scientific reports

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Bromodomain and extra-terminal protein inhibitors modulate natural killer cell function and differentiation

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Natural killer (NK) cells are integral to the innate immune system, playing a crucial role in immune surveillance and the rapid response to virally infected and tumor cells. Epigenetic gene expression regulation significantly influences NK cell function and differentiation. Using a high-throughput small-molecule drug screening approach, we identified bromodomain and extra-terminal domain (BET) inhibitors (BETi) as potent modulators of NK cell function, reducing proinflammatory cytokine secretion while increasing markers of NK cell maturation and cytotoxicity. During NK lineage specification from hematopoietic stem cells, we demonstrated that BETi reduced NK cell fate and promoted increased myeloid cell differentiation. Moreover, differentiated NK cell types had more functionally differentiated gene expression programs. Thus, BET proteins are crucial for both mature NK cell functions and controlling NK cell lineage development from progenitors in the bone marrow. These findings suggest that BETi can fine-tune NK cell responses, offering promising therapeutic potential for cancer immunotherapy and the treatment of inflammatory and autoimmune diseases. Our study underscores the critical role of BET inhibitors in regulating NK cell function and opens new avenues for targeted immune modulation.

Keywords Natural killer cells, Innate immunity, Single-cell genomics, Epigenetic regulation

Natural killer (NK) cells are a critical component of the innate immune system, responsible for the rapid response to virally infected cells and tumor cells[1–4]. They also play a pivotal role in control of immune responses and the maintenance of tissue homeostasis through immunoregulation of other immune cell types[5–8]. While NK cell immunosurveillance is an essential aspect of our immune system, prolonged or overactivation of NK cells can lead to detrimental effects. Increased NK cell activity can result in excessive inflammation, contributing to the pathogenesis of various inflammatory and autoimmune diseases[7,9,10]. Moreover, excessive NK cell-mediated cytotoxicity can disrupt normal tissue homeostasis and impair the function of other immune cells, potentially leading to immune dysregulation and increased susceptibility to infections[11–14]. Therefore, while NK cells are crucial for immune defense, their activity must be tightly regulated to prevent harmful consequences.

Long-term functional states of NK cells can be imprinted through epigenetic rewiring of the NK cell gene expression networks[15–18]. This is often induced through exposure to stimuli such as cytokines or during viral infections where NK cells bearing particular receptors are activated by ligands on infected cells, which leads to lasting changes in the epigenetic landscape of NK cells[19,20]. Epigenetic regulation of cell fate and function is a critical interface between environmental stimuli and cellular gene expression patterns that influence function. For example, pre-activation with specific cytokines (IL-12, IL-15, and IL-18) resulted in the demethylation of conserved non-coding sequences in key genes like *IFNG*, enhancing the transcriptional activity of interferon-

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gamma (IFN-γ)[21,22]. These epigenetically imprinted NK cells, often referred to as memory-like or adaptive NK cells, exhibit altered effector functions. Adaptive NK cells expanded during cytomegalovirus infections showed reduced responsiveness to innate cytokines alone or stimulation with autologous activated cells, but mediated enhanced antibody-triggered cytotoxic activity against infected or tumor targets. While innate cytokine-induced memory NK cells exhibit enhanced responsiveness to innate cytokines in addition to enhanced antitumor and antiviral activities[19–22]. These memory-like or adaptive NK cells maintain their functional competence over extended periods[23–27]. Thus, epigenetic memory allows NK cells to "remember" previous activations, ensuring a rapid and robust response upon re-exposure to similar stimuli.

Epigenetic regulation is also critical for the maturation of NK cells from NK precursor cells in the bone marrow[28,29]. Alterations of histone modifications have been shown to influence NK cell fate commitment from hematopoietic precursors. Inhibition of the methyltransferase EZH2 or treatment with IL-15 increased generation of NK cell lineage specification and led to mature NK cells with enhanced cytotoxicity against tumor cells[30,31]. Understanding and harnessing this epigenetic imprinting process is crucial for developing effective NK cell-based therapies for cancer and infectious diseases. Importantly, epigenetic modifications can be targeted and reversed. Recent advances in epigenetic targeting drugs have opened new avenues for modulating immune cell functions, offering potential therapeutic strategies for various diseases[15,18,32–34].

In this study, we conducted a small-molecule drug screen using epigenetic targeting compounds to identify drugs that could modulate NK cell functions. This screen included inhibitors of the bromodomain and extra-terminal domain (BET) family of proteins (BETi). The BET family of proteins, which includes BRD2, BRD3, BRD4 and BRDT recognize acetylated lysine residues on histone tails, influencing chromatin structure and transcriptional activity[35–38]. BET proteins are involved in various cellular processes including cell cycle regulation, apoptosis and inflammation. BETi have been shown to have direct antitumor properties by disrupting the transcription of oncogenes and inhibiting tumor growth and progression[39–41]. BETi have also been shown to have significant anti-inflammatory properties and are being developed as therapies for reducing inflammation in arthritis, colitis, sepsis and central nervous system disease inflammation[42–47]. Our screen revealed that BETi are critical regulators of NK cell activity, in line with limited prior studies that identified that BETi could modulate NK cell cytolytic and inflammatory function[48,49]. Furthermore, we found that BETi not only influences NK cell function but also plays a pivotal role in the differentiation of NK cells from bone-marrow-derived stem cells. These results were further validated through transcriptome studies, which provided insights into the molecular mechanisms underlying the effects of BETi on NK cells.

The ability to control NK cell function has significant translational implications. By modulating NK cell activity, we can potentially suppress NK cell-mediated inflammation, which is a critical factor in various inflammatory and autoimmune diseases. This study highlights the therapeutic potential of BET inhibitors in regulating NK cell functions and opens new pathways for the development of targeted therapies aimed at controlling immune responses. The findings suggest that BET inhibitors could be leveraged to fine-tune immune responses, offering new hope for patients with conditions characterized by excessive inflammation or inadequate immune surveillance.

Methods

Human subjects

Natural killer cells were isolated by immunomagnetic negative selection from peripheral blood mononuclear cells (PBMCs) from deidentified healthy donors that were purchased through IQBiosciences. Bone marrow CD34 + cells were isolated by immunomagnetic positive selection from human bone marrow aspirate purchased through StemCell Technologies. Both cell types were cryopreserved and purity of cell types and viability were determined for each lot by the source provider. Acceptance criteria for each specimen included cell count, cell viability (≥ 70%) and purity determined by flow cytometry. The threshold for purity for both cell types was ≥ 90% determined by percentage of $CD34^+$ of $CD45^+$ cells for the bone marrow cells and percentage of expression of $CD56^+$ and $CD3^-$ for mature NK cells. Both types of primary cells were accompanied by lot-specific Certificates of Analysis from the vendor that included quality control details and verification that the specimens met the minimum quality thresholds. Specimens were obtained in compliance with applicable federal, state, and local laws, regulations, and guidance using Informed Consent Forms and protocols approved by either an Institutional Review Board, the Food and Drug Administration (FDA), The U.S. Department of Health and Human Services, and/or an equivalent regulatory authority.

