Megakaryocyte maturation involves activation of the IRE1α-dependent adaptive unfolded protein response

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Abstract

Endoplasmic reticulum stress triggers the unfolded protein response (UPR) to promote cell survival or apoptosis. Transient endoplasmic reticulum stress activation has been reported to trigger megakaryocyte production, and UPR activation has been reported as a feature of megakaryocytic cancers. However, the role of UPR signaling in megakaryocyte biology is not fully understood. We studied the involvement of UPR in human megakaryocytic differentiation using PMA (phorbol 12-myristate 13-acetate)-induced maturation of megakaryoblastic cell lines and thrombopoietin-induced differentiation of human peripheral blood-derived hematopoietic stem cells. Our results demonstrate that an adaptive UPR mediated by IRE1α (inositol-requiring enzyme 1α) endonuclease activity is required for megakaryocyte differentiation. Differentiation did not alter the response to the canonical endoplasmic reticulum stressors DTT or thapsigargin. However, thapsigargin, but not DTT, inhibited differentiation, consistent with the involvement of Ca²⁺ signaling in megakaryocyte differentiation.
Keywords: megakaryopoiesis, endoplasmic reticulum (ER) stress, unfolded protein response (UPR), calcium, IRE1α, XBP-1
Introduction

The endoplasmic reticulum (ER) is a vital cellular organelle involved in folding and post-translational modification of secreted and transmembrane proteins, storage of calcium, and metabolism of carbohydrates, with disruptions of these processes leading to ER stress [1-4]. In response to ER stress, eukaryotic cells trigger a tightly coordinated intracellular signaling cascade known as the unfolded protein response (UPR) to maintain protein homeostasis. There are three key UPR signal activators: IRE1α (inositol-requiring enzyme one alpha), PERK (protein kinase R-like ER kinase), and ATF6 (activating transcription factor 6). Once these UPR signal molecules are activated, the continued accumulation of unfolded proteins is prevented by upregulating the expression of chaperones to promote folding, repressing mRNA translation to temporarily inhibit global protein synthesis, and initiating ER-associated degradation (ERAD) pathways. However, if the UPR is unable to restore protein homeostasis, UPR signaling events may cause apoptosis [5, 6].

Previous studies have demonstrated that ER stress and the UPR signaling events are involved in the differentiation of many cell types, including fibroblasts [7], osteoblasts [8], neutrophils [9], plasma cells [10] and mammary epithelial cells [11]. It has been proposed that ER stress activation triggers an apoptotic-like phase of megakaryocyte differentiation in human megakaryoblastic cell lines, and may drive proplatelet formation [12, 13]. Recent studies report that UPR-specific gene expression is upregulated in calreticulin (CALR)-mutated megakaryocyte progenitors, increasing survival of the progenitors to contribute to the abnormal accumulation of bone marrow megakaryocytes in myeloproliferative neoplasms [14-18]. Despite these studies showing evidence of UPR activation during normal and abnormal megakaryopoiesis, the precise role of UPR in megakaryocyte biology is not understood.

In this study, we investigated the involvement of UPR signaling and examined the consequences of UPR inhibitors and activators during in vitro maturation of megakaryocytic cell lines and human peripheral blood (PB) derived hematopoietic stem cells (HSC).

Materials and Methods

Cell culture and differentiation

Human megakaryoblastic leukemic cells, MEG-01 (ATCC CRL-2021) [19] and K-562 (ATCC CRL-243) [20], were maintained in RPMI-1640 (Gibco; 3180002) medium supplemented with...
10% fetal bovine serum (FBS), sodium bicarbonate (2 g/L), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO₂ humidified atmosphere. The identity of the cell lines was confirmed by STR analysis (DNA Diagnostics, NZ) and the cells were confirmed to be mycoplasma free. In vitro megakaryocytic maturation was induced by treating K-562 or MEG-01 cells (2.5 x 10⁵ cells/mL) in a 6-well plate with 0.5 nM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich).

CD45+ cells were isolated and purified from PB mononuclear cells from individual donors as previously described [21, 22]. Isolated CD45+ cells (1 x 10⁵ cells/ml) were cultured in StemSpan™ medium (StemCell Technologies, Vancouver, Canada) supplemented with 10 ng/ml thrombopoietin (TPO), interleukin (IL) 6 and IL 11 (StemCell Technologies) at 37°C in a humidified 5% CO₂ incubator for 14 days with media changes on days 5, 8, and 11.

