicin-treated THP-1 cells was measured using the CaspGLOW Fluorescein Active Caspase-9 Staining Kit (Thermo Fisher Scientific). Approximately 5×10^5 cells in 0.3 mL IMDM + 10% FBS were stained with 1 µL FITC-LEHD-FMK and incubated for 30 min in a CO_2 incubator before washing twice with the supplied wash medium and analysis with flow cytometry. Both untreated and treated, but not stained THP-1 cells, were used as negative controls. At least 25,000 gated cells were collected.

4.10. Mitochondrial Membrane Potential

Mitochondrial membrane potential was assessed using the MitoProbe DiIC1(5) Assay Kit (Thermo Fisher Scientific). THP-1 cells were cultured with or without PMPs for 24 h before 5×10^5 cells in 1 mL IMDM + 10% FBS were stained with DiIC1(5) using carbonyl

cyanide 3-chlorophenylhydrazone-treated cells as a correction for background signal and incubated for 30 min following the manufacturer's instructions. After doublet discrimination, MFI (mean fluorescence intensity) values of gated cells were compared using the APC channel on the flow cytometer. See Figure S3A and B for gating strategy. At least 30,000 gated cells were collected.

4.11. Cell Cycle Analysis

THP-1 cells were incubated for 24 h in IMDM + 10% FBS with or without PMPs. Cells were washed and 5×10^5 cells were stained with 10 µM Vybrant Dye Cycle Green Stain (Thermo Fisher Scientific) and incubated for 30 min in a 37 °C water bath. Immediately after incubation, the cells were analyzed using the FITC channel on the flow cytometer. 2N cells, representing G0/G1 cell phase, were gated out after doublet discrimination. See Figure S3A and C for gating strategy. At least 10,000 gated cells were collected.

4.12. Flow Cytometry Proliferation Analysis

Proliferation analysis was performed with the Cell Trace Far Red Proliferation Kit (Thermo Fisher Scientific) and analyzed with the APC channel on the flow cytometer. On day 0, THP-1 cells at a concentration of 1×10^6 per mL were stained with 5 µM Far Red reagent in DPBS and incubated briefly for 5 min in a 37 °C water bath to avoid excessive cell toxicity. The stained cells were washed with IMDM + 20% FBS and cultured as previously described. Medium with or without PMPs was added on days 2 and 4 to keep concentration of cells below 8×10^5 per mL. A sample of the cells was analyzed on day 0 to identify baseline MFI. At least 25,000 gated cells were collected.

4.13. Flow Cytometry Immunophenotyping

THP-1 cells were cultured under aforementioned conditions with or without PMP co-incubation and harvested after 48 h. Approximately 1×10^6 cells were washed in DPBS, resuspended in 98 µL of DPBS containing 0.5% BSA, and labeled with 2 µL of anti-CD14 APC (clone REA599; Miltenyi Biotec). The cells were incubated for 10 min at 4 °C and washed before analysis. An unstained sample was used to determine background signal. At least 30,000 gated cells were collected.

4.14. Measurement of CDK4 by Indirect Intracellular Flow Cytomtery

THP-1 cells were cultured for 24 h before harvest and analyzed for intracellular protein using the published protocol by Ludwig and colleagues [60]. Briefly, cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). Then, cells were incubated with unconjugated anti-CDK4 (clone DCS-31; Thermo Fisher Scientific) and labeled with the proper conjugated secondary antibody. Dilution and incubation time can be found in Table S2. A "no primary antibody" sample was used to subtract background signal. At least 25,000 gated cells were collected.

4.15. Measurement of Cell Cross-Sectional Area

For measurement of the cross-sectional area, cultured cells were transferred to a Bürker chamber to minimize the physical cell membrane manipulation and assessed under an inverted phase-contrast microscope. Four representative fields per technical replicate at 100× magnification were captured using an Olympus Pen Lite E-PL5 camera (Olympus, Tokyo, Japan). Pictures were analyzed using the particle analysis function of the ImageJ software ver. 1.52k [61]. Image optimization and thresholding was performed as described in the Supplementary Methods.

4.16. Serum Starvation

In separate experiments, analysis of daunorubicin-induced apoptosis and cell death, cell cycle, and mitochondrial membrane potential were performed in serum-starved THP-1 cells without PMP co-incubation. Cells in the exponential growth phase were washed,

resuspended, and kept for 48 h in IMDM before further analysis, as described in the separate sections. For measurement of proliferation rate, cells were resuspended at a concentration of 4×10^5 per mL IMDM, with or without 10% FBS, and counted using the flow cytometer after 24, 48, and 72 h.

4.17. Statistical Analysis

Statistical analyses were performed using the IBM SPSS 26 software (IBM Corp., Armonk, NY, USA). Comparison between experimental groups was performed using tests for paired or independent data when appropriate. The data were checked for normality using P-P plots, Shapiro–Wilks test, and Kolmogorov–Smirnov test. A p value < 0.05 was considered significant. Mean values are reported with a 95% confidence interval unless otherwise specified. "n" denotes technical replicates.

5. Conclusions

We show that PMP co-incubation decreases mitochondrial membrane potential, inhibits cell cycle progression, decreases proliferation, and induces differentiation toward macrophages in THP-1 cells. This differentiation effect, combined with decrease in cell activity, may explain the observed protection against daunorubicin-induced cell death, which is also evident in primary AML cells.

