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Acute myeloid leukemia driven IL-3-dependent upregulation of BCL2 in non-malignant hematopoietic stem and progenitor cells increases venetoclax-induced cytopenias

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**Data availability:** Detailed data available on request.
TO THE EDITOR:

The BH3 mimetic venetoclax, in combination with low dose cytarabine, decitabine or azacitidine has shown clinical efficacy in newly diagnosed acute myeloid leukemia (AML) patients over 75 or those ineligible for intensive induction chemotherapy (1, 2). This has been a significant advance, particularly in the treatment of older AML patients who historically have been difficult to treat (3-5). Venetoclax selectively inhibits the BCL2 protein which is overexpressed in AML to activate intrinsic apoptosis of AML cells. However, this treatment regime is associated with higher incidences of cytopenias, including clinically relevant neutropenia, febrile neutropenia and thrombocytopenia (2, 6). The underlying cause of cytopenia in the context of venetoclax-treated AML patients remains unexplained. Here, we show that AML drives IL-3-dependent upregulation of BCL2 in non-malignant hematopoietic stem and progenitor cells (HSPC), which is targeted by venetoclax and causes cytopenias.

To model AML \textit{in vivo} we have previously used the syngeneic AML mouse model in which the MN1 oncogene is overexpressed in progenitor cells (7, 8). All \textit{in vivo} work in this study followed the institutional and national guidelines for the care and use of laboratory animals. To determine if this model recapitulates the clinical cytopenias associated with venetoclax treatment, MN1 cells were engrafted into non-conditioned female C57BL/6 mice and 100 mg/kg venetoclax or vehicle control were administered for 7 consecutive days by oral gavage (Fig 1A). Blood and BM samples were taken following sacrifice of mice and analysed on BD FACSymphony™ A1 (BD Biosciences, Berkshire, UK). Flow cytometry analysis demonstrated that monocytes and neutrophils as well as B and T cells are depleted in venetoclax treated MN1 engrafted mice compared to vehicle control treated MN1 engrafted mice (Fig 1B and Supplementary Fig 1A). B and T cell depletion was also observed in control mice treated with venetoclax, but monocyte and neutrophil counts were not reduced (Fig 1C). We next investigated the effect of venetoclax on non-malignant HSPCs in the BM, specifically hematopoietic stem cell (HSC), multipotent progenitor (MPP) and LSK (Lin$^-$ Sca1$^+$ CD117$^+$) populations (Supplementary Fig 1B). HSC, MPP and LSK populations were all depleted by venetoclax in MN1 engrafted mice compared to vehicle control treated MN1 engrafted mice (Fig 1D).

Further, we assessed the BCL2 expression profile in non-malignant LSKs in the presence of AML. MN1 cells were injected into non-conditioned female C57BL/6 mice. At day 14, LSKs were FACS purified from the BM (Fig 2A). Real-time qPCR analysis determined that
LSKs in MN1 engrafted mice exhibit increased BCL2 gene expression compared to control mice (Fig 2B). Elevated BCL2 protein expression was confirmed in LSKs in MN1 engrafted mice relative to control mice using flow cytometry (Fig 2C). To determine the mechanism for increased BCL2 expression these findings were modelled in vitro by co-culturing LSKs with either MN1 or MEIS1/HOXA9 AML subtypes. MEIS1/HOXA9 cells were retrovirally generated and labelled with mCherry as previously described (7, 8). Real-time qPCR analysis confirmed that LSKs have significantly elevated BCL2 gene expression under AML co-culture conditions (Fig 2D). This was further confirmed at the protein level using flow cytometry for BCL2 (Supplementary Fig 1C). To determine if a secretory factor from AML is responsible for inducing BCL2 upregulation in LSKs, we cultured LSKs with cell-free conditioned media from MN1 cells (Fig 2E). Real-time qPCR confirmed that BCL2 is upregulated in LSKs treated with AML conditioned media (Fig 2F) and demonstrates that an AML mediated secretory factor is triggering BCL2 overexpression in LSKs.

Previous research has suggested roles for interleukins (IL-) 1,3 and 6 and TNF (9-12) as inducers of BCL2 transcription in different cell types (11) (Fig 3A). Therefore, to interrogate the mechanism, BCL2 expression was assessed in LSKs treated with IL-3, IL-6, IL-1β and TNF by real-time qPCR. Notably, results showed that BCL2 was significantly upregulated in LSKs in response to an IL-3 stimulus (Fig 3B), but not IL-6, IL-1b or TNF. Studies have previously shown that IL-3 levels are increased in the serum of patients with AML (13). Furthermore, IL-3 has been shown to directly affect HSPC function, driving proliferation and differentiation but impairing long-term repopulation and self-renewal potential (14, 15). IL-3 therefore appears to play a role both in AML disease pathogenesis and in normal HSC function. In this paper we focus on the impact of IL-3 on BCL2 expression and the subsequent influence of venetoclax on HSPC function rather than the overall impact of IL-3 on HSPC function, which is beyond the scope of this paper.