**ER stress modulators**

The conventional ER stressors thapsigargin (Tg) (T9033; Sigma-Aldrich) or dithiothreitol (DTT) (D0632; Sigma-Aldrich), were used to induce ER stress at the indicated concentrations and time points. The following compounds were used as UPR inhibitors: PF429242-dihydrochloride (SML0667; Sigma-Aldrich), an ATF6 inhibitor [23], AMG-PERK-44 (HY-12661A, MedChemExpress), a PERK inhibitor [24], and SN34221 (Supplementary data), an IRE-1α endonuclease inhibitor [25]. The chemicals were dissolved in DMSO or distilled water, and DMSO used as a control when appropriate.

**Analysis of megakaryocytic maturation by flow cytometry**

Cells were harvested, washed with Dulbecco’s PBS, resuspended in 100 µL of Dulbecco’s PBS, and stained for 30 min in the dark at room temperature. Antibodies used were anti-CD61 phycoerythrin (PE) (1:20; 1075384; BD Biosciences), mouse IgG1 PE (isotype control; 7223589; BD Biosciences), anti-CD41a V450 (1:20; 1011309; BD Biosciences), V450 IgG1 (isotype control; 7128864; BD Biosciences). Live cells were determined with Zombie NIR (1:100; B312060; BioLegend) or Zombie Green (1:100; B334686; BioLegend). After incubation, the cells were washed with Dulbecco’s PBS or wash buffer (1X Dulbecco’s PBS, 1 mM EDTA, 3% FBS), resuspended in FACS buffer (0.01% sodium azide, 0.1% BSA in Dulbecco’s PBS) analyzed using the Guava® easyCyte 5 HPL benchtop flow cytometer (MerckMillipore) or BD LSRFortessa™ cell analyzer (BD Biosciences). Data analysis was performed using FlowJo.
version 10 (FlowJo, Ashland, USA). Data reported are geometric mean fluorescence intensity (MFI) of the live cell (Zombie negative) population.

**RNA isolation and RT-qPCR**

Total RNA was isolated from K-562 and MEG-01 cells using TRIzol (Invitrogen). The concentration of the RNA was measured using Qubit™ RNA HS Assay Kit (ThermoFisher Scientific). First-strand cDNA synthesis was performed with 1 µg of total RNA in 20 µL reaction volume using SuperScript™ IV VILO™ (SSIV VILO) master mix. Real-time quantitative RT-PCR reactions were run on LightCycler™ 480 Instrument II System (Roche Life Science) using PowerUp™ SYBER™ Green Master Mix (Thermo Fisher Scientific). All the procedures were followed as recommended by the manufacturer. The qPCR primer sequences are shown in Table S1. RT-qPCR was performed in triplicate, and relative expression was quantified using the Pfaffl method [26]. Data were normalized to hypoxanthine phosphoribosyl transferase 1 (HPRT) and glyceraldehyde 3-phoshate dehydrogenase (GAPDH) using basket normalization [27].

**Semi-quantitative PCR analysis for XBP-1 splicing detection**

To detect XBP-1 splicing, XBP-1 mRNA was amplified using XBP-1 primers (forward 5’CCTTGTAGTTGAGAACCAGG 3’ and reverse 5’ AGGGGCTTGGTATATATGTGG 3’), and the reactions were carried out in the C1000 Touch™ thermal cycler (Bio-Rad). Briefly, first-strand cDNA was synthesized with 2 µg of total RNA in 20 µL reaction volume. To amplify XBP-1 mRNA, 5 µL of undiluted cDNA was used as a template in a 50 µL reaction mixture with an initial denaturation step for 3 min at 94 ºC, followed by 30 cycles of denaturation for 30 s at 94 ºC, annealing for 30 s at 54 ºC, and extension for 60 s at 72 ºC, and a final extension step for 10 min at 72 ºC. PCR products were run on 2% (w/v) agarose gel, stained with 0.5 µg/mL ethidium bromide, and visualized using the Gel Doc™ XR+ Imaging System (Bio-Rad).

**Protein extraction and western blotting**

Cells were lysed in RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich; MSSAFE). Total protein (20 µg) was resolved by SDS PAGE under reducing conditions and transferred to nitrocellulose membrane. Membranes were stained with REVERT™ 700 total protein stain (LI-COR Biosciences), washed and imaged.
on the Odyssey Fc Imaging System (LI-COR Biosciences). The membrane was then blocked with Intercept® Blocking Buffer (LI-COR Biosciences) for 1 h at room temperature and incubated overnight with 1:500 anti-GRp78 (BiP) antibody (sc-1050; Santa Cruz) at 4 ºC. IRDye® 680-LT donkey anti-goat (C60127-06, LI-COR Biosciences) was used as a secondary antibody, and the blots were visualized in the Odyssey Fc Imaging System. Band intensities were quantified with Image Studio Lite version 5.2 (LI-COR Biosciences). Briefly, signals from the total protein stain and target protein were measured, the lane normalization factor (LNF) was calculated for total protein in each sample, and the target protein band intensities were normalized using the LNF.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA). The comparison of means between different groups was performed using one sample t-test, one-way or two-way ANOVA, or two-tailed Student’s t-test. *P* < 0.05 was considered significant.

**Results**

**The unfolded protein response is activated during megakaryocytic maturation of K-562 and MEG-01 cells**

We chose K-562 and MEG-01 cell lines to study the role of UPR activation in megakaryocytic maturation because both are myeloid cell lines that mature towards megakaryocytes upon PMA treatment [28, 29]. We confirmed that treatment with 0.5 nM PMA caused an increase in the expression of the megakaryocytic maturation markers CD61 and GATA1 over 3 days without changing cell viability (Figure S1).

To determine whether the UPR is activated during PMA-induced maturation of K-562 and MEG-01 cells we examined the expression of UPR target genes. The expression of CHOP (DDIT3), ERdj4 (DNAJB9), HERPUD1, and XBP1 mRNA was significantly increased in K-562 and MEG-01 cells at day 3 (Figure 1). This suggests that there is activation of the IRE1α and/or ATF6 arms of the UPR during maturation. PERK/ATF6-dependent ATF4 and GADD34 (PPP1R15A) expression was also significantly upregulated in K-562 cells, but not MEG-01 cells, at day 3 of maturation (Figure S2A,B). BiP (HSPA5), which is both a UPR sensor and a target, was not induced in MEG-01 cells, and showed a significant decrease in K-562 cells (Figure 1).
As this was inconsistent with the other UPR targets we also analyzed BiP protein expression and saw no change upon maturation (Figure S2C). Overall, these results indicate that activation of UPR signaling pathways occurs during PMA-induced megakaryocytic maturation of K-562 and MEG-01 cells.

Figure 1. UPR target gene expression is upregulated during PMA-induced maturation of K-562 and MEG-01 cells into megakaryocytes. K-562 (A) or MEG-01 (B) cells were treated with 0.5 nM PMA and expression of CHOP, ERdj4, HERPUD1, XBP1 and BIP mRNA at days 0, 1, 2 and 3 was determined by RT-qPCR. Relative expression was calculated using the value at day 0 as 1. N=3 ± SD. *P < 0.05, **P < 0.01 compared to day 0 (one sample t-test).

Inhibition of IRE1α endonuclease activity, but not PERK or ATF6, inhibits megakaryocytic maturation

Having shown that the UPR is activated during megakaryocytic maturation of K-562 and MEG-01 cells, we next wanted to determine whether activation of one or more arms of the UPR is required for maturation. To do this we used inhibitors of the IRE1α, PERK and ATF6 UPR pathways. In the BiP-dependent IRE1α pathway, activation of the IRE1α endonuclease induces splicing of XBP1 mRNA [30], with the resulting XBP-1 transcription factor driving gene expression. We first confirmed that novel IRE1α endonuclease inhibitor SN34221 [25] dose dependently inhibited DTT-induced XBP1 splicing in K-562 cells, with complete inhibition at 50 μM (Figure S3). Next the impact of SN34221 on PMA-induced K-562 maturation was
determined. There was an approximate 25% decrease in CD61 MFI in the live cell population (Figure 2A) and a decrease in GATA1 expression (Figure 2B) at day 3 with 50 μM SN34221, with a small decrease in cell viability (Figure S4A). This suggests that activation of the IRE1α UPR pathway may contribute to K-562 megakaryocytic maturation. Consistent with inhibition of maturation, SN34221 also decreased the maturation-associated increase in expression of the XBP-1 target ERdj4 but not the PERK/ATF6 targets CHOP, ATF4, GADD34 or HERPUD1 (Figure 2C). In contrast, inhibition of the PERK UPR pathway with AMG-PERK44 [24] significantly increased CD61 MFI at day 3 in the live cell population (Figure 2D), and inhibited expression of the PERK targets CHOP, ATF4 and HERPUD1 (Figure 2E). We also observed a dose-dependent decrease in cell viability (Figure S4B). Our results with the PERK inhibitor suggest that the PERK branch of the UPR could have an inhibitory effect on PMA-induced megakaryocytic maturation but plays a vital role in megakaryocyte survival. Finally, the site-1 protease inhibitor PF-429242 [23] was used to inhibit the ATF6 UPR pathway but no change in CD61 expression (Figure S4C) or cell viability was observed (Figure S4D) although the expression of CHOP, ATF4 and HERPUD1 was significantly decreased (Figure S4E).
Figure 2. The effect of pharmacological inhibition of UPR on K-562 maturation and HSC differentiation. A and D. K-562 cells were treated with 0.5 nM PMA in the presence of SN34221 (A) or AMG-PERK44 (C) and expression of CD61 determined (fold change MFI compared to day 0). N=3 ± SD. *P < 0.05, ***P < 0.001, ****P < 0.0001 (two-way ANOVA). B and D. K-562 cells were treated with 0.5 nM PMA in the presence (white bars) or absence (grey bars) of 50 μM SN34221 (B) or 2 μM AMG-PERK44 (D) and relative gene expression measured at day 3. N=3 ± SD. *P < 0.05, ** P<0.01, ***P<0.001 (unpaired t-test). E. % CD61+ (light grey bars) or CD61+/CD41+ cells (dark grey bars) at
days 11 and 14 of TPO-induced megakaryocyte differentiation of HSC in the presence of DMSO or 50 μM SN34221. N=3 ± SD *P < 0.05 (one-way ANOVA).

Having observed the potential involvement of the IRE1α UPR pathway in megakaryocytic maturation of K-562 cells, we next wanted to determine whether this pathway was also involved in TPO-induced differentiation of HSC isolated from human peripheral blood. The IRE1α endonuclease inhibitor SN34221 significantly decreased the percentage of partially mature (CD61+) and fully mature (CD61+/CD41+) megakaryocytes at day 14 (Figure 2E). Consistent with inhibition of differentiation there was no evidence of proplatelet formation or membrane blebbing at day 14 (Figure S5C). These results indicate that the IRE1α signaling branch of UPR is involved in megakaryocyte differentiation of HSC.

**Activation of ER stress by thapsigargin, but not DTT, inhibits PMA-induced maturation of K-562 cells**

Our data suggests that activation of the IRE1α pathway is involved in megakaryocyte maturation. We next determined whether activation of the IRE1α pathway by an exogenous ER stress had any impact on megakaryocyte maturation. We confirmed that the ER stressors DTT, a small-molecule redox reagent for reducing protein disulfide bonds, and thapsigargin, a sarco/endoplasmic reticulum ATPase (SERCA) inhibitor, induced IRE1α activation as shown by the splicing of XBP1 mRNA (Figure S6). Exogenous induction of the UPR by DTT had no impact on K-562 megakaryocytic maturation (Figure 3A) or viability (Figure 3B). In contrast, treatment with thapsigargin caused a dose-dependent reduction in PMA-induced maturation of K-562 cells (Figure 3C) with no change in cell viability (Figure 3D). Thapsigargin also inhibited TPO-induced HSC differentiation (Figure 3E) and subsequent proplatelet production (Figure S5D). Overall, these data indicate that while exogenous IRE1α activation does not enhance megakaryocyte maturation, ER Ca²⁺ homeostasis plays a central role in megakaryocyte differentiation.
Figure 3. The effect of UPR activation on K-562 maturation and PBMC differentiation. A-D. K-562 cells were treated with 0.5 nM PMA for 2 days in the presence of DTT (A and B) or Tg (C and D) and analyzed for expression of CD61 (fold change MFI compared to day 0) (A and C) or cell viability (B and D). N=3 ± SD. *P < 0.05, **P < 0.01 (one way ANOVA). E. % CD61⁺ (light grey bars) or CD61⁺/CD41⁺ cells (dark grey bars) at days 11 and 14 of TPO-induced megakaryocyte differentiation of HSC in the presence of DMSO or 1 μM Tg. N=3 ± SD *P < 0.05 (one-way ANOVA).