Our results warrant further research to explore the in vivo effects of platelet microparticles in AML, both as anti-apoptotic agents, and as modulators of the disease, as they represent possible therapeutic targets through the use of platelet inhibitors.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13081870/s1, Figure S1: Resistance to daunorubicin (DNR)-induced cell death in primary acute myelogenous leukemia (AML) cells, individual data, Figure S2: Gating strategy for flow cytometric apoptosis assay, Figure S3: Gating strategy for flow cytometric analyses, Table S1: TaqMan assays used in this study, Table S2: Description of antibodies used in this study.

Author Contributions: Conceptualization, D.C.; Methodology, D.C.; Software, D.C.; Validation, D.C.; Formal Analysis, D.C.; Investigation, D.C.; Resources, D.C., H.R. and T.H.; Writing—Original Draft Preparation, D.C.; Writing—Review and Editing, D.C., H.R., O.N., P.M. and T.H.; Visualization, D.C.; Supervision, T.H.; Project Administration, D.C. and T.H.; Funding Acquisition, D.C. and T.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the regional ethics committee, *Regional Etisk Komite Vest* (ref 8144) on 16 May 2017.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Paper III

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Article

Platelet Microparticles Decrease Daunorubicin-Induced DNA Damage and Modulate Intrinsic Apoptosis in THP-1 Cells

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Abstract: Platelets can modulate cancer through budding of platelet microparticles (PMPs) that can transfer a plethora of bioactive molecules to cancer cells upon internalization. In acute myelogenous leukemia (AML) this can induce chemoresistance, partially through a decrease in cell activity. Here we investigated if the internalization of PMPs protected the monocytic AML cell line, THP-1, from apoptosis by decreasing the initial cellular damage inflicted by treatment with daunorubicin, or via direct modulation of the apoptotic response. We examined whether PMPs could protect against apoptosis after treatment with a selection of inducers, primarily associated with either the intrinsic or the extrinsic apoptotic pathway, and protection was restricted to the agents targeting intrinsic apoptosis. Furthermore, levels of daunorubicin-induced DNA damage, assessed by measuring gH2AX, were reduced in both 2N and 4N cells after PMP co-incubation. Measuring different BCL2-family proteins before and after treatment with daunorubicin revealed that PMPs downregulated the pro-apoptotic PUMA protein. Thus, our findings indicated that PMPs may protect AML cells against apoptosis by reducing DNA damage both dependent and independent of cell cycle phase, and via direct modulation of the intrinsic apoptotic pathway by downregulating PUMA. These findings further support the clinical relevance of platelets and PMPs in AML.

Keywords: acute myelogenous leukemia; platelets; microparticles; apoptosis

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1. Introduction

Platelets were originally discovered in the late 19th century as a key player in hemostasis [1]. It is now clear, however, that they serve a broader role in both health and disease [2–7]. Platelets contain many different biologically active molecules, which include proteins [8,9], regulatory microRNAs [10,11], and long RNA sequences, such as ribosomal RNAs and protein-coding transcripts inherited from parental megakaryocytes [12,13]. The long RNA sequences are prone to time-dependent decay [12,14], and correlation with the proteome is weak [13], suggesting only a limited protein synthesis capacity, which may be confined to reticulated platelets [12].

Bioactive substances can be secreted from platelets as paracrine or endocrine factors that are able to modify various cancers [15–17]. These bioactive molecules can also be transferred via platelet microparticles (PMPs), which in turn have been shown to be internalized by many different cancer cells, altering crucial functions of the cells, namely invasiveness, proliferation, and viability [18–20]. The pro-tumoral properties of platelets are further supported by retrospective and observational data showing an association between platelet inhibition and decreased risk for development of cancer, and increased cancer-specific survival [21–24]. However, the mechanism underlying this potential effect remains unknown, and the data from the few prospective studies that have been done are less convincing [25–27].

Acute myelogenous leukemia (AML) is a bone marrow disease affecting hematopoietic stem and progenitor cells [28,29]. The genomic landscape of AML has been thoroughly analyzed and the first study performing whole-genome sequencing in AML was already published in 2008 [30]. AML usually has a lower frequency of somatic mutations than most other cancers [31,32], with a median of 13 different coding mutations per case [33,34]. Despite the low mutational burden, there are large variations in transcriptomic and proteomic signatures in AML cells, compared with healthy hematopoietic progenitors, and even between different subclones [35–37]. Despite increased knowledge in the genomics of AML, treatment strategies have essentially remained unchanged for several decades, with a few exceptions [38]. Curative treatment, which is restricted to younger patients, consists of intensive chemotherapy with consolidating hematopoietic stem cell treatment for high-risk cases. Despite this, median survival is just 11 months when including all age groups [39], underscoring the need for a more profound understanding of progression of the disease and development of treatment resistance, in addition to established genomic mechanisms.

Targeting apoptosis in cancer is a novel treatment strategy that is finally maturing into clinical use. Apoptosis can be divided into two separate pathways, which are interlinked with common feedback mechanisms [40]; the death receptor initiated extrinsic pathway (FAS/CD95, TNFR1, TRAIL-R1, TRAIL-R2, DR3, and DR6), and the intrinsic, or mitochondrial, pathway. Dysregulation of the latter has proven to be an important feature in cancer biology [41]. The regulatory and anti-apoptotic proteins in the BCL2-family are also known to be upregulated in hematological malignancies [42,43]. Hence, numerous drugs that target major apoptotic regulators, such as BCL2 or MCL1, are currently either under development, or have just been approved, to treat a variety of hematological malignancies, including AML [41].