For experiments using flow cytometry-based readouts to assess the function of NK cells within total human PBMCs, leukocyte cones obtained with appropriate ethical approval from healthy human donors who provided written informed consent were purchased from the NH Blood and Transplant Service, Oxford, UK. All work was compliant with institutional guidelines. All samples were provided without donor-identifying information, and donor age, biological sex, ethnicity and other demographic information was unknown.

Cell line culture

NK-92 cells were purchased from ATCC (CRL-2407). Cells were passaged twice after thawing before any experiments were performed. NK-92 Media composition consisted of Alpha Minimum Essential Medium with L-glutamine and sodium bicarbonate (Gibco), 0.2 mM myo-inositol (Alfa Aesar), 0.1 mM 2-mercaptoeathnol (Gibco), 20 µM folic acid (Alfa Aesar), 150 U/µL recombinant IL-2 (Gibco), 12.5% Horse Serum (Thermo Fisher Scientific), 12.5% Fetal Bovine Serum (Thermo Fisher Scientific). Media was replenished every 2 days and cells passaged every 3 days.

CD4.221 cells, a version of the 721.221 B lymphoblastoid cell line that expresses CD4 (CD4.221) was a kind gift from Professor Masafumi Takaguchi (Kumamoto University, Japan), and were grown in in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% non-essential amino acids (Gibco), 1%

penicillin/streptomycin (Gibco), 1% sodium pyruvate (Sigma-Aldrich) and 0.1% 2-mercaptoethanol (Gibco) (R10). Cells were passaged every 3–4 days.

Small-molecule drug screen of NK cell function

Epigenetics Screening Library featuring 158 epigenetic drugs was purchased from Cayman Chemical (Item No. 11076). Drugs were diluted to 25 μM and 100 nM in NK-92 media prior to addition to cells. NK-92 cells were plated at 200,000 cells/well. 48 h after drug addition, NK-92 cells were stimulated with IL-12 (30 ng/mL) and IL-15 (100 ng/mL) for 24 h. Supernatant was collected.

Cell viability

Drugs were diluted to 25 μM and 100 nM in NK-92 media prior to addition to cells. NK-92 cells were plated at 25,000 cells/well. Cell titer glo (Promega) was administered according to manufacturer recommended protocol and recorded 48 h after drugs were applied.

Mulitplexed binding assay

Cytokine levels associated with inflammation were measured via a 17plex CD8 + T-Cell magnetic bead-based multiplex assay (Millipore) based on the Luminex xMAP technology, following the manufacturer's recommended protocol. The kit provided a set of 17 antigen conjugated beads (GM-CSF, sCD137, IFNγ, IL-10, Granzyme A, IL-13, Granzyme B, sFas, IL-2, IL-4, IL-5, IL-6, sFasL, MIP-1α (CCL3), MIP-1β (CCL4), TNFα, and Perforin. Concentrations were calculated based on 7-serial dilution of known standards and 2 quality controls.

Enzyme-linked immunosorbent assay

Detection and quantification of IFN-γ was performed using a Human IFN-γ ELISA kit (R&D Systems) following the standard manufacturer's protocol with supernatant dilution of 1:10.

Analysis of the effects of BETi on the phenotypic and functional activation of NK cells within total PBMCs

Healthy donor PBMCs were isolated from leukapheresis cones by centrifugation on a Histopaque-1077 gradient. PBMCs isolated from leukapheresis samples were cryopreserved prior to use. PBMCs from individual donors (500,000 cells per well) were cultured in 96-well U-bottom plates (Corning) in R10 medium. Cells were stimulated with 10ng/ml recombinant IL-12 (130-129-720, Miltenyi) plus 100ng/ml recombinant IL-18 (B001-5, R&D Systems) and with 100nM CPI-203 (15479, Cayman Chemical) or 100nM AZD 5153 (15479, Cayman Chemical), or cultured in medium only for 48 h at 37 °C. PBMCs were harvested after 48 h, and were then incubated with the CD4.221 MHC class I low cell line (5:1 E: T ratio) for 5 h at 37 °C to stimulate NK cell activation in the presence of CD107a APC-H7 antibody (BD Biosciences, Cowley, UK) and GolgiPlug (containing brefeldin A, 1/1000 final concentration, BD Biosciences). Following incubation cells were washed and were Fc blocked with Human TruStain FcX (Biolegend) prior to extracellular staining at room temperature for 15 min with saturating concentrations of CD14 BV510 (Biolegend), CD19 BV510 (Biolegend), CD56 BV605 (Biolegend), CD3 BV650 (Biolegend), CD16 PE-Cy5 (Biolegend), NKG2A Pe-Cy7 (Miltenyi Biotec), and NKG2C BV421 (BD Biosciences), CD57 Alexa Fluor 488 (BD Biosciences), in the presence of Aqua fixable live/dead stain (Invitrogen). Antibodies to KIR2DL2/L3/S2 APC [CD158b1/b2.j] (Beckman Coulter), KIR2DL1/2DS5 APC IgG1 [CD158a] (R&D systems) and KIR3DL2 APC (R&D systems) were also included in the extracellular staining panel in some experiments. Cells were then washed and fixed and permeabilized with the FoxP3 intranuclear staining buffer kit (eBioscience) or BD Cytofix/Cytoperm™ fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions, followed by intracellular staining with PLZF PE/DazzleTM 594 (BD Biosciences), granzyme B PE (Biolegend), Perforin Alexa700 (Biolegend), TNF-α BV711 (Biolegend), IFN-γ BV785 (Biolegend) and CD3 BV650 (Biolegend). Cells were then washed and samples were acquired on a BD Fortessa X20 using BD FACSDiva8.0 (BD Bioscience). Data were analyzed using FlowJo 10 (TreeStar), with statistical analysis being performed using Graphpad Prism v10.

RNA-seq of primary NK cells and ex vivo differentiated CD34 + bone marrow cells

Primary NK cells were treated with BETi drugs (100nM) or control for 48 h followed by stimulation with IL-12 (30 ng/mL) and IL-15 (100 ng/mL) for 24 h. NK cells were harvested and processed for RNA extraction using the RNeasy Micro Kit from (Qiagen). RNA concentrations were determined using the Qubit RNA High Sensitivity kit on a Qubit fluorometer (Thermo Fisher Scientific). RNA libraries were prepared with the TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Kit (Illumina). Libraries were run on the Illumina Nova-Seq 6000 in the 2 × 151 bp paired-end format. After sequencing, initial quality check for library complexity and per-base sequence quality throughout all reads was done using FastQC (v0.11.7) software. Next, trimming of low-quality bases (< 20), short reads (< 30), and adaptor sequences was done with the fastqc-mcf (v1.05). Reads were then aligned to GRCh38/hg38 using STAR (v2.7). htseq-count (v0.6.0) was used to generate the counts table for the features. Differential gene expression analysis was carried out using R package DESeq2 (v1.26.0). GSEA v4.1.0 was used for gene set enrichment analysis. Raw counts matrix obtained after running htseq-count were normalized using DESeq2 to generate expression dataset. Next, phenotype file was prepared based on group information for the samples. Hallmark gene sets (v7.4) and Human_HGNV_ID_MiSigDB (v7.4) chip hosted on the GSEA-MsigDB file servers were used as Gene sets and Chip annotation file respectively. Genes weren't collapsed and "gene_set" parameter was used for permutation. While rest of the options were run with default settings.