To determine the effect of IL-3 inhibition on BCL2 expression, LSKs cultured with MN1 conditioned media were treated with IL-3 neutralising antibody MP2-8F8 (Bio X Cell, NH, USA). Real-time qPCR analysis confirmed that IL-3 neutralisation prevents upregulation of BCL2 in LSKs cultured in MN1 conditioned media (Fig 3C). BCL2 gene expression was also reduced in MN1 and MEIS1/HOXA9 cells treated with IL-3 neutralising antibody (Fig 3D), suggesting that the effect of IL-3 inhibition on BCL2 expression is non-specific. Despite this, blocking IL-3 does not make AML cells resistant to BCL2 inhibition (Supplementary Fig
1D). Next, to determine if IL-3 treatment results in post-translational changes in BCL2 in LSKs, both total and phosphorylated BCL2 protein levels were measured by flow cytometry. Results demonstrate that both total and phosphorylated BCL2 levels are increased to similar levels after IL-3 treatment (Supplementary Fig 1E). Of note, venetoclax was found to have no direct effect on BCL2 expression in mature haematopoietic lineages in the presence of AML, suggesting that the observed cytopenias are driven by changes in the HSPCs (Supplementary Fig 1F).

Finally, to assess the effect of IL-3 inhibition on cytopenias secondary to venetoclax treatment in vivo, mice were engrafted with MN1 cells and treated with 100 mg/kg venetoclax and 50 μg/kg IL-3 neutralising antibody daily for 7 days (Fig 3E). Results show that both monocyte and neutrophil counts recovered in AML engrafted mice treated with both venetoclax and IL-3 neutralising antibody compared to venetoclax only treated mice (Fig 3F). Taken together we show that AML induces an IL-3-dependent upregulation of BCL2 in HSPCs, which in turn increases HSPC sensitivity to venetoclax and causes cytopenias. Understanding of this mechanism could provide insight for a treatment strategy with venetoclax to reduce the incidence of cytopenias observed in AML patients.
REFERENCES


Figure legends

Fig 1 – Venetoclax treatment causes depletion of neutrophils and monocytes in AML engrafted mice. A) Experimental schematic. MN1 cells were retrovirally generated and subsequently tagged with GFP to allow distinction from non-malignant cells as previously described (7, 8). 2.5x10^6 MN1 cells were injected into female non-conditioned C57BL/6 mice aged 12-14 weeks. At day 10, 100 mg/kg venetoclax or vehicle control was administered by oral gavage for 7 days. Peripheral bloods (PB) and BM were assessed by flow cytometry following sacrifice. B) Cells counts for B cells, T cells, monocytes and neutrophils per mL of PB in MN1 engrafted mice treated with venetoclax (n = 8) compared to AML control mice (n = 8). C) Cells counts as above but in control mice with venetoclax treatment (n = 5) and without (n = 5). D) Cell counts per 100,000 BM cells for HSC, MPP and LSK in MN1 engrafted mice treated with venetoclax (n = 8) compared to AML control mice (n = 8). All data in B-D are represented as median + interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001 using Mann-Whitney U Test.

Fig 2 – AML drives an upregulation of BCL2 in non-malignant LSKs. A) Experimental schematic. 2.5x10^6 MN1 cells were injected into female non-conditioned C57BL/6 mice aged 12-14 weeks. LSKs were sorted by FACS on day 14. B) Real-time qPCR assessed BCL2 gene expression in LSKs in MN1 engrafted mice (n = 5) compared to control mice (n = 5). qPCR assay was performed with the SYBR-green technology (PCR biosystems, UK) (8) on QuantiStudio 5 PCR system using BCL2 (Mm_Bcl2_vb.1_SG QuantiTect Primer Assay, GeneGlobe ID QT01057224) and GAPDH (Mm_Gapdh_3_SG QuantiTect Primer Assay, GeneGlobe ID QT01658692) primers from Qiagen. C) BCL2 protein level in LSKs quantified by mean fluorescence intensity (MFI) in MN1 engrafted mice (n = 6) and control mice (n = 6) using flow cytometry. D) LSKs were isolated from BM of young C57BL/6 mice and 5x10^4 cells were co-cultured in transwells with either MN1 or MEIS1/HOXA9 cells for 48h in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Foetal bovine serum (FBS), 1% Penicillin Streptomycin (Pen-Strep). Real-time qPCR assessed BCL2 gene expression in LSKs co-cultured with AML cells (n = 4) compared to LSK-only controls (n = 4). E) Schematic for cell-free conditioned media extraction from MN1 cells. Isolated LSKs were cultured with MN1 conditioned media for 48h. F) Real-time qPCR assessed BCL2 gene expression in LSKs with MN1 conditioned media (n = 4) versus LSK-only controls (n = 4). All data in B, C, D and F are represented as median + interquartile range. *p < 0.05 using Mann-Whitney U Test.

Fig 3 – AML drives an IL-3 mediated overexpression of BCL2 in non-malignant LSKs which is reversed by IL-3 neutralisation, and restores neutrophil and monocyte counts in combination with venetoclax in AML engrafted mice. A) Schematic depicting potential mechanisms for BCL2 transcription in non-malignant LSKs. B) 5x10^4 LSKs were cultured in DMEM supplemented with 10% FBS and 1% Pen-Strep and treated with 100 ng/mL IL-3, IL-6, IL-1B or TNF for 24h. Real-time qPCR assessed BCL2 gene expression in treated LSKs (n = 4) compared to non-treated LSKs (n = 4). C) 5x10^4 LSKs were next
cultured with MN1 conditioned media and treated with 5 μg/mL anti-IL-3 (IL-3 neutralising antibody MP2-8F8, Bio X Cell, NH, USA) for 48hr. Real-time qPCR assessed BCL2 gene expression in LSKs cultured with MN1 conditioned media and anti-IL-3 antibody (n = 6), LSKs cultured with MN1 conditioned media only (n = 6) and LSK-only controls (n = 6). D) 3x10^5 MN1 and MEIS1/HOXA9 cells were treated with 5 μg/mL anti-IL-3 for 48h. Real-time qPCR assessed BCL2 gene expression in anti-IL-3 treated AML cells (n = 5) compared to non-treated AML cells (n = 6). E) Experimental schematic. 2.5x10^6 MN1 cells were injected into male non-conditioned C57BL/6 mice aged 12-14 weeks. At day 10, 100 mg/kg venetoclax was administered by oral gavage alongside 50 μg/kg anti-IL-3 by intraperitoneal injection, both daily for 7 days. Peripheral bloods (PB) were assessed by flow cytometry following sacrifice. F) Cell counts for B cells, T cells, monocytes and neutrophils per mL of PB in MN1 engrafted mice treated with venetoclax plus anti-IL-3 antibody (n = 7) compared to venetoclax only (n = 7). All data in A-F excluding A, E are represented as median + interquartile range. *p < 0.05, **p < 0.01, ****p < 0.0001 using Mann-Whitney U Test or Kruskal-Wallis test for multiple comparisons.
Figure 2.

A

MN1 cells → Young C57BL/6 mice → Sorted LSKs

B

BCL2

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C

Count

BCL2 (Pe Cy7)

D

BCL2

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E

MN1 culture → Centrifugation 2000 rpm 2 min → Supernatant → Centrifugation 14000 rpm, 10 min → MN1 conditioned media → Isolated LSKs

F

BCL2

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Supplementary Fig 1 – Flow cytometry gating, BCL2 expression and cell viability.

A) Gating strategy for B cells (CD4+ CD8+ CD19+), T cells (CD4+ CD8+ CD19+), monocytes (CD11b+ Ly6 G+) and neutrophils (CD11b+ Ly6 G+). B) Gating strategy for non-malignant HSC (Lin- Sca1+ CD117+ CD150+ CD48-), multipotent progenitor (MPP) (Lin- Sca1+ CD117+ CD150+ CD48+), and LSK (Lin- Sca1+ CD117+). C) BCL2 protein expression is elevated in LSKs co-cultured with both MN1 and MEIS1/HOXA9 AML subtypes. LSKs were isolated from BM of young C57BL/6 mice and 5x10^6 cells were co-cultured in transwells with either MN1 or MEIS1/HOXA9 cells for 48h. BCL2 protein level was quantified by mean fluorescence intensity (MFI) in LSKs co-cultured with AML cells (n = 6) compared to LSK-only controls (n = 5) using flow cytometry. **p < 0.01 using Mann-Whitney U Test. D) Blocking IL-3 does not make AML cells resistant to venetoclax. 5x10^4 MN1 and MEIS1/HOXA9 cells were cultured with 10 μM venetoclax, 5 μg/mL anti-IL-3 or both for 24h. Percentage cell viability was quantified using the CellTiter-Glo® luminescent assay in MN1 and MEIS1/HOXA9 cells remaining untreated (n = 6), treated with venetoclax (n = 6), treated with anti-IL-3 (n = 6) and treated with venetoclax and anti-IL-3 (n = 6). E) Total BCL2 and phosphorylated BCL2 protein levels are elevated in LSKs treated with IL-3. 2x10^5 LSKs were cultured in DMEM supplemented with 10% FBS and 1% Pen-Strep and treated with 100 ng/mL IL-3 for 24h. Total BCL-2 protein level and phosphorylated BCL2 within the BCL2-positive population was quantified by mean fluorescence intensity (MFI) in LSKs treated with IL-3 (n = 3) compared to LSK-only controls (n = 3) using flow cytometry. Data are non-significant using Mann-Whitney U Test. F) BCL2 protein levels were quantified by MFI in the lineage positive cell population of MN1 engrafted mice treated with venetoclax (n = 9) compared to control MN1 engrafted mice (n = 8). Data are non-significant using Mann-Whitney U Test. All data in C-F are represented as median ± interquartile range.