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Review Article

Platelet intrinsic apoptosis

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ABSTRACT

In a healthy individual, the lifespan of most platelets is tightly regulated by intrinsic, or mitochondrial, apoptosis. This is a special form of programmed cell death governed by the BCL-2 family of proteins, where the prosurvival protein BCL-X_L maintains platelet viability by restraining the prodeath proteins BAK and BAX. Restriction of platelet lifespan by activation of BAK and BAX mediated intrinsic apoptosis is essential to maintain a functional, haemostatically reactive platelet population. This review focuses on the molecular regulation of intrinsic apoptosis in platelets, reviews conditions linked to enhanced platelet death, discusses *ex vivo* storage of platelets and describes caveats associated with the assessment of platelet apoptosis.

1. Introduction

Platelets are small anucleate blood cells critical for haemostasis and wound healing and are involved in a range of other processes [1]. The platelet precursor cells, the megakaryocytes, are large polyploid cells mostly residing in the bone marrow. They produce around 100 billion platelets per day. Newly generated immature platelets, also called reticulated platelets, possess RNA that is rapidly lost as platelets age [2,3]. The lifespan of platelets in circulation is brief, 8 to 10 days in humans [4] and close to 5 days in mice [2]. In a healthy individual, platelet count is kept within tight physiological ranges varying from 150 to 400 $\times$ 10^9/L blood. Reduced platelet number in disease can be influenced by a range of processes including impaired platelet production, enhanced platelet destruction/clearance, abnormal spleen sequestration, and increased haemostatic consumption. Antibody-mediated platelet destruction/clearance, and modulation of platelet surface glycans are examples of established mechanisms of enhanced platelet clearance in disease [5]. In the last 15 years, insights from pharmacological developments and genetically manipulated mouse models have helped characterising the components of the intrinsic, or mitochondrial, apoptosis pathway that controls steady state platelet lifespan. This review describes the molecular regulation of platelet lifespan by intrinsic apoptosis and potential caveats associated with the laboratory assessment of platelet apoptosis.

2. Intrinsic and extrinsic apoptosis pathways in nucleated cells

Intrinsic apoptosis regulates cell numbers during development and haematopoiesis, maintaining homeostasis and allowing unwanted cells to be swiftly removed in the absence of inflammation [6,7]. Cancer and autoimmune diseases can arise if this process is perturbed. The overexpression of prosurvival BCL-2 proteins has been identified as a mechanism for increased cell survival in certain malignancies and is therefore a target for cancer therapy. In contrast, excessive apoptosis is associated with neurodegeneration and thrombocytopenia. Two convergent apoptotic pathways of programmed cell death exist; the intrinsic (non-receptor mediated) and extrinsic (receptor mediated) pathways. The apoptotic process was first described by Kerr and colleagues as morphological changes that included pyknosis (chromatin condensation), nuclear decrease and membrane blebbing [8]. Nowadays, apoptosis is clearly defined on a molecular level and is not to be mixed up with other programmed cell death pathways such as necroptosis [9], pyroptosis [10] or ferroptosis [11].

Intrinsic apoptosis can be initiated by a range of cellular stresses such as intracellular injury, cytokine depletion, or treatment with chemotherapeutic drugs. The apoptotic activity is strictly regulated by prosurvival and prodeath BCL-2 family members, which balance the commencement or inhibition of apoptosis. The prosurvival protectors MCL-1, BCL-2, BCL-X_L, BCL-W, A1(BFL-1) and BCL-B [6] sequester the prodeath proteins BAK and BAX in healthy cells. However, the balance