Megakaryocytic maturation of K-562 cells does not alter the response to an exogenous ER stress.
Finally, we examined whether maturation alters the ability of the K-562 or MEG-01 cells to respond to ER stress. K-562 cells were treated with DTT or thapsigargin before or 3 days after PMA treatment, and the expression of UPR target genes was quantified by RT-qPCR. DTT treatment activated the UPR as indicated by a strong induction of \(XBP1\) splicing, and \(CHOP\), \(BIP\) and \(ATF4\) (but not \(GADD34\)) expression, in immature (untreated) and mature (PMA treated) K-562 cells (Figure 4A). Similarly, thapsigargin treatment induced \(XBP1\) splicing and increased \(CHOP\), \(BIP\) \(ATF4\) and \(GADD34\) expression in immature K-562 cells, with all but \(ATF4\) also showing increased expression in the mature cells (Figure 4B). Similar results were obtained with MEG-01 cells (Figure S7). These results indicate that even though UPR pathways are activated during PMA-induced maturation, this does not alter the ability of the cells to further activate canonical UPR pathways in response to an exogenous ER stressor.
Figure 4. PMA treated and untreated K-562 cells are similarly responsive to ER stress induced by DTT or thapsigargin. Immature (no PMA) or mature (PMA) K-562 cells were treated with 10 mM DTT for 0-2 h (A) or 0.1 µM Tg for 0-12 h (B). Expression of XBP1s and XBP1t, CHOP, BIP, ATF4, and GADD34 mRNA was quantified by RT-qPCR. Expression is relative to 0 h. N=3 ± SD. *P < 0.05, **P < 0.01 (one sample t-test).

Discussion

UPR signaling pathways have been found to play a vital role in organellar development and cellular differentiation in a variety of cell types [7-11, 31, 32]. Furthermore, CALR mutations, the second most common driver mutations in classical Philadelphia chromosome-negative MPN myeloproliferative neoplasms [33, 34], are associated with activation of UPR target genes, leading to increased survival and abnormal accumulation of megakaryocyte progenitors in patients’ bone marrow [14-18]. However, the role of UPR signaling in normal megakaryocyte differentiation is unclear. Here we have used the K-562 and MEG-01 cell lines and primary HSC to determine how UPR signaling contributes to megakaryocyte differentiation.

UPR signaling can be divided into an adaptive response, allowing a cell to survive in a new environment, and a terminal response, which triggers cell death [5, 6]. The balance between these two responses depends upon the nature of the initial stress, and the success of the adaptive response. Early reports suggested that localized caspase activation is required for megakaryopoiesis [35, 36], and more recently it has been suggested that UPR signaling drives caspase activation via the intrinsic apoptosis pathway during maturation [12, 13]. However, the role of caspase activation in megakaryopoiesis is uncertain, with genetic studies in mice convincingly showing that intrinsic apoptosis must be suppressed for successful differentiation [37, 38]. Studies suggesting a role for ER stress-induced apoptosis in megakaryopoiesis have mostly used PMA-induced maturation of various cell lines. However, the PMA concentrations used to induce maturation and draw conclusions about the role of UPR signaling also resulted in extensive cell death [12, 13]. Therefore, it is unclear whether UPR activation in these studies was a specific feature of megakaryocyte maturation or a generalized response to cell stress. In the current study we used a low concentration of PMA that induced megakaryocytic maturation of K-562 and MEG-01 cell lines in the absence of cell death, clearly demonstrating that induction of apoptosis is not required for PMA-induced megakaryocytic maturation. Under these conditions maturation was accompanied by increased transcription of several UPR target genes:
CHOP, XBP1, ERDj4, HERPUD1, ATF4 and GADD34. Expression of these genes in the absence of cell death is consistent with activation of the adaptive UPR. The terminal stages of megakaryopoiesis involve endomitosis and polyploidization with an accompanying high demand for synthesis of proteins and membranes prior to proplatelet formation and platelet release [39]. Therefore, it is unsurprising that the adaptive UPR is activated in maturing megakaryocytes. This would then assist terminal megakaryopoiesis by increasing the biosynthesis of chaperone proteins, increasing membrane synthesis, and boosting the protein folding capacity of the ER. Indeed XBP-1 is an important regulator of specialist secretory cells which require a high level of protein synthesis [32, 40].

To determine which arm of the adaptive UPR was activated, we inhibited IRE1α endonuclease activity (SN34221), PERK autophosphorylation (AMG-PERK44) and ATF6 cleavage (PF-429242). SN34221 inhibited PMA-induced maturation of K-562 cells and TPO-induced differentiation of HSC, implicating the IRE1α pathway in the adaptive UPR. IRE1α is an endonuclease located in the ER membrane. There are two outcomes of IRE1α endonuclease activity, XBP1 splicing to induce expression of proteins involved in ER protein translocation, folding and secretion, and regulated IRE1-dependent decay (RIDD) [6, 30, 41-44]. Both XBP1 splicing and RIDD have been implicated in differentiation of a range of cell types as well as in C. elegans and Drosophila development [32, 45-52]. The specific role of XBP1 splicing in the hematopoietic compartment has been investigated using a Vav-1 promoter-driven XBP1 knockout mouse model, finding a specific defect in eosinophil differentiation, but the number of bone marrow megakaryocytes and circulating platelets was not determined [53]. Our observation that both immature and mature K-562 cells show similar responses to DTT and thapsigargin, and that DTT has no impact on maturation, further suggests that the IRE1α-mediated adaptive response is distinct from the canonical ER stress response to accumulation of unfolded proteins. Understanding the specific role of XBP1 splicing and/or RIDD in megakaryopoiesis requires further investigation.

Treatment with thapsigargin inhibited both PMA-induced maturation of K-562 cells and TPO-induced differentiation of HSC. Thapsigargin blocks SERCA, inducing protein misfolding and the UPR, and altering Ca^{2+} homeostasis [54-56]. Since DTT had no impact on megakaryocytic maturation, it is likely that dysregulation of Ca^{2+} homeostasis underlies inhibition of maturation by thapsigargin. This is consistent with the known roles of intracellular Ca^{2+} in
megakaryopoiesis. Activation of phospholipase C generates inositol trisphosphate (IP3) triggering release of Ca\(^{2+}\) from the ER via the IP3 receptor. Subsequent store-operated calcium entry (SOCE) induces expression of nuclear factor of activated T cells (NFAT), a transcriptional regulator implicated in megakaryopoiesis [57-59]. Additionally, Ca\(^{2+}\)-entry through a membranous Ca\(^{2+}\) ion channel supports megakaryocyte differentiation [60, 61]. Ca\(^{2+}\)-dependent activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK) induces transcription of CREB (c-AMP response element-binding protein) to activate the genes with CRE elements, including megakaryocyte-lineage specific genes such as FLI1, HOXC6, MXD3, PRDM16, FOXB1, TBX6, HDAC11 and NPAS1 [58, 59, 62]. Finally, changes in intracellular Ca\(^{2+}\) homeostasis occur in myeloproliferative neoplasms and may contribute to disease pathogenesis through ER stress [63-66].

Megakaryocyte differentiation is a complex process that requires the activation of several synergistic pathways. Our findings indicate that the IRE-1-mediated adaptive UPR, rather than an apoptotic response, is involved in megakaryocyte differentiation, and provide further evidence for the importance of calcium signaling in megakaryopoiesis.

**Statements and Declarations**

**Funding:** This work was supported by Leukaemia & Blood Cancer New Zealand (to ECL), University of Otago School of Biomedical Sciences Dean’s Bequest Fund (to ECL), and Auckland Medical Research Foundation (to MK-Z). Author MF was supported by a University of Otago Doctoral Scholarship. Authors MPH and DCS were supported by Cancer Society Auckland/Northland.

**Competing interests:** The authors have no competing interests to declare.

**Author contributions:** ECL and MK-Z conceived the study. ECL, MK-Z and MF designed the study. Experimental work was performed by MF. All authors contributed to data analysis. The first draft of the manuscript was written by MF and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data availability:** Data and materials are included within the article or Supplementary Information are available from the authors on request.

**Ethics approval:** The study was approved by the University of Otago Human Ethics Committee (NZ) (H20/132).

**Consent to participate:** Informed consent was obtained from all individual participants included in the study.

**Acknowledgements:** Not applicable
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