The intrinsic apoptotic pathway is initiated by several factors, including DNA damage or cellular stress, which is accompanied by upregulation of the pro-apoptotic BH3-only proteins (including BAD, BID, NOXA, HRK, BMF, PUMA, BIM), which then activate the effector proteins BAK and BAX directly or through inhibition of anti-apoptotic regulator proteins [44,45]. Upon activation, the predominantly mitochondrial outer membrane (MOM)-bound BAK, and predominantly cytosolic BAX protein, oligomerize in the MOM, leading to cytochrome c leakage from the mitochondria [46,47]. Cytochrome c then forms an apoptosome with apoptotic protease activating factor-1 (APAF1), which recruits procaspase 9, both activating and regulating its function [48,49]. Caspase-9 activates caspase-3, where the intrinsic and extrinsic pathways converge. Caspase-3 has multiple substrates [50], including a caspase-dependent DNase, which leads to DNA degradation upon activation by caspase-3 [51].

Our group has previously shown that co-incubation of the monocytic AML cell line, THP-1, or primary AML samples, with platelet microparticles, protects against daunorubicin (DNR)-induced apoptosis and cell death, at least partially via a decrease in cell activity [52]. We also found that miR-125a and miR-125b levels were elevated in THP-1 cells after PMP co-incubation. These microRNAs have been associated with chemotherapy resistance [53,54]. However, whether the PMP-associated increase in resistance to DNR is caused solely by protection against DNR-induced cell damage, or a modulation of the intrinsic apoptotic pathway regulators, remains unknown. Thus, we sought to further examine the anti-apoptotic effects of PMPs in the monocytic AML cell line THP-1.

2. Results

2.1. PMPs Offered Protection from Apoptosis Induced by Multiple Agents

We have previously demonstrated that PMPs increase resistance to DNR-induced apoptosis and cell death [52]. To investigate whether co-incubation with PMPs provided a general anti-apoptotic effect, we compared apoptosis and cell death after treatment with several agents associated with inducing apoptosis, primarily through intrinsic (alantolactone, staurosporine, MG 132), or extrinsic (piceatannol, TRAIL) apoptosis. Co-incubation of PMPs with THP-1 cells decreased the relative frequency of dead and apoptotic cells

induced by alantolactone, staurosporine, and MG 132, but not piceatannol (Figure 1). In our experiments 50 ng/mL TRAIL was not sufficient to induce apoptosis in THP-1 cells, but it slightly potentiated the apoptotic effect of piceatannol. Surprisingly, PMP co-incubation increased the relative frequency of dead and apoptotic cells in the case of the combination of piceatannol and TRAIL ($p = 0.003$). However, as this effect was marginal (mean difference of 1.89 percentage points; SD 0.43), it could be biologically insignificant. From these analyses, we suggest that PMPs may provide general protection from apoptosis, but seemingly only against agents that primarily activate the intrinsic apoptotic pathway.

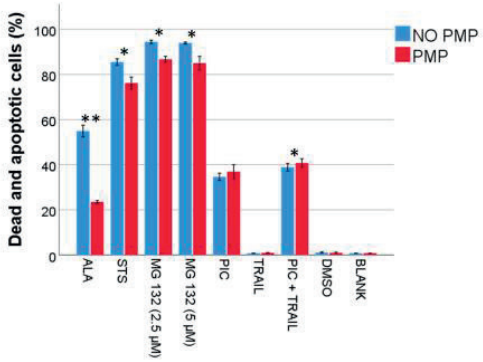

Figure 1. Apoptosis inhibition by platelet microparticles (PMPs). THP-1 cells with or without PMP co-incubation for 24 h were treated with an apoptosis-inducing molecule at a concentration and an incubation time as described in Table S1 ($n = 4$). Relative frequency of dead and apoptotic cells were analyzed by flow cytometry, and gated out in a single gate (annexin V vs. propidium iodide). Data were compared using the paired-sample t-test for data pairs. * $p < 0.05$, ** $p < 0.001$. ALA, alantolactone. STS, staurosporine. Pic, piceatannol. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

2.2. PMPs Reduced Both Caspase-8 and Caspase-9 Activation Induced by DNR

The cytotoxic effect of DNR is commonly associated with an increase in DNA damage, i.e., an intrinsic stimulus. However, it is also suggested to activate the extrinsic apoptotic pathway [55]. To evaluate activation of intrinsic and extrinsic apoptosis after DNR-treatment, we measured levels of active caspase-8 and caspase-9 by flow cytometry, and gated the cells in "lo", "mid", and "hi" populations. In the case of caspase-8, it was not possible to accurately discriminate between the "mid" and "hi" populations, and consequently these populations were gated as one. Our analyses indicated that both caspases were highly activated after DNR-treatment, but this was partially inhibited by PMP co-incubation (Figure 2A, Figure S3). In addition, fluorescence of the respective caspases were decreased for all subpopulations in PMP co-incubated cells (Figure 2B, Figure S3). The relative decrease in frequency of caspase-8 or caspase-9 "mid/hi" cells associated with PMP co-incubation were equal (Figure 2C; $p = 0.756$). These findings indicated that activation of caspase-8 is important in DNR-induced apoptosis, and is most likely inhibited by PMPs via an upstream mechanism common with caspase-9 activation.