Ex vivo NK cell lineage commitment culture from CD34 + cells

Cryopreserved bone marrow CD34⁺ cells (StemCell Technologies) were thawed and cultured in StemSpan SF media with 10% FBS, penicillin and streptomycin antibiotic mixture (100X; Gibco), Gentamicin (25 µg/ml; Gibco), SCF (20 ng/mL; Peprotech), Flt3L (20 ng/mL; Peprotech), IL-7 (20 ng/mL; Peprotech), IL-15 (20 ng/mL; Peprotech), and IL-21 (20 ng/mL; Peprotech) formulated to promote NK cell development from progenitors[31,50]. For each culture condition, 250,000 CD34 + cells were cultured in a 24-well tissue culture plate with 500 µl of culture media. At Day 0, cells were treated with control (PBS), AZD5153 (100nM, Caymen Chemicals), or CPI-203 (100nM, Caymen Chemicals). For undifferentiated controls 250,000 CD34⁺ cells were stored for RNA isolation. Half of the media in each well was removed and replaced with fresh media every 4 days of culture. On day 20 cells were isolated for analysis.

Quantitative PCR of NK cell lineage markers

RNA was isolated using the RNeasy 96 kit (Qiagen). cDNA synthesis was performed with Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Taqman probes for *NCAM1* (Hs00941830_m1), *KLRK1* (Hs00183683_m1) and *18s* (Hs99999901_s1) RNA were purchased (Thermo Fisher Scientific). Taqman Fast Advanced Master Mix (Thermo Fisher Scientific) was used following manufacturer recommended protocol and samples were run on Applied Biosystems QuantiStudio 12 K Flex. Input RNA was normalized across samples and relative expression was determined using the 18s housekeeping gene expression.

scRNA-seq of differentiated NK cells

CD34⁺ cells cultured in conditions to promote NK cell lineage commitment were isolated at day 20 of culture and single-cell RNA-seq libraries were prepared by separating single cells into droplets with barcoded gel beads using the 10x Chromium instrument following the Chromium Single-cell 3' v4 reagent kit and library protocol (10x Genomics). Indexed samples were sequenced using an Illumina NovaSeq. Fastq file generation and alignment to the human genome (GRCH38-2020-A) were performed using the CellRanger analysis pipeline (v8.0.1). Individual libraries were aggregated using CellRanger Aggr resulting in 61,109 mean reads per cells with 4,697 median genes per cell. Downstream analysis of cell matrices was done using the Seurat package (v4.3.0). Cells with ≥15% mitochondrial RNA composition were filtered from the dataset. Azimuth version 0.4.6 (bonemarrowref, version 1.0.0) was utilized to identify cell types. Differential expressed genes between cell clusters or groups were determined using Seurat by the nonparametric Wilcoxon rank sum test. Graphs and plots were generated using the Seurat and ggplot 2 (v.3.3.3) R packages and Graphpad Prism version 10.

Statistical analysis

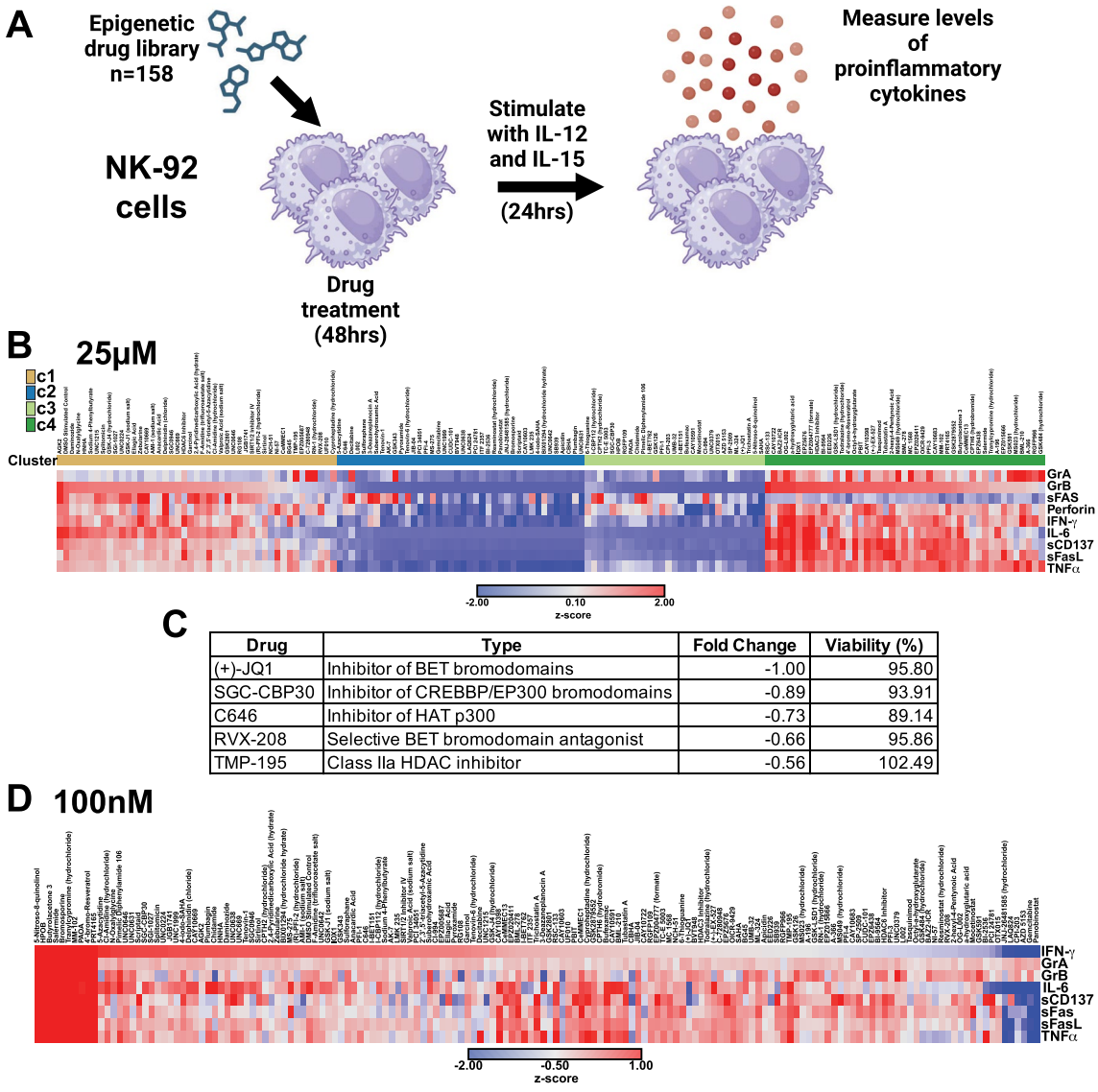
Statistical analysis performed using Graphpad Prism version 10 unless otherwise noted.