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between prosurvival and prodeath members varies depending on cell type. Apoptosis can be triggered in situations of cellular stress where a third group of BCL-2 proteins, the BH3-only proteins (BAD, BIK, BIM, BMF, NOXA, PUMA, and HRK), can hinder the prosurvival proteins. This mechanism activates the prodeath molecules BAK and BAX, directly or indirectly [12–15], to cause irreversible mitochondrial injury through mitochondrial outer-membrane permeabilization (MOMP). Cytochrome *c* gets released from the mitochondria and participates in apoptosome assembly, leading to Caspase-9 activation. Moreover, the released SMAC (second mitochondria-derived activator of caspases) protein keeps the caspase inhibitor XIAP (X-linked inhibitor of apoptosis protein) in check. Next, effector caspases (Caspase-3/7) convert from pro-caspases to their active forms and Xk related protein 8 (Xkr8) [16] facilitates phosphatidylserine (PtdSer) translocation from the inner to the outer leaflet of the cell surface membrane. PtdSer works as an "eat me" signal enabling rapid detection and removal of apoptotic bodies by phagocytic cells.

Ligand binding to death receptors, expressed on the cell surface, triggers extrinsic apoptosis. The surface death receptors comprise members of the tumour necrosis factor receptor (TNFR) family of which the FAS receptor (FASR) is a member. Importantly, downstream activation of Caspase-8 ensues which is mediated by FAS-associated death domain protein (FADD) and in some situations by TNFR-associated death domain protein (TRADD). In so-called type I cells, effector caspases (Caspase-3/7) can be directly activated downstream of Casapse-8 activation [17]. Alternatively, in type II cells, activated Caspase-8 triggers BID cleavage to produce tBID, which then activates BAK and BAX connecting the extrinsic and intrinsic pathways [18]. Both pathways end in effector caspase activation, PtdSer exposure, and the generation of apoptotic bodies.

3. Regulation of platelet lifespan by BCL-X_L, BAK and BAX

It has been known for quite some time that platelets contain parts of the apoptotic machinery, based on RNA or protein expression [19–23]. However, in the last 15 years, understandings from pharmacological advances and genetically manipulated mouse models have helped defining the elements of the intrinsic apoptosis pathway that regulates steady state platelet lifespan. In 2007, ground-breaking studies from Mason et al. and Zhang et al. using genetic and pharmacological tools (BH3-mimetics) uncovered that BCL-X_L is the major prosurvival protein needed to uphold platelet lifespan [24,25] (Fig. 1). The first hint for this discovery came in 2000 from Wagner and co-workers who observed thrombocytopenia in mice after deletion of *Bclx* in the haematopoietic system, secretory tissues, and skin using *MMTV-Cre* [26]. It has later been demonstrated by Josefsson and colleagues that megakaryocyte lineage-restricted loss of *Bclx*, mediated by platelet factor 4 (*Pf4*)-*Cre*, diminished platelet lifespan in mice from ~5 days to ~5 h, dropping platelet counts to ~2 % of wild-type levels [27,28].

Tumour cells often overexpress BCL-2 family prosurvival proteins, where the specific prosurvival member(s) and levels vary depending on