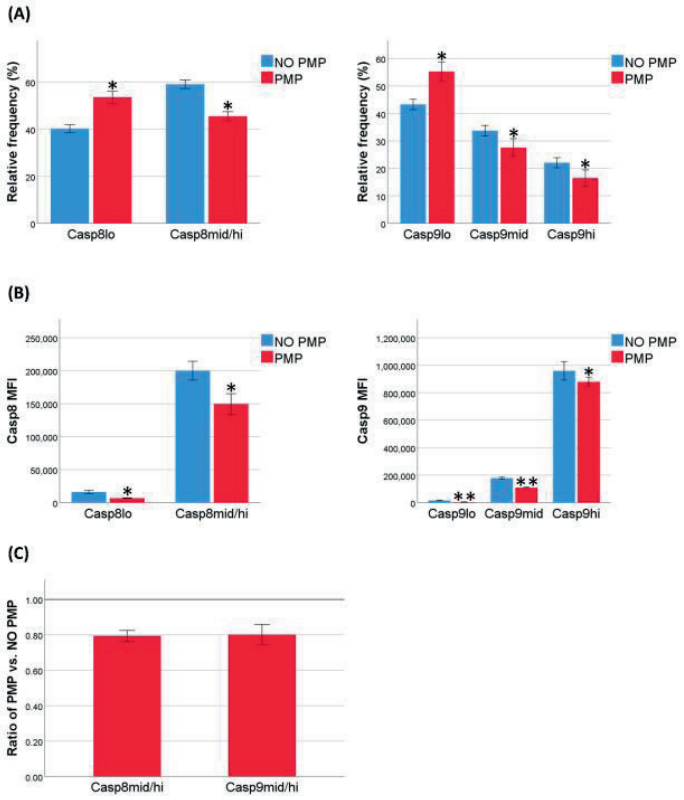

Figure 2. Caspase-8 and caspase-9 activation after daunorubicin (DNR)-treatment. (**A**) Active caspase-8 and caspase-9 were analyzed by flow cytometry after 24 h, with or without platelet microparticle (PMP) co-incubation, and an additional 24 h with DNR-treatment at 0.5 μM (n = 4). Events were gated as either "lo", "mid", or "hi", according to fluorescence intensity. Data are presented as the relative frequencies of the populations. (**B**) Background corrected mean fluorescence intensity (MFI) of the respective caspases for different populations. (**C**) Ratio of the relative frequencies of the subpopulations "mid" and "hi" combined for respective caspases between THP-1 cells, with versus without PMP co-incubation. Data were compared using the paired-sample t-test for data pairs. * $p < 0.05$. ** $p < 0.001$. Casp8, caspase-8. Casp9, caspase-9.

2.3. PMP Co-Incubation Downregulated Pro-Apoptotic PUMA Protein

To further investigate if PMPs could independently affect intrinsic apoptosis, we analyzed levels of BCL2-family proteins with and without PMP co-incubation, and both with and without DNR. Gating strategy is summarized in Figure 3. For the cell population

only visible with DNR-treatment (P2), levels of BAK, BCL2, MCL1, and PUMA were relatively less increased with PMP co-incubation (Figure 4), when compared to non-DNR-treated THP-1 cells (P1), and the decrease seen in BMF levels was relatively less. We also identified the P1 population in DNR-treated cells, and antibody fluorescence intensity was more or less unaffected, except for BMF, which had a somewhat higher level than the P1 population in non-DNR-treated cells. The relative change in fluorescence intensity accompanying PMP co-incubation was as anticipated, and followed the expected trend of protection from DNR-induced cell damage with PMP co-incubation. For example, the fluorescence intensity of BAK increased with DNR in both groups, but the increase was less with PMP co-incubation than without. However, in the case of PUMA we found a reduced signal intensity with PMP co-incubation in all cell populations, independent of DNR. Thus, we suggest that PMP co-incubation may protect THP-1 cells against DNR-induced cell death, at least partially through downregulation of the pro-apoptotic PUMA protein.

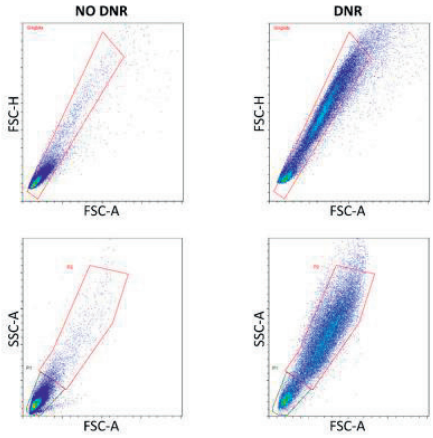

Figure 3. Gating strategy for intracellular flow cytometry of BCL2-family proteins. Doublets were first discriminated in FSC-A vs. FSC-H plots. P1 represents the population of daunorubicin (DNR)-treated cells gated in FSC-A vs. SSC-A plots that aligned well with non-DNR-treated cells. P2 represents a population generated by DNR-treatment and with increased light scatter.

2.4. Inhibitors of Caspase-9 and BAX Protected Against DNR-Induced Cell Death, but Less so with PMP Co-Incubation

As PMPs can decrease PUMA protein levels, DNR-induced apoptosis in cells co-incubated with PMPs may be less driven by the intrinsic apoptotic pathway. We investigated whether the protective effect of two inhibitors of intrinsic apoptosis, iMAC1 (BAX) and Q-LEHD-Oph (caspase-9), was affected by PMP co-incubation prior to DNR-treatment. We found a lower relative reduction in the relative frequency of dead and apoptotic cells, both for iMAC1 and Q-LEHD-Oph, with PMP co-incubation, which may indicate that caspase-9 activation was a weaker driver in apoptosis (Figure 5A). In addition, inhibiting the activity of caspase-9 or BAX with Q-LEHD-Oph and iMAC1 only yielded a reduction in levels of active caspase-9 in the "NO PMP" setting (Figure 5B). Thus, inhibitors of the

intrinsic apoptotic pathway were less effective when THP-1 cells were co-incubated with PMPs, suggesting a direct modulation of this pathway.

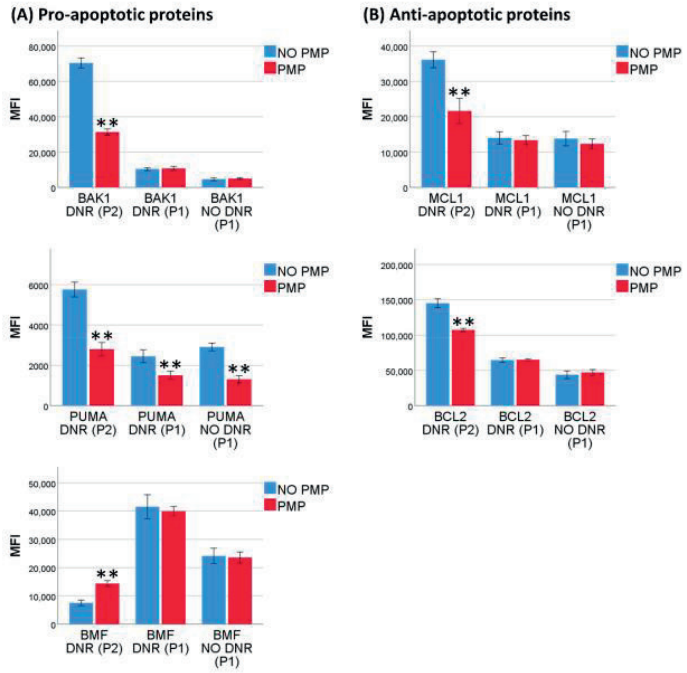

Figure 4. Levels of BCL2-family proteins before and after daunorubicin treatment. (**A**) THP-1 cells were co-incubated with or without platelet microparticles (PMPs) for 24 h before treatment with or without 0.5 µM daunorubicin (DNR) for an additional 24 h. Cells were then analyzed by intracellular flow cytometry. Data were collected as mean fluorescence intensity (MFI) levels corrected for a "no primary antibody" sample of pro-apoptotic BCL2-family proteins (n = 5). P1 represents the population determined by viable DNR untreated cells, but also visible in DNR-treated cells, with minimal changes of protein expression. P2 represents the population generated by DNR-treatment. (**B**) MFI levels corrected for a "no primary antibody" sample of anti-apoptotic BCL2-family proteins (n = 5). Data were compared using the paired-sample t-test for data pairs. ** $p < 0.001$.

2.5. PMP Co-Incubation Reduced DNA Damage After DNR-Treatment Independently of Cell Cycle Phase

To evaluate the anti-apoptotic effect of PMP co-incubation, we indirectly analyzed double-stranded DNA-breaks (DSBs) through measurement of phosphorylated histone H2AX, or gH2AX, after four h of DNR-treatment. As the process of apoptosis increases DSBs, we first investigated if apoptosis was induced within this time frame. We found that after four h apoptosis was still at the baseline level (Figure 6A). As expected, fluorescence of gH2AX was increased after DNR-treatment in 4N cells compared to 2N cells for both

groups, and the relative frequency of 2N cells was increased with PMP co-incubation (Figure 6B,C). Additionally, the fluorescence of gH2AX was decreased, both for 4N cells, and more surprisingly, for 2N cells with co-incubation of PMPs, compared to the "NO PMP" setting (Figure 6B). These findings indicated that PMP co-incubation protected THP-1 cells against DNR-induced apoptosis by decreasing the amount of DNA damage produced by DNR-treatment, both dependently and independently of cell cycle inhibition.

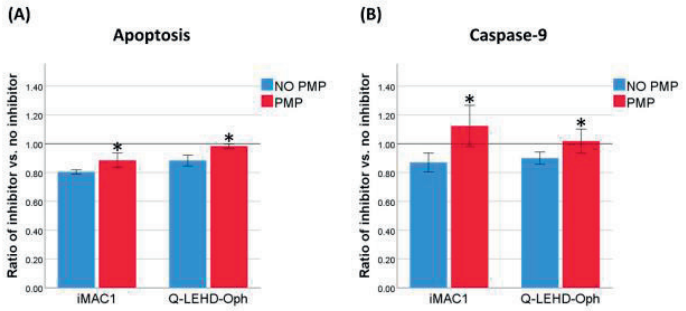

Figure 5. Effects of BAX and caspase-9 inhibitors on apoptosis and caspase-9 activation. (**A**) THP-1 cells were incubated with or without platelet microparticles (PMPs) for 23 h, and then with or without iMAC1 (BAX inhibitor), or Q-LEHD-Oph (caspase-9 inhibitor), for 1 h before treatment with 0.5 μM daunorubicin (DNR). Ratio of relative frequency of dead and apoptotic cells with or without inhibitor was calculated after 24 h ($n = 3$). (**B**) Ratio of relative frequency of caspase-$9^{mid/hi}$ cells after 24 h of DNR-treatment with or without inhibitor ($n = 3$). Data were compared using the paired-sample t-test for data pairs. * $p < 0.05$.

3. Discussion

Platelets are now recognized as an important contributor in cancer biology through several mechanisms involving immune evasion, metastasis, and development of cancer microenvironments [15,56–60]. We have previously shown that platelet microparticles increase resistance to DNR in acute myelogenous leukemia cells as a result of decreasing cell activity [52]. Here we provide evidence that this effect is multifactorial. We showed that PMPs protected equally against caspase-8 and caspase-9 activation in DNR-induced apoptosis, and that PMPs decreased DNR-induced DNA damage, not just by inhibiting cell cycle progression. The PMPs also directly modulated intrinsic apoptosis via the downregulation of the pro-apoptotic PUMA protein.

The anti-apoptotic effect of PMPs was evident with alantolactone, staurosporine, and MG 132, all primarily associated with activation of the intrinsic apoptotic pathway in THP-1 cells [61–63]. On the other hand, PMPs did not protect THP-1 cells against piceatannol or a combination of TRAIL + piceatannol, which are known to activate death receptor 5 and the extrinsic apoptotic pathway [64]. This indicates that PMPs may have broader anti-apoptotic properties, albeit restricted to intrinsic apoptosis. However, it yields no insight into the distinct mechanisms, which could be a common upstream effect on the intrinsic apoptotic pathway, e.g., cell cycle inhibition decreasing induced cellular stress or DNA damage. Interestingly, MG 132 has been shown to induce apoptosis in THP-1 cells arrested in either G1 or G2/M phases, but not when macrophage differentiation is induced [63]. Previously we have shown that PMPs inhibit cell cycle progression, and stimulate differentiation towards macrophages [52]. Thus, the latter could represent an anti-apoptotic mechanism independent of cell cycle inhibition by PMPs.

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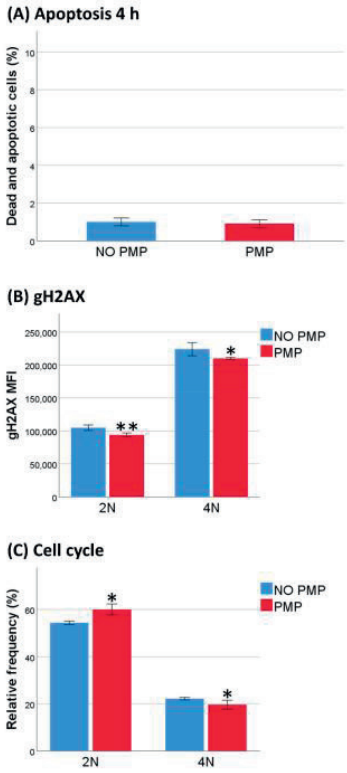

Figure 6. DNA damage after daunorubicin (DNR) treatment. (**A**) THP-1 cells were co-incubated with or without platelet microparticles (PMPs) for 24 h and treated with 0.5 µM DNR for 4 h before analysis with flow cytometry. Relative frequency of dead and apoptotic cells (n = 3). (**B**) Mean fluorescence intensity (MFI) of gH2AX corrected for both an unstained sample and background corrected MFI of representative experiment without DNR (n = 4). (**C**) Relative frequency of 2N (G1) and 4N (G2/M) cells (n = 4). Data were compared using the paired-sample t-test for data pairs. * $p < 0.05$.

Apoptosis induction by DNR is generally believed to be a result of inhibition of topoisomerase II (Top2) enzyme activity, leading to a rise in DNA-Top2 cleavage complexes [65]. The dependence of intact p53 protein for apoptosis induction by doxorubicin, a related Top2 poison, suggests a strong reliance on the activation of the intrinsic apoptotic pathway for this class of chemotherapeutics [66]. However, there is evidence that DNR-treatment upregulates death receptors and activate caspase-8 in multiple leukemic cell lines, thereby

inducing extrinsic apoptosis [55]. In our experiments, caspase-8 and caspase-9 activation was equally inhibited by PMPs, suggesting that PMPs interfere with a common activation mechanism. However, this does not completely rule out a skew in upstream initiation of these pathways, as the levels of active caspase-8 and caspase-9 are also regulated by the downstream caspase-3 and caspase-7 as important feedback mechanisms [40].

We showed that not only was the relative frequency of caspase-9 positive cells lower with PMP co-incubation, but the potency of caspase-9 and BAX inhibitors was also reduced. Both these findings suggest a weaker drive from the intrinsic apoptotic pathway in PMP co-incubated cells, but also correlated with a reduction in the ultimate function of these molecules, which is the inhibition of caspase-9 activation. iMAC1 inhibits conformational activation of BAX, and maybe BAK, without competing with BH3 only proteins [67,68]. The anti-apoptotic effect of iMAC1 is also known to decrease with higher levels of BAX [69], but this should increase chemosensitivity [70], which is the opposite of the effects associated with PMPs. We suggest a common mechanism to explain the reduction in potency of both inhibitors. LEHD (leu-glu-his-asp)-sequence based peptides block the catalytic activity of caspase-9 [71]. iMAC1 will also lead to a decrease in caspase-9 activity by inhibiting mitochondrial outer membrane permeabilization [72]. Thus, both inhibitors ultimately lead to a decrease in the activation of caspase-3, which is not only essential for apoptosis induction, but also for caspase-9 activation in a feedback loop [40]. Thus, if the intrinsic apoptotic pathway is inhibited by PMP co-incubation, the relative contribution of this pathway to caspase-3 activation is reduced compared to the extrinsic pathway, which is also activated by DNR. This should lead to a relative reduction in efficiency of apoptosis inhibition through the intrinsic apoptotic pathway, as extrinsic apoptosis is presumably unaffected by both PMPs and the inhibitors. However, one important caveat for this conclusion is the selectivity of the caspase-inhibitor, which, at least in the older generation inhibitors, is proven to be poor [71]. There are some indications that the second generation inhibitor Q-LEHD-Oph also inhibits caspase-8, but this has not been analyzed in a cell-free system and it was less extensive then the caspase-9 inhibition [73]. Furthermore, our conclusion is supported by results involving two independent inhibitors of separate stages in the intrinsic apoptotic pathway.

The inhibitory effect of PMPs on cell cycle progression is a possible mechanism for increased DNR-resistance, since Top2 poisons are believed to be most effective in proliferative cells [65]. We have previously provided evidence for this, showing that serum starvation of THP-1 cells significantly reduces DNR-induced apoptosis [52]. By measuring gH2AX we showed that PMP co-incubation decreased the level of DNA damage after DNR-treatment. Phosphorylation of histone H2AX is induced by double-stranded DNA-breaks as a DNA damage response [74]. Thus, the level of gH2AX is a widely used proxy for DSBs in biological research [75–77]. As expected, gH2AX levels increased more in dividing 4N cells (G2/M), compared with non-dividing 2N cells (G1) across both groups. As PMPs inhibit cell cycle progression, this would necessarily decrease the level of DNA damage. However, we identified a relative decrease in the signal intensity of gH2AX with PMP co-incubation for both cell phases, suggesting a de facto protective mechanism against the effects of DNR, independent of cell cycle inhibition. Somewhat surprisingly, gH2AX levels were also lower with PMP co-incubation in cells in the G1 cell phase. Previously we have found a decrease in mitochondrial membrane potential associated with PMP co-incubation [52], which may decrease the level of reactive oxygen species (ROS). Significant DNA damage in cells in G1 cell phase is also found in doxorubicin-treated U2OS osteosarcoma cells [78]. This probably has a different etiology compared to the mechanism in the G2/M cell phase and may be explained by an increase in ROS [79,80].

An important question regarding the anti-apoptotic effect of PMPs is if they can directly modulate the apoptotic response. We measured anti-apoptotic (BCL2 and MCL1) and pro-apoptotic (BAK, BMF, and PUMA) BCL2-familiy proteins, both in response to DNR and at baseline, in a "NO DNR" setting. The decreased levels of PUMA associated with PMP co-incubation probably represent a de facto downregulation, as it was present

in all cell populations both with and without DNR. This could be due to increased levels of microRNAs, miR-125a and miR-125b, which are transferred by PMPs [52], and proven to downregulate the protein at the translational level, inducing chemoresistance [53,54]. Furthermore, the "readiness" for activation of intrinsic apoptosis in AML cells has clinical relevance as it is a predictor of outcome with conventional treatment [81]. The other proteins analyzed were also altered, but not in the viable, non-DNR-treated cells, and always in sync with an expected decrease in apoptosis and cell damage associated with PMP co-incubation. Thus, it cannot be stated that these proteins were directly downregulated as a result of PMP-internalization. These differences could be a result of an altered regulation of BCL2-family proteins caused by downregulation of other proteins, such as PUMA. However, they may also stem from a shift in ratio of apoptotic to dead cells, which we did not discriminate. Surprisingly, DNR increased the fluorescence intensity for both the anti-apoptotic proteins tested (BCL2 and MCL1); one would expect a decrease in the level of anti-apoptotic proteins when apoptosis is induced. However, this pattern has been observed for some anti-apoptotic proteins in select leukemic cell lines and is presumably transitory [82].

PUMA is regulated by several factors, including different transcription factors and proteins like forkhead box O (FOXO) and p53 family members [83]. However, these mechanisms may be shared with other pro-apoptotic BCL2-familiy proteins [84], and therefore do not coincide with our observations of isolated PUMA downregulation. PUMA is also post-translationally regulated by phosphorylation and proteosomal degradation, which is proven to be induced by interleukin-3 and HER2 [85,86], but none of these proteins are considered to be a part of the platelet granule or releasate proteome [8,9]. In addition, there are other microRNAs that are present in PMPs, like miR-221 and miR-222 [11], which also are known to downregulate PUMA [87]. However, this has not been investigated in THP-1 or other acute myelogenous leukemia cell lines.

The evidence provided here supplements our previously published work that PMPs have anti-apoptotic properties in acute myelogenous leukemia. This effect could stem partially from inhibition of cell cycle progression and cell activity, making the cells less susceptible to damage induced by chemotherapy. In addition, we showed that PMPs may modulate the intrinsic apoptotic pathway through downregulation of PUMA, as a mechanism independent of cell cycle inhibition. The mechanistic findings from this study were derived solely from one cell line and need to be confirmed in primary AML cells. Nonetheless, translational research with PMPs in AML is warranted, as the indications for platelet inhibition to decrease PMP production, and thus potentially increase chemosensitivity, are further supported.

4. Materials and Methods

4.1. Cell Line

The THP-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) + 10% FBS (Sigma Aldrich, St. Louis, MO, USA). Culture medium was partially replaced approximately every second day to keep the total cell concentration in the range of 2–6 $\times$ 10^5 per mL, and cells were only used in experiments once the exponential growth phase was reached. Cultures were kept for less than three months.

4.2. Platelet Concentrate

Platelet concentrates pooled from four donors were produced using the automated Tacsi system (Terumo BCT, Lakewood, CO, USA) and were provided by the Department of Immunology and Transfusion Medicine, Stavanger University Hospital (Stavanger, Norway). The platelet concentrations were 0.94–1.06 $\times$ 10^9 per mL. Leukocytes were removed by filtration to a residual level of <1.00 $\times$ 10^6. The storage medium for the

platelets was approximately 35% plasma and 65% additive solution (PAS-III; Baxter, Lake Zurich, IL, USA). Written consent was obtained from all donors.

4.3. Platelet Releasate

Platelet releasate was produced by adding human thrombin (Sigma Aldrich) at a final concentration of 1 U/mL to the platelet concentrates in 50 mL tubes, and incubating for one hour in a 37 °C water bath, as described in [52]. The releasates were mixed by gentle shaking every 5 min. To separate the releasate from the clot, the tubes were centrifuged for 10 min at 900 g and the supernatant was transferred to new 50 mL tubes. The samples were stored at −80 °C. Fibrin clots that appeared after thawing were removed using a 10 mL serological pipette.

4.4. Platelet Microparticle Production

Platelet microparticles were isolated as previously described [52]. Briefly, platelet releasate was centrifuged at 15,000 g for 90 min at room temperature, and the supernatant carefully poured off. The PMPs were then resuspended in IMDM + 10% FBS and transferred to cell culture, thoroughly mixing with the cells by pipetting. The final concentration of PMPs was 1.5×10^7 per mL culture medium in all experiments. The wells were mixed again by pipetting 2 h after the PMPs were added to the cell cultures.

4.5. Platelet Microparticle Quantitation

One mL of platelet releasate, washed with 9 mL of Dulbecco's phosphate-buffered saline (Sigma Aldrich), was centrifuged as described above and the supernatant carefully poured off. The platelet microparticles were resuspended in 400 µL of 0.22 µm filtered Annexin V Binding Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), and 200 µL of the solution was transferred to a second tube. The solution was then stained with 20 µL of Annexin V FITC (Milteny Biotec), and 2 µL of anti-CD61 APC (clone Y2/51; Miltenyi Biotec), or 22 µL of 0.22 µm filtered Annexin V Binding Buffer for a negative control and incubated for 15 min at room temperature. Finally, 278 µL of 0.22 µm filtered Annexin V Binding Buffer and 50 µL CountBright beads (Thermo Fisher Scientific) were added before analysis. Microparticle gates were set using Megamix-PLUS FSC beads (bead size range: 0.3 to 0.9 µm; BioCytex, Marseille, France), according to our previous report [52]. At least 2500 bead events were collected. This, and all other flow cytometric analyses, were performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) using CytExpert ver. 2.4 acquisition and analysis software (Beckman Coulter). An example of gating strategy for PMP quantitation can be found in Figure S1 (see Supplementary Materials).

4.6. Apoptosis Assay

Approximately 5×10^5 cells per mL THP-1 cells were cultured with or without PMPs for 24 h. The cells were then treated with an apoptosis inductor at a concentration and time interval as indicated in Table S1. Cell viability was analyzed with the Annexin V FITC Kit (Miltenyi Biotec), strictly following the manufacturer's instructions. Dead and apoptotic cells were analyzed using flow cytometry and gated out in a single gate using a dot plot of FITC-A versus PerCP Cy 5.5-A after doublet discrimination. At least 20,000 gated cells were collected. An example of gating strategy can found in Figure S2.

4.7. Apoptosis Inhibition

For select experiments, after the initial 23 h of incubation with or without PMPs, the THP-1 cells were pretreated with either 20 µM of the caspase-9 inhibitor, Q-LEHD-Oph (Abcam, Cambridge, UK), or 10 µM of the BAX inhibitor, iMAC1 (Sigma Aldrich), and incubated for one hour before adding DNR, as described in the previous section.

4.8. Caspase Activity

Caspase-8 and caspase-9 activity in THP-1 cells after 24 h of DNR-treatment was measured using the CaspGLOW Fluorescein Active Staining Kit for the respective caspases (Thermo Fisher Scientific). Approximately 5×10^5 cells in 0.3 mL IMDM + 10% FBS were incubated with 1 µL of either FITC-IETD-FMK or FITC-LEHD-FMK for 30 min in a CO_2 incubator before washing twice with the supplied wash medium, and analyzing with flow cytometry. Both untreated and treated, but not stained, THP-1 cells were used as controls to determine low, medium, and high caspase populations. At least 20,000 gated cells were collected.

4.9. gH2AX

Measurement of gH2AX by flow cytometry was performed according to Darzynkiewicz et al. [75]. After PMP co-incubation, cells were fixed with 1% methanol free formaldehyde for 15 min on ice, then fixed and permeabilized in 70% ethanol, and stored overnight. Fixed and permeabilized cells were stained with FITC conjugated anti-phospho-Histone H2A.X (Ser139) antibody (clone JBW301; 1 µg/100 µL; Sigma Aldrich), and propidium iodide solution (5 µg/mL; Thermo Fisher Scientific) containing DNase free RNASE A/T1 cocktail (25 U/1000 U per mL; Thermo Fisher Scientific).

4.10. BCL2-Family Proteins

THP-1 cells were cultured for 24 h with or without PMPs, and an additional 24 h with or without treatment with DNR, before analysis of intracellular proteins using the published protocol by Ludwig et al. [88]. Briefly, cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). Cells were then incubated with unconjugated antibodies and labeled with the proper conjugated secondary antibodies. A list of the antibodies used, dilutions, and incubation times, can be found in Table S2. A "no primary antibody" sample was used to subtract the background signal. At least 25,000 gated cells were collected.

4.11. Statistical Analyses

Statistical analyses were performed using the IBM SPSS 26 software (IBM Corp, Armonk, NY, USA). All figures show mean values with 95% confidence intervals. A comparison of means was performed using tests for paired data, or one-sample tests, when appropriate. The data were checked for normality using PP plots, the Shapiro–Wilks test, and the Kolmogorov–Smirnov test. A p value < 0.05 was considered significant. "n" denotes technical replicates.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22147264/s1.

Author Contributions: Conceptualization, D.C.; methodology, D.C.; software, D.C.; validation, D.C.; formal analysis, D.C.; investigation, D.C.; resources, D.C. and T.H.; writing—original draft preparation, D.C.; writing—review and editing, D.C., O.N., P.M. and T.H.; visualization, D.C.; supervision, O.N., P.M., T.H.; project administration, D.C. and T.H.; funding acquisition, D.C. and T.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the regional ethics committee, Regional Etisk Komite Vest (ref 8144) on 16.05.2017.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author.

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Conflicts of Interest: The authors declared no potential conflict of interest.

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Appendix C

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