Results

Identification of small molecule epigenetic-targeting compounds that regulate proinflammatory cytokine programs in natural killer cells

To identify epigenetic regulators of NK cells, we utilized a high-throughput epigenetics screening library that consisted of 158 small molecule drug compounds that are known to modulate the activity of methyltransferases, demethylases, histone acetyltransferases, histone deacetylases and acetylated lysine reader epigenetic proteins. We treated a NK cell line (NK-92) with the drug library at a 25µM concentration to identify which drugs affected proinflammatory cytokine secretion after NK cell stimulation with the cytokines IL-12 and IL-15 for 24 h (Fig. 1A). We measured the levels of 9 cytokines and immune molecules associated with inflammation and cellular cytotoxicity (Granzyme A, Granzyme B, soluble FAS, Perforin, Interferon gamma, IL-6, soluble CD137, soluble FAS ligand and TNF alpha) using a multiplexed bead-based cytokine assay. At the 25µM drug treatment concentration we identified 78 and 93 drugs that reduced the levels of IFN-γg and IL-6 by greater or equal to 50% compared to control treatment, respectively (Supplemental Fig. 1A). Using k-means clustering, we identified four clusters that represented different patterns of cytokine levels after stimulation (Fig. 1B). Clusters 1 and 4 had high cytokine levels after stimulation, differing by reduced soluble FAS and increased granzyme A/B levels in cluster 4 compared to cluster 1. Clusters 2 and 3 had reduced proinflammatory cytokine levels after NK cell activation (Fig. 1B). We also measured the impact of each drug on cell death of the NK-92 cells using an LDH cytotoxicity assay to identify drugs with immune cell toxicity. We found that over 53% (84/158) of the drugs alone at 25µM concentration reduced the viability of NK cells to less than 80% compared to control (Supplemental Fig. 1B). When we selected drugs that reduced cytokine production and did not have a significant effect on NK cell viability, there were five drugs that met these criteria (Fig. 1C). Three of the five drugs targeted inhibition of bromodomain containing proteins ((+)-JQ1, SGC-CBP30, RVX-208), while the other two were inhibitors of HAT p300 (C646) and HDACs (TMP-195; Fig. 1C).

We performed the drug screen again, but utilizing 100nM drug concentrations to reduce direct drug toxicity to the NK cells (Fig. 1D). We identified 12 drugs that reduced IFN-γg or IL-6 cytokine levels by at least 2-fold compared to controls (Fig. 1D & Supplemental Fig. 1C). Moreover, we observed significantly less drug-induced toxicity to the NK cells, with only 12 of the 158 drugs reducing NK cell viability ≤ 80% (Supplemental Fig. 1D). Of the 12 drugs that were identified to reduce cytokine levels after NK cell activation, 5 did not significantly affect NK cell viability at 100nM (Fig. 1E). Surprisingly, all 5 of the drugs that reduced IFN-γ and IL-6 levels, but did not impact cell viability, were BET inhibitors (AZD5153, CPI-203, (+)-JQ1, OTX015, IBET762). Thus, the BET family of proteins played a key role in regulating proinflammatory cytokine secretion in NK cells after activation, and could be inhibited by multiple BET inhibitor (BETi) small-molecular drugs that did not have a significant impact on overall NK cell viability.



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Drug	Type	Fold Change	Viability (%)
(+)-JQ1	Inhibitor of BET bromodomains	-1.00	95.80
SGC-CBP30	Inhibitor of CREBBP/EP300 bromodomains	-0.89	93.91
C646	Inhibitor of HAT p300	-0.73	89.14
RVX-208	Selective BET bromodomain antagonist	-0.66	95.86
TMP-195	Class IIa HDAC inhibitor	-0.56	102.49

E

Reduced IFNγ or IL-6 at 100nM

Drug	Type	Viability
AZD 5153	Inhibitor of BET bromodomains	**87.23%**
CPI-203	Bioavailable inhibitor of BET bromodomains	**90.26%**
(+)-JQ1	Inhibitor of BET bromodomains	**113.95%**
OTX015	Inhibitor of BET bromodomains	**109.22%**
I-BET762	Inhibitor of BET bromodomains	**118.15%**
Panobinostat	pan-HDAC inhibitor	4.34%
JNJ-2648158	pan-HDAC inhibitor	4.72%
LAQ824	A hydroxamate-based HDAC inhibitor	5.50%
Gemcitabine	Metabolite of cisplatin (Chemo)	9.48%
PCI 24781	HDAC inhibitor	61.42%
BI-2536	Inhibitor of Plk1	23.85%
LMK 235	Selective inhibitor of HDAC4 and HDAC5	73.93%

Fig. 1. Small-molecule drug screen identified key epigenetic regulators of NK cell function. (**A**) Overview of strategy to screen the effects of 158 small-molecule drugs targeting epigenetic regulators on secreted cytokine and cytolytic protein levels after NK-92 cell activation with IL-12 and IL-15. (**B**) Heatmap of concentrations of cytokines and cytolytic proteins in cell culture supernatants, displayed as normalized z-scores at 25μM drug treatment concentration. K-means clustering utilized to group the drugs by protein pattern into four clusters (c1-c4). (**C**) Drug names and type that induced both ≤ 0.5 fold change in analyte levels and did not reduce cell viability ≤ 80%. (**D**) Heatmap of concentrations of cytokines and cytolytic proteins in cell culture supernatants, displayed as normalized z-scores at 100nM drug concentration treatment. (**E**) Drug names and type that mediated ≤ 0.5 fold change in either IFNγ or IL-6 levels along with cell viability. Arrows highlight drugs that did not reduce cell viability ≤ 80%. All five selected drugs targeted BET proteins.

BETi reduce proinflammatory cytokine activation and functional markers of primary human NK cells

To confirm that BETi could have an impact on immune responses of primary human NK cells, we isolated NK cells from healthy donor-derived PBMCs ($n = 4$). On day 1, NK cells were treated with 100nM BETi (AZD5153 or CPI-203) or mock drug treated (control). On day 3, NK cells were stimulated with IL-12 and IL-15. Cells and supernatant were both isolated and analyzed on Day 4. As a negative control, NK cells were also untreated and unstimulated. First, we measured the levels of IFN-γ secreted into the supernatant. No IFN-γ was detected in the NK cells that were not stimulated with IL-12 and IL-15 (unstimulated; Fig. 2A). In the mock drug treated NK cells (control), we detected IFN-γ release after stimulation with cytokines (median = 2,077 pg/ml). Similar to the results of the drug screen in NK-92 cells, there was significant reduction of IFN-γ levels after stimulation in the primary NK samples treated with either of the BETi small molecule drugs (median AZD5153 = 316.8 pg/ml, median CPI-203 = 360.7 pg/ml; Fig. 2A).