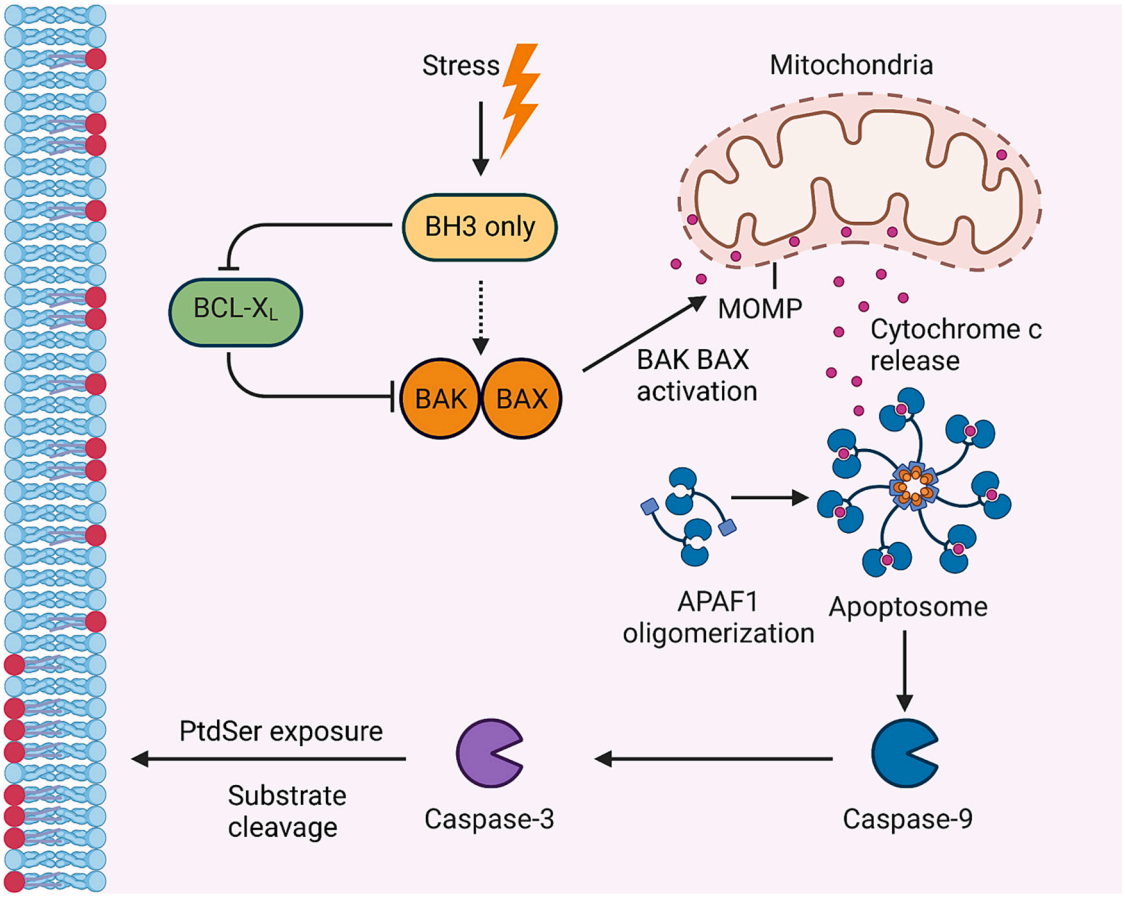

Fig. 1. Platelet intrinsic apoptosis.
Schematic illustration of platelet intrinsic apoptosis. In healthy platelets, prosurvival BCL-X_L operates by restraining BAK and BAK, the critical facilitators of cell death. Stress signals activate the BH3 only proteins (BAD, BID, BIM, BIK, BMF and PUMA) which can block and displace pro-survival proteins thus activating BAK and BAX to cause mitochondrial outer membrane permeabilisation (MOMP), with subsequent Cytochrome c release and assembly of Apaf-1 and Cytochrome c into the apoptosome. Next, the apoptosome can recruit and activate the inactive pro-caspase-9. Downstream activation of effector caspase-3 occurs and subsequent phosphatidylserine (PtdSer) surface exposure.
Adapted from "Apoptosis Extrinsic and Intrinsic Pathways", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

the tumour type. BH3 mimetic drugs are designed to trigger apoptosis in cancer cells by mimicking the activity of BH3-only proteins. In early clinical trials the combined inhibition of BCL-X_L, BCL-2 and BCL-W with BH3 mimetic ABT-263 (Navitoclax) [29], an orally available analogue of ABT-737 [30], caused dose-limiting thrombocytopenia when treating haematological malignancies [31,32]. The induction of rapid-onset platelet apoptosis with ensuring thrombocytopenia was shown to be an on-target effect of BCL-X_L inhibition [24,31,33–35]. The more recently developed specific BCL-2 inhibitor, Venetoclax (ABT-199), efficiently induced apoptosis of leukemic cells known to express BCL-2, while sparing platelets [36] and was approved in 2016 for the treatment of chronic lymphocytic leukemia. While there is evidence that platelets in adults and neonates express BCL-2 protein [20,22,24,25,27,37–41] (Table 1), megakaryocyte lineage specific deletion of *Bcl-2* in mice did not alter platelet lifespan or platelet count [38] consistent with the clinical results from the use of the BCL-2 inhibitor (Venetoclax). Hence, BCL-2 is not needed for platelet survival. Fascinatingly, overexpression of prosurvival BCL-2 in mice was shown to modestly prolong platelet lifespan [42].

BAK and BAX are the two central mediators of mitochondrial apoptosis. It is well-known that nucleated cells deficient in BAK and BAX are resistant to apoptotic triggers, and hence, do not present with cytochrome *c* release or downstream caspase activation [43]. Similarly, BAK and BAX play important roles in regulating platelet apoptosis. Loss of *Bak* in mice doubles platelet lifespan with resultant significantly elevated platelet counts [24,27]. However, conditional (*Pf4*-Cre) loss of the other central pro-death molecule, *Bax*, only modestly extends platelet lifespan and does not notably influence platelet counts in mice [27]. Hence, BAK is considered as the key controller of platelet lifespan at steady state, while BAX has a secondary role (Fig. 1). Dual deletion of *Bak* and *Bax* doubles platelet lifespan in mice [27] to the same level as *Bak* loss, rescues the thrombocytopenia caused by *Bcl-x* deletion [27,39] and makes platelets unaffected by pharmacological BCL-X_L inhibition [33]. In agreement with the murine findings, human genome-wide-association reports have revealed common variants in the *BAK1* gene correlating with platelet number [44,45].

4. The MCL-1 protein is not expressed in platelets

Do additional BCL-2 family members, others than BCL-X_L, BAK and BAX, participate in the regulation of platelet lifespan? MCL-1 is a short-lived prosurvival protein critical for the survival of haematopoietic stem cells and multipotent haematopoietic progenitors [7]. BCL-X_L and MCL-1 coordinately regulate megakaryocyte survival (reviewed in [46]), where dual conditional loss in the megakaryocytic lineage (*Pf4*-Cre-mediated) resulted in embryonic death [47,48]. But, neither conditional megakaryocyte lineage deletion nor haematopoietic overexpression of *Mcl-1* changed murine platelet number or lifespan [42,47,48]. The MCL-1 protein is known to have a short half-life, in part because of rapid proteosomal degradation. Even though expressed in megakaryocytes,

Table 1
Intrinsic apoptosis RNA transcript and protein expression in platelets.

Intrinsic apoptosis RNA and proteins expressed in platelets						
	RNA		**Protein**		**Human** (references)	**Mouse** (references)
	Yes	No	Yes	No		
Prosurvival						
A1 (BFL-1)	√*		?			*A1b and A1d but not A1a RNA are expressed in murine platelets.[51]
BCL-2	√		√		[20, 22, 25, 37, 39-41]	[24, 27, 38, 39, 51]
BCL-B	?		?			
BCL-W	√		√		[25, 39]	[39, 51]
BCL-X_L	√		√		[25, 34, 37, 39-41, 100, 101]	[24, 39, 51, 64]
MCL-1	√			√	[25, 34]	[24, 47, 48, 51]
Prodeath						
BAK	√		√		[20, 25, 34, 39-41, 58]	[24, 27, 39, 51, 55]
BAX	√		√		[20, 22, 25, 34, 39-41, 57, 58, 101]	[24, 27, 39, 51, 55]
BAD	√		√		[41]	[51, 54]
BID	√		√		[34, 39, 41, 56-58, 101]	[24, 39, 51, 55]
BIM	√		√		[39]	[39, 51]
BIK		√*	√*		*Protein detected in human platelets.[41]	*RNA transcript not detected in murine platelets.[51]
BMF	√		?			[51]
BOK		√		?		[51]
HRK		√		?		[51]
NOXA		√		√	[34]	[51]
PUMA	√*			√	[34, 41]	[39] *RNA transcript present in murine platelets.[51]
Other						
Apaf-1	√		√		[101, 102]	
Caspase-3	√		√		[19-21, 33, 34, 37, 39, 41, 56, 57, 101, 103]	[39, 55, 69]
Caspase-7		√		√	[21]	
Caspase-9	√		√		[19, 34, 37, 57, 101]	[69]
Cyt c	√		√		[25, 34, 37, 57, 101]	[104]
PtdSer	√		√		[21, 25, 33, 34]	[33, 55, 69]
Xkr8	?		?			

This table gives an overview of studies reporting presence or absence of RNA transcript and/or protein expression of intrinsic apoptosis members in human and murine platelets. Cytochrome *c* (Cyt c), phosphatidylserine (PtdSer), and Xk-related protein 8 (Xkr8).

MCL-1 protein is lacking in platelets [24,25,27,39,47,48] (Table 1). Moreover, MCL-1 has been found in murine platelets subsequent to proteasome inhibitor treatment *in vivo* [48] implying that some residual protein could possibly exist in newly produced platelets, where RNA is present.

Regardless of its existence in platelets, the pro-survival protector BCL-W [25,39] (Table 1) is not likely to play a key role in controlling platelet lifespan as systemic *Bcl-w* knock out did not affect platelet counts in mice [49,50]. Furthermore, A1 is an additional prosurvival molecule identified in young platelets by RNA expression [21,51], but it is presently unclear if the protein is expressed in platelets (Table 1). Systemic *A1a* deletion in mice did not lead to an altered platelet phenotype [52], consistent with the lack of *A1a* RNA in murine platelets [51]. Nevertheless, in mice there are four *A1* genes. Results from a study that targeted all A1 isoforms in mice showed that platelet counts were unchanged [53]. As described above, anucleate platelets can undergo mitochondrially-driven apoptosis, however, their inability to sustain MCL-1 protein levels makes them separate to their precursor cells, and most other cell types, and dependent on only one prosurvival protector, BCL-X_L.

5. Roles of BH3-only proteins in platelets – do they trigger apoptosis?

Limited information is available on the role of death initiator BH3-only proteins in platelets. The presence of BAD [41,54], BID [24,34,39,41,55–58], BIK [41], and BIM [39] protein have been demonstrated in platelets, while NOXA [34], PUMA [34,39,41], and HRK [51] seem to be lacking (Table 1). Moreover, BMF RNA transcript has been shown to be present in young platelets [51]. Kodama and colleagues engineered mice with combined deletion of *Bim* and *Bid*, but this did not modulate platelet count [39]. Currently, BAD is the lone BH3-only initiator protein proven to affect platelet lifespan. However, its deletion in mice only led to a modest lengthening in platelet lifespan [54]. Hence, the effect of BAD on steady state platelet lifespan is minimal in comparison to loss of *Bak* or *Bak/Bax*. A study by Zhao and co-workers proposed that BAD is a protein kinase A substrate, allowing BAD to start platelet apoptosis by sequestering BCL-X_L when protein kinase A is downregulated [59]. Furthermore, multiple studies have proposed that PI3/AKT-mediated inactivation of BAD safeguards platelets from apoptosis [60–63] indicating that several cues can influence platelet lifespan. Nevertheless, conclusive genetic evidence is currently missing since the effects of BAD deletion on platelet lifespan is modest compared to the removal of BAK/BAX.

It needs to be determined if additional BH3-only members than BAD contribute to mediating platelet apoptosis, in what combination and how they incorporate extra- and intracellular signals. Alternatively, changes in BCL-X_L could trigger platelet apoptosis. Initially a "molecular clock" model was proposed with BCL-X_L levels declining over time relative to BAK/BAX [24], but this theory was later revised when *in vivo* aged platelets in mice did not exhibit notably reduced BCL-X_L levels [64]. Another hypothesis that requires to be tested is if the activity of BCL-X_L is modified as platelets age, and whether this modulation would lead to impairment of BCL-X_L-dependent restriction of BAK [46]. Future studies are needed to decipher the mechanism for initiation of physiological platelet apoptosis.

6. Death receptor mediated extrinsic apoptosis

In stark contrast to megakaryocytes, currently it is unclear whether extrinsic apoptosis can be initiated by death receptors in platelets. Actually, platelets lack surface expression of the FAS receptor [21,55,65] and TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 and 2 [65]. Nevertheless, platelets contain intracellular factors of this pathway including Caspase-8 [21,55–57,66], FADD [55] and BID. Caspase-8 is the key regulator of extrinsic apoptosis. Strikingly, in mice platelet number or lifespan were not affected by conditional loss of Caspase-8 in the megakaryocytic lineage [55]. Several studies have reported Caspase-8 activation in platelets but not linked to classical death receptor ligand binding [56,66–68]. In summary, evidence of canonical death receptor facilitated extrinsic apoptosis in platelets is at present missing.

7. Apoptotic caspases

Along with Caspase-8, platelets also contain Caspase-9 [19,34,37,57,69] and effector Caspase-3, but appear to lack Caspase-7 (Table 1). Caspase activation is an established characteristic of apoptosis, mediating DNA fragmentation in nucleated cells, and degradation of cytoskeletal proteins. However, caspase activation is downstream of mitochondrial injury in the intrinsic pathway, and not vital for cell death and the removal of cells in circulation [70]. Steady state platelet count and platelet lifespan were normal in chimeric mice missing *Caspase-9* in haematopoietic cells. Nevertheless, platelets lacking Caspase-9 exhibited delayed PtdSer-exposure after induction of intrinsic apoptosis *in vitro* [69]. Combined loss in haematopoietic cells of *Caspase-3* and *-7*, or *Apaf-1* did not either affect steady state platelet numbers [55] indicating that Caspase-9, -3, -7 and Apaf-1 are superfluous for the regulation of platelet lifespan. Nonetheless, caspase activation in haematopoietic cells has been demonstrated to be an essential mechanism in order to maintain apoptosis immunologically silent. Genetic or pharmacological inhibition of Caspase-9, -3 or -7 initiates innate immune signalling within dying cells with subsequent production of interferon-β [71].

8. Two mitochondrial pathways to platelet PtdSer exposure

There are at least two distinct mitochondrial pathways to platelet PtdSer exposure [33] 1) the intrinsic apoptosis pathway and 2) procoagulant platelet formation. Procoagulant platelets are a subpopulation of highly activated platelets, that express coagulation-promoting activity by PtdSer exposure, and play a role in haemostasis [72]. They are reliant on mitochondrial cyclophilin D and Ca^{2+}-dependent scramblase activation pathways, where TMEM16F (ANO6) is a key player [73,74]. PtdSer exposure facilitates formation of the coagulation factor tenase and prothrombinase complexes and their subsequent formation of Factor Xa and thrombin, respectively. Next, fibrinogen gets converted to fibrin leading to clot stabilization. Procoagulant platelet formation is most efficiently generated *in vitro* with the dual-agonists thrombin (or PAR-4 peptide or thrombin PAR-1 receptor activating peptide (TRAP)) and a GPVI agonist (convulxin, collagen, or collagen related peptide (CRP-XL)). Features of procoagulant platelets include mitochondrial depolarization, cyclophilin D dependence, PtdSer exposure, FXa/FVa binding, thrombin generation, α-granule release, ballooning, extracellular vesicle release, and increased membrane permeability [75,76]. Several of these characteristics are also associated with intrinsic platelet apoptosis comprising mitochondrial depolarization, PtdSer exposure, thrombin generation, increased membrane permeability, and extracellular vesicle release.

There are, however, key disparities when comparing apoptotic and procoagulant platelets. Once platelets become apoptotic *in vitro* due to BCL-X_L inhibition (ABT-737 or Navitoclax treatment), platelet mitochondrial damage is BAK/BAX dependent, cytochrome *c* is released from damaged mitochondria, blebbing occurs and PtdSer exposure is caspase-dependent, while there is no or negligible α-granule release. In procoagulant platelets, there is cyclophilin D dependence, PtdSer exposure is Ca^{2+}-dependent by activation of TMEM16F, ballooning occurs and there is significant α-granule release.

In vivo, platelet apoptosis induced by pharmacological inhibition or conditional loss of *Bcl-x*, led, in addition to thrombocytopenia, to a slight but noteworthy increase in circulating PtdSer positive platelets [27,35]. However, a recent study in mice demonstrated that this pathway is

unlikely to support thrombin generation *in vivo* [77]. Xkr8 is recognised as the enzyme catalysing caspase-mediated lipid scrambling in nucleated cells [16]. Whereas this protein is ubiquitously expressed [16], it has yet to be detected in platelets.

9. Caveats when investigating platelet apoptosis

There are several caveats when assessing platelet apoptosis. Certain classical apoptosis assays such as TUNEL staining of DNA fragmentation or the use of nuclear dyes to separate early apoptotic from necrotic cells cannot be employed in the anuclear platelet. Furthermore, platelets are in general tricky cells to work with as prone to rapid activation and aggregation when exposed to shear stress, certain materials, and agonists. While flow cytometric assessment of platelet surface receptors is common practice and relatively straight forward, intracellular staining protocols that require permeabilization can be difficult to optimise by flow cytometry and are often avoided. Moreover, platelets contain relatively low levels of BCL-2 family proteins which requires a concentrated sample when performing immunoblotting.

Several of the markers and/or methods used to measure apoptotic and procoagulant platelets are the same such as mitochondrial depolarization, PtdSer exposure recognised by annexin V (annexin A5) or lactadherin, thrombin generation, and extracellular vesicle release. However, the time differs, where the generation of apoptotic platelets often takes hours, it only takes minutes to generate procoagulant platelets. There is a need for harmonization in this area as many of the markers and methods used to assess apoptotic platelets are not specific when used in isolation but are also associated with procoagulant platelets. The International Society on Thrombosis & Haemostasis (ISTH) platelet physiology and vascular biology scientific and standardization committees therefore initiated a collaborative project "Identification of markers that can distinguish procoagulant platelets from apoptotic platelets". The results were presented at the ISTH 2022 Congress in London and a communication will be published in the near future.

10. Platelet storage for transfusion – is inhibition of apoptosis beneficial?

The brief platelet lifespan represents a major logistical challenge in transfusion medicine. It is established that platelets stored for transfusion should not be refrigerated (cold-stored), as they are rapidly cleared upon transfusion [78]. Nevertheless, platelets are described as more haemostatically effective post refrigeration and they are potentially making a comeback to stop uncontrolled bleeding [79]. On the other hand, storage at 37 °C causes swift loss of platelet viability [21], with the apoptotic elements of BCL-X_L degradation, caspase-9, −3 activation and cleavage of cytoskeletal proteins [37,77]. Hence, the current practice is room temperature storage of platelet concentrates at 20 °C to 24 °C. This permits platelets to be retained for 5 to 7 days depending on the collection method, storage practice, and pathogen reduction protocol [80]. The functional decay and morphological alterations that occur during storage is known as "the platelet storage lesion" (PSL) and correlates with reduced platelet lifespan and loss of haemostatic function post-transfusion [81]. The PSL holds features of both platelet activation and cell death.

Would inhibition of intrinsic apoptosis be of potential value for extending platelet survival during storage? Pharmacological caspase inhibition did not improve platelet function during *ex vivo* storage of human platelets [37], but inhibition of caspases does not prevent BAK/BAX mediated mitochondrial injury. Kodama et al. observed that Caspase-3/7 activity was lessened in stored murine platelets after *Bak/Bax* deletion or BCL-X_L overexpression [39], backing the existence of an apoptotic feature. A study by Pleines and co-workers that also included *Bak/Bax* deficient platelets (resistant to intrinsic apoptosis) demonstrated that although intrinsic apoptosis was rapidly induced during storage at 37 °C, it was not detected when platelets were kept at the standard storage temperature of 22 °C [77]. Remarkably, platelet loss of *Bak* and *Bax* did not prevent the development of the PSL at either temperature [77]. Hence, inhibiting intrinsic apoptosis in blood banked platelets is unlikely to yield significant benefit. It will, however, be interesting to see if blocking of intrinsic apoptosis during platelet cold-storage will prove beneficial in future studies.

11. Acquired thrombocytopenias with elements of apoptosis

Certain thrombocytopenias linked to viral and bacterial infections have features of megakaryocyte and/or platelet apoptosis. Platelets from bacterial-infected sepsis patients [82], and platelets exposed to *Helicobacter pylori* [83] have shown elements of apoptosis. In mice, platelet extrinsic apoptosis has recently been shown to be triggered by LPS in a sepsis model, where mice with caspase-8 deficient platelets were transiently protected from thrombocytopenia [68].

Thrombocytopenia is a feature of severe COVID-19 viral infections [84]. The low platelet count can partially be attributed to platelet apoptosis and the inclusion of platelets into microthrombi and severe thrombotic events. Severe COVID-19 is associated with increased antibody-mediated procoagulant platelets as well as apoptotic platelets [84,85]. Furthermore, megakaryocytes from HIV patients [86,87], and megakaryocytes and platelets from dengue virus-infected patients [88–90] have elements of apoptosis. In mice infected with lymphocytic choriomeningitis virus (LCMV), a mouse model of HIV, conditional deletion of intrinsic (*Bak/Bax*) and extrinsic apoptosis (*Caspase-8*) in the megakaryocyte lineage safeguarded megakaryocytes from death [55]. However, in the same report platelets were not protected from LCMV induced death. While platelet apoptosis might be one factor in virally induced thrombocytopenia; it is likely one of many.

Signs of megakaryocyte and/or platelet apoptosis have also been described in immune thrombocytopenia [91–93], myelodysplastic syndrome [91,94,95] acute coronary syndromes [96], and conditions of intravascular haemolysis [97].

12. What are the consequences of extending platelet survival *in vivo*?

Are platelets functionally normal in the absence of apoptosis? A study from Pleines et al. investigated the role of BAK/BAX-mediated apoptosis in haemostasis and thrombosis [77]. Mice with *Bak/Bax*-deficient platelets exhibited increased bleeding times and unstable thrombus formation. Platelets from these mice are unable to undergo intrinsic apoptosis and hence the *in vivo* lifespan was doubled when compared to wild-type. This phenotype was not caused by impaired procoagulant function (PtdSer exposure), but was associated with a defect in granule release and reduced mitochondrial spare respiratory capacity in aged platelets. Strikingly, rejuvenation of *Bak/Bax*-deficient platelets *in vivo* fully rescued the observed haemostatic defects. Thus, apoptotic culling of old platelets from the bloodstream is essential to maintain a functional, haemostatically reactive platelet population [77].

13. Conclusions and future perspectives

In a healthy individual, intrinsic apoptosis is a key controller of platelet lifespan in circulation. In fact, apoptotic removal of old platelets from the bloodstream is critical to maintain a functional, haemostatically reactive platelet population. Several thrombocytopenias linked to bacterial and viral infections exhibit elements of megakaryocyte and/or platelet apoptosis. The balance between BCL-2 family prosurvival and prodeath proteins dictates cell fate and varies in different cell types. Megakaryocyte survival is coordinately regulated by prosurvival BCL-X_L and MCL-1. But since MCL-1 is a short-lived protein that requires constant *de novo* protein synthesis, BCL-X_L is the key pro-survival protein required to maintain platelet lifespan, restraining prodeath BAK and

BAX. However, many of the markers and methods used to assess apoptotic platelets are not specific when used in isolation but are also associated with procoagulant platelets. Hence there is a need for harmonization in this area with the identification of markers that can distinguish apoptotic from procoagulant platelets.

BCL-X_L inhibition triggers rapid platelet intrinsic apoptosis with ensuing thrombocytopenia. The information obtained from the characterisation of the constituents of the platelet intrinsic apoptotic machinery could have future therapeutic effects. Reducing platelet number in a controlled way, by triggering intrinsic apoptosis could potentially be beneficial in certain settings of platelet excess (e.g., conditions including atherosclerosis in diabetes and certain cancers [98,99]).

Declaration of competing interest

E.C.J. has received royalties from WEHI related to Venetoclax.

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