To gain further insight into the impact of the BETi on the functional activation of NK cells, PBMCs from $n = 6$ healthy donors were pre-treated for 48 h with BETi (AZD5153 or CPI-203) or incubated in medium only, setting up replicate cultures in medium with or without IL-12/IL-18; then all cells were co-cultured with/without HLA-I-deficient CD4.221 target cells for 5 h, and NK cell IFN-γ and TNF-α production, degranulation (CD107a expression) and Granzyme B expression were analyzed by flow cytometry, using the gating strategy in Supplemental Fig. 2 to identify NK cells. We did not detect any significant effect of the BETi on overall PBMC viability in either unstimulated or IL-12/IL-18 stimulated conditions, or on NK cell frequencies within PBMCs (Fig. 2B). Stimulation of PBMCs with IL-12/IL-18 increased granzyme B expression in NK cells, but the IL-12/18-induced increase in granzyme B expression was diminished in the BETi treated NK cells, with granzyme B levels in AZD5135-treated IL-12/IL-18 stimulated NK cells being significantly lower than those in cells stimulated with IL-12/18 in medium only (Fig. 2C). Exposure of PBMCs pre-treated with medium only with/without IL-12/18 to target cells resulted a significant increase in the percentage of NK cells undergoing degranulation relative to that in non-target-cell-stimulated NK cells (Fig. 2D). However, pre-treatment of PBMCs with BETi precluded target cell triggering of significant increases in the % NK cells undergoing degranulation relative to that in non-target-cell-exposed conditions, and the % IL-12/IL-18-pre-treated NK cells undergoing degranulation in the presence of target cells was significantly lower in BETi-treated versus non-inhibitor-treated conditions (Fig. 2D). Without IL-12/IL-18 pre-treatment there was also a modest increase in the % NK cells producing IFN-γ and TNF-α in target cell-stimulated versus non-target-cell-exposed conditions, whilst IL-12/IL-18 pre-treatment triggered spontaneous IFN-γ production from a high % of NK cells (that was modestly increased by target cell stimulation), and led to a significant increase in the % NK cells producing TNF-α in target cell-stimulated versus non-target-cell-exposed conditions (Fig. 2E,F). Notably, in the presence of the BETi the % IL-12/IL-18 pre-treated NK cells undergoing spontaneous IFN-γ production was significantly lower than in the absence of the inhibitor (Fig. 2E), paralleling the IL-12/IL-15-stimulated IFN-γ release data in Fig. 2A. Furthermore, whereas target cell stimulation of PBMCs pre-treated with IL-12/IL-18 triggered a significant increase in the percentage of NK cells producing TNF-α relative to that observed in the absence of target cells, inclusion of the BETi during the pre-treatment step reduced the target cell stimulation response that it was no longer significantly different from those observed in the absence of target cells (Fig. 2F). However, this reduction in TNF-α was not statistically significantly lower than the IL-12/IL-15 and target cells stimulation condition. Overall, these data showed that BET inhibitors reduce innate cytokine-stimulated proinflammatory cytokine production by primary human NK cells and limit target cell-triggered functional NK cell activation, without affecting cell viability.

BETi alter transcriptome of primary human NK cells

We isolated RNA and performed RNA-seq on the 100nM BETi treated and control treated primary NK cells after IL-12 + IL-15 activation. After sequencing and analysis, we determined genes that were differentially expressed between BETi treated groups compared to control. We defined genes that were significant up- or downregulated (negative Log_{10} adjusted p value ≥ 1.5), and were changed by ≤ -1.5 or ≥ 1.5 $\log_2$ fold change, as significantly changed in expression. This identified 362 (26 upregulated and 336 downregulated) and 445 (119 upregulated and 326 downregulated) genes that were changed compared to control for AZD5153 and CPI-203, respectively (Fig. 3A & Supplemental Data 1 & 2). When we compared differentially expressed genes between the two BETi (AZD5153 vs. CPI-203) treatment groups, we only identified a single significantly different gene (*H3F3AP6*), indicating that each drug had similar effects on the NK cell transcriptome compared to controls. Both BETi drugs had more downregulated genes as compared to upregulated ones, which suggested a more overall reduction in the gene expression of genes during BET protein inhibition.

Next, we examined the expression levels of genes associated with known NK cell functions (Fig. 3B). We did not observe any significant changes in gene expression of *PRF1*, which encodes perforin a protein in cytotoxic granules important for NK cell-mediated cytotoxicity. *KLRK1* encodes NKG2D, an important activating receptor of NK cells that recognizes receptors expressed by stressed, infected and transformed cells. We found that BETi increased the levels of NKG2D gene expression in the NK cells (Fig. 3B). Conversely, BETi decreased the expression of the gene that encodes CD16 (*FCGR3A*), that is important for mediating antibody-dependent cell-mediated cytotoxicity (ADCC). Treatment of NK cells with BETi also reduced the expression of several inhibitory immune checkpoint molecules (genes that encode for PD-1 (*PDCD1*), LAG3, and TIGIT; Fig. 3B). Finally, we performed gene set enrichment analysis (GSEA, Broad Institute) of the genes significantly changed in NK cells treated BETi (Fig. 3C). We did not identify any significant pathways that were positively associated with the BETi treatment groups using this analysis, however, there were hallmark pathways that were significantly negatively associated with the BETi treatment group (enriched in the control group; Supplemental Data 3 & 4). This most likely is the result of the significant number of downregulated genes in the NK cell treated with BETi

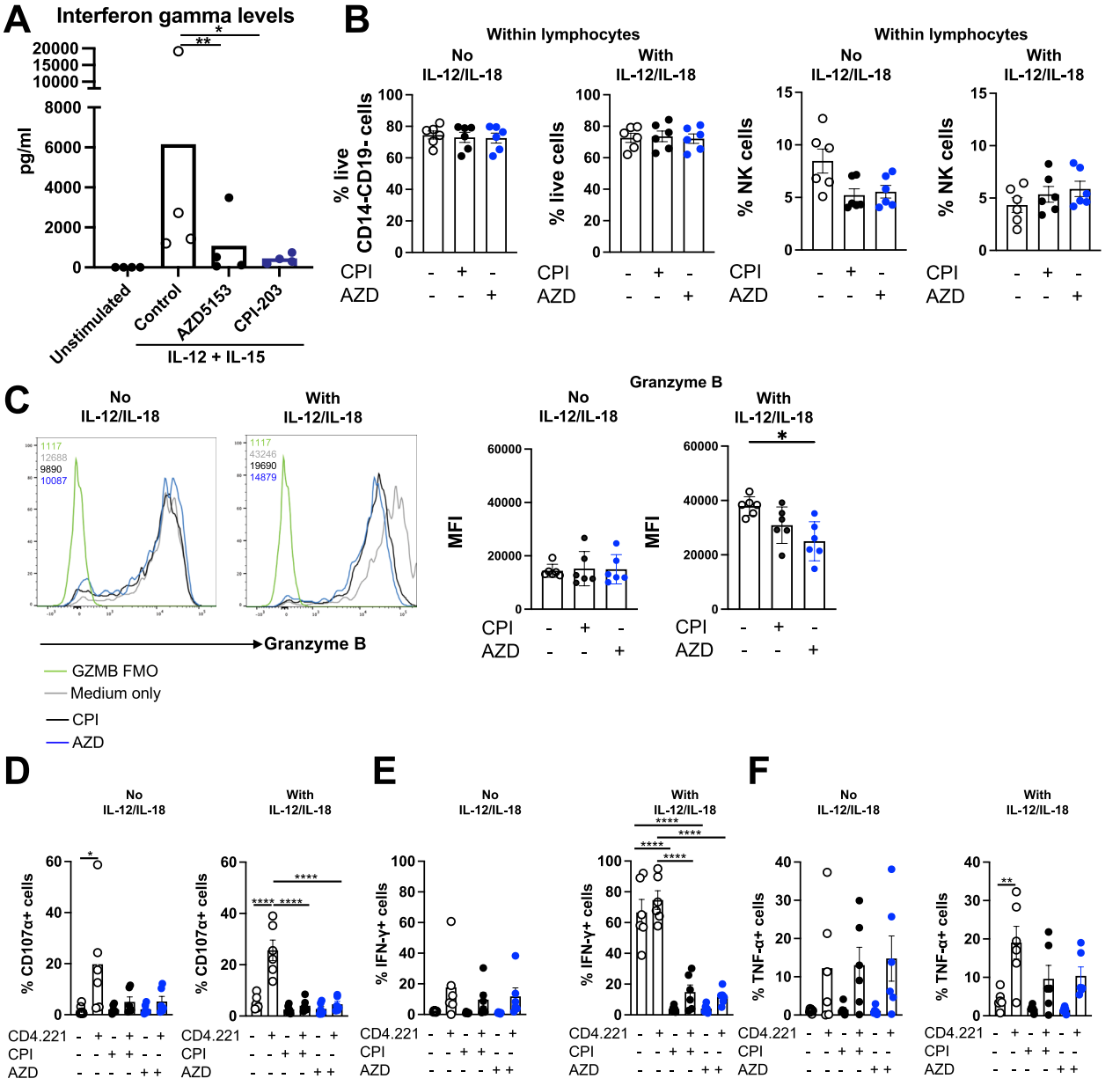


Fig. 2. BETi impact function of primary human NK cells. (**A**) Human primary NK cells isolated from healthy donors ($n = 4$) unstimulated or stimulated with IL-12 + IL-15 for 24 h with prior (24 h) treatment with BETi (AZD5153 or CPI-203) compared to no drug treatment control. Bar graph of levels of IFNγ secreted into the cell culture supernatant measured by ELISA reported in pg/ml. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, Ratio paired t-test. (**B**) Percentage of live $CD14^{-}CD19^{-}$ cells and of total NK cells (live $CD14^{-}CD19^{-}CD56^{+}CD16^{+}$) within the lymphocyte gate (Supplementary Fig. 2) in PBMCs from healthy donors ($n = 6$) incubated in medium only (open circles) or with CPI-203 (black circles) or with AZD5153 (blue circles), in the presence or absence of IL-12 plus IL-18, for 48 h, and then co-cultured with CD4.221 cells for a further 5 h and analyzed. (**C**) Representative histogram overlays illustrating expression of granzyme B in NK cells in the indicated conditions, and bar graphs showing the median fluorescence intensity (MFI) of granzyme B staining in NK cells after culture of PBMCs from healthy donors ($n = 6$) for 48 h in medium with or without IL-12 + IL-18 in the absence (open circles) or presence of BET inhibitors CPI-203 (black circles) or AZD5153 (blue circles), followed by co-culture for 5 h with CD4.221 cells in the presence of Brefeldin A. (**D**–**F**) Percentages of $CD107a^{+}$ (**D**), of IFN-γ^{+} (**E**) and of TNF-α^{+} cells (**F**) in total NK cells within PBMCs from healthy donors ($n = 6$) incubated in medium only (open circles) or with CPI-203 (black circles) or with AZD5153 (blue circles), in the presence or absence of IL-12 plus IL-18, for 48 h, and then co-cultured with CD4.221 cells in the presence of Brefeldin A for a further 5 h and analyzed. In (**B**–**F**), each symbol represents data from a single donor; error bars indicate mean ± sem of $n = 6$ samples. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.00001$; one way-ANOVA with Tukey's post-test.

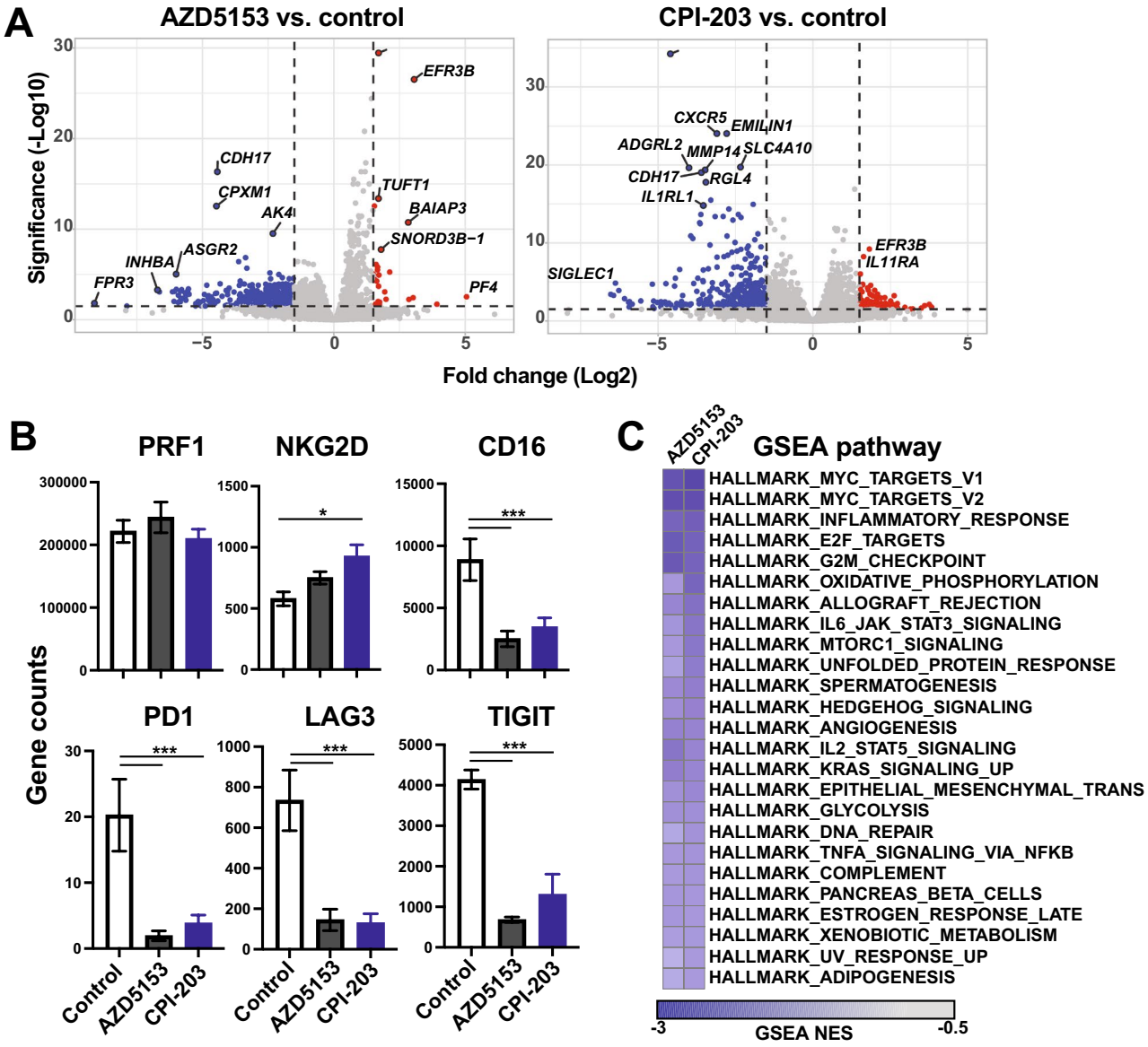


Fig. 3. BETi alter the transcriptome of human primary NK cells after activation. (**A**) Volcano plot of Log2 fold-change and significance of differentially expressed genes from BETi-treated (AZD5153 or CPI-203; 48 h treatment) compared to control treated following 24 h stimulation with IL-12/IL-15 determined by RNA-seq of primary human NK cells ($n = 4$ per group). Blue dots represent significantly downregulated genes, and red dots represent significantly upregulated genes. Select individual genes are labeled. (**B**) Bar graphs of selected genes that encode PRF1, NKG2D, CD16, PD1, LAG3 and TIGIT showing normalized gene counts in each sample. Error bars indicate mean ± sem. Adjusted p value determined by DeSeq2 analysis with Bonferroni correction (***$P \leq 0.001$, *$P \leq 0.05$). (**C**) Heatmap of Normalized Enrichment Scores (NES) calculated by Gene Set Enrichment Analysis (GSEA) for gene pathways that were negatively enriched with BETi treatment.

compared to control as opposed to upregulation, or activation, of gene expression. The top pathways negatively associated with BETi treatment in NK cells were negative enrichment for genes targeted by the transcriptional regulator MYC, and gene expression programs involved in the inflammatory response. There were also other pathways critical for cellular inflammation, metabolism and proliferation that were also negatively associated with the BETi treatment groups (Fig. 3C). Thus, BETi treatment of NK cells led to increased NKG2D expression, decreased CD16 gene expression, and decreased inhibitory checkpoint molecule expression, and negatively impacted pathways related to inflammation and metabolism, suggesting that BET proteins control a complex functional regulatory network in NK cells.

Epigenetic regulation of NK cell lineage fate commitment from bone marrow hematopoietic stem cells

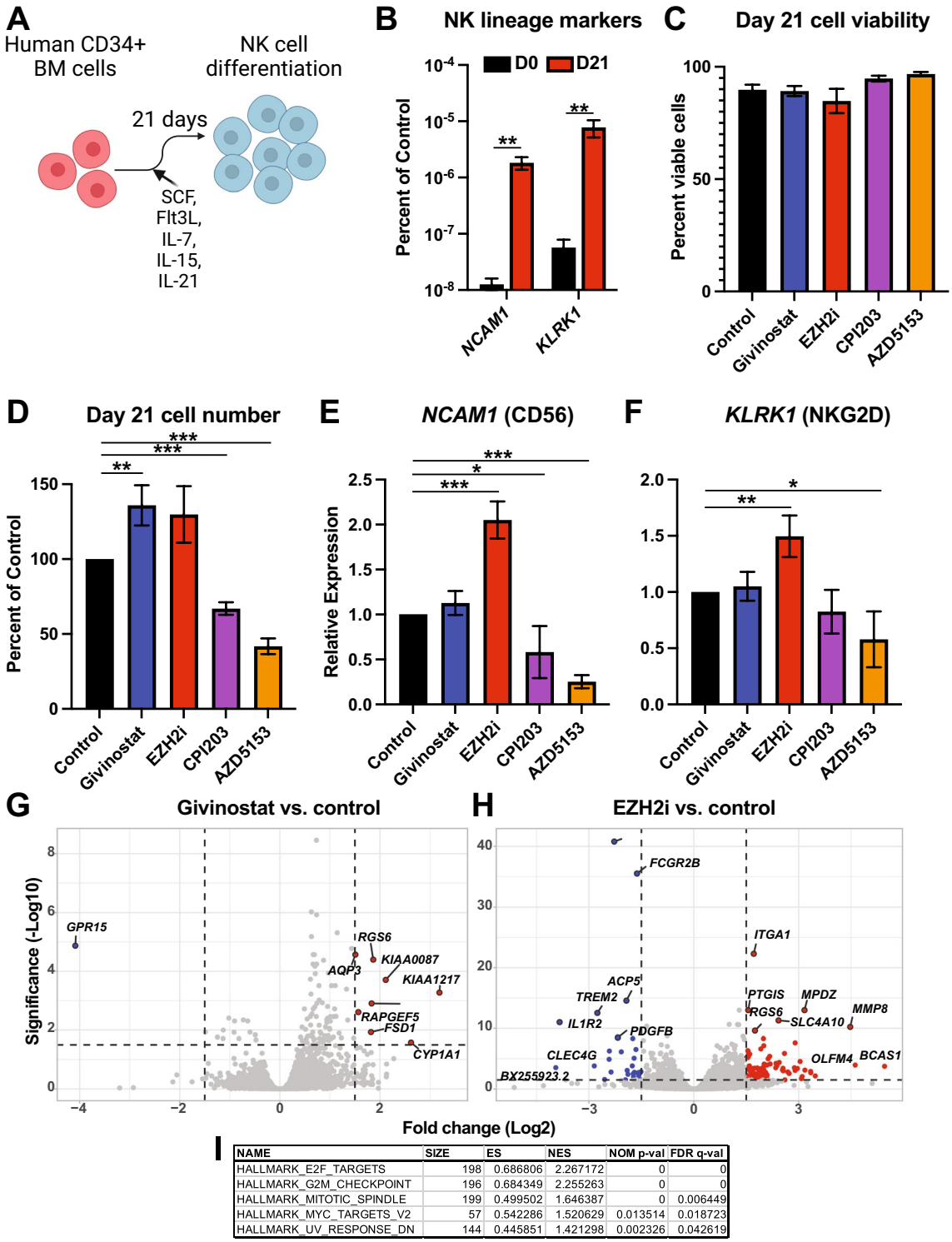
NK cells develop from bone marrow progenitor cells which are common lymphoid precursors. Prior studies have shown that alterations in histone modifications in multipotent precursors can influence the fate and phenotype of mature NK cells. Specifically, inhibition of EZH2 has been shown to not only give rise to increased numbers of NK precursors, but the mature NK cells also had enhanced cytotoxicity against tumor cells[31]. Moreover, the HDAC inhibitor givinostat has been shown to reduce proinflammatory signaling in immune cells, but the role in NK cell function and differentiation has not been explored. To determine the impact of epigenetic modification of HSPCs on NK cell fate, we used an ex vivo cell culture system where human NK cell fate is promoted from hematopoietic progenitor $CD34^+$ HSPCs isolated from human bone marrow through culturing them for 21 days with soluble SCF, Flt3L, IL-7, IL-15 and IL-21 (Fig. 4A). At day 0, the $CD34^+$ HSCs express low levels of NK cell lineage markers *NCAM1* (CD56) and *KLRK1* (NKG2D). At day 21, after NK cell differentiation, *NCAM1* and *KLRK1* have significantly increased gene expression indicating promotion of NK cell fate (Fig. 4B).

We utilized this system to determine the effect of treating the $CD34^+$ HSCs at day 0 with small-molecule drugs targeting epigenetic regulators. In addition to the BETi, we found that the HDAC inhibitor givinostat could also reduce the levels of proinflammatory cytokine levels after stimulation in primary human NK cells (Supplementary Fig. 3). We sought to determine the impact of givionstat, EZH2i and BETi treatment of HSC at the start of culture on the long-term development of NK cells from progenitor precursors. We treated the cells at day 0 with no drug (control), givinostat, EZH2i and the two BETi (CPI-203 and AZD5153). At day 21 after NK cell differentiation, we did not observe any significant difference in cell viability between the treatment groups (Fig. 4C). At day 21, givinostat and EZH2i treatment groups had significantly more cell numbers in culture, whereas the two BETi (CPI-203 and AZD5153) had significantly reduced cell numbers (Fig. 4D). This indicated that early drug treatment could have effects on long-term proliferation of the cells during culture. Next, we determined differences in NK cell lineage marker gene expression between the groups at day 21. Compared to control, there was no significant difference in *NCAM1* or *KLRK1* in the cells treated with givinostat. While EZH2i treatment resulted in significantly increased expression of both *NCAM1* and *KLRK1*, both BETi (CPI-203 and AZD5153) had significantly reduced levels of *NCAM1*, and AZD5153 had a significant decrease in *KLRK1* expression (Fig. 4E,F). These data demonstrate that early treatment of bone-marrow-derived $CD34^+$ HSC at day 0 with epigenetic targeting drugs, resulted in different phenotypes of differentiated cells at day 21 in an ex vivo culture. None of the drugs significantly impacted cell viability. Givinostat treatment resulted in an increased number of cells, but no difference in *NCAM1* or *KLRK1* marker gene expression. As was previously reported[31], EZH2i treatment increased the number of NK cells and increased levels of both of these NK lineage markers. Treatment with BETi reduced cell numbers and gene expression of *NCAM1* or *KLRK1* genes associated with the NK cell lineage.

To gain further insights into the molecular gene networks involved in promoting NK cell maturation, we performed bulk cell population RNA-seq of the day 21 NK cells from control, givinostat and EZH2i treated groups. Givinostat treatment resulted in minimal alterations to gene expression, with only 9 genes (8 upregulated and 1 downregulated) having statistically significant changes compared to the control group (Fig. 4G & Supplemental Data 5). Two of the upregulated genes (*FSD1* and *RAPGEF5*) have been associated with NK cell movement and migration, while the others had no known roles in NK cell function. The only downregulated gene, *GPR15*, encodes a G-protein couple receptor also involved in lymphocyte homing. Thus, givionstat treatment resulted in minimal differences in the transcriptomic network of differentiated NK cells. EZH2i treatment resulted in the gene expression changes of 121 transcripts (93 upregulated and 28 downregulated) compared to the control group (Fig. 4H & Supplemental Data 6). Interestingly, there was significant downregulation of genes that are negative regulators of NK cell function (*MMP9*, *FCGR2B*, *IL1R2*), but also downregulation of genes associated with IL-12, IL-15 and IL-18 responsiveness in NK cells (*CLEC4G*, *TREM2* and *PDGFB*; Fig. 4H). *BCAS1* and *PTGIS* were upregulated and have been associated with an immunomodulatory role of NK cells. Similar to the upregulated genes during givinostat treatment, there was an upregulation of genes associated with cell movement and migration (*MMP8* and *ITGA1*; Fig. 4H). Gene set enrichment analysis of the genes altered by EZH2i treatment identified pathways significantly enriched that were associated with cellular proliferation (Fig. 4I). Epigenetic regulation of NK cell lineage fate commitment from bone marrow hematopoietic stem cells demonstrates that early treatment with epigenetic targeting drugs influences the proliferation, viability, and gene expression of differentiated NK cells, with EZH2 inhibition enhancing NK cell numbers and cytotoxicity markers, while BET inhibitors reduced cell numbers and NK lineage marker expression. This suggested that BET proteins could be important controllers of NK cell lineage commitment in addition to regulating mature NK cell function.

Single-cell RNA-seq reveals impact of BETi on NK cell lineage commitment from bone marrow progenitors

At day 21 after promoting NK cell lineage commitment from bone marrow $CD34^+$ cells described above, we performed scRNA-seq on 65,105 cells from two control-treated sample pools that consisted of equally-pooled cells from two independent donors each (16,865 cells, control-1; 16,848 cells, control-2) and two BETi-treated sample pools that consisted of equally-pooled cells from two independent donors each (16,742 cells, AZD5153; 14,650 cells, CPI-203; Fig. 5A & Supplemental Fig. 4A). We detected 4,697 median genes per cell and removed cells with high levels of mitochondrial gene expression as a marker of stressed and/or dying cells ($\geq$ 15%; Supplemental Fig. 4A). We identified 32 distinct clusters of cells based on the transcriptome of each cell population (Supplemental Fig. 4B). We found that 14 clusters had higher frequencies of cells from the BETi-treated conditions (clusters 12, 8, 15, 19, 28, 26, 3, 20, 16, 30, 1, 22, 2, 7; Supplemental Fig. 4C). Moreover, we identified upregulated genes in each distinct cluster (Supplemental Fig. 4D & Supplemental Data 7). To further



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NAME	SIZE	ES	NES	NOM p-val	FDR q-val
HALLMARK_E2F_TARGETS	198	0.686806	2.267172	0	0
HALLMARK_G2M_CHECKPOINT	196	0.684349	2.255263	0	0
HALLMARK_MITOTIC_SPINDLE	199	0.499502	1.646387	0	0.006449
HALLMARK_MYC_TARGETS_V2	57	0.542286	1.520629	0.013514	0.018723
HALLMARK_UV_RESPONSE_DN	144	0.445851	1.421298	0.002326	0.042619

identify and classify immune cell populations in the NK cell lineage culture, we utilized a reference single cell atlas from human bone marrow (Azimuth) (Fig. 5A). We identified cell populations of hematopoietic stem progenitor cells (HSPC), monocytes (Mono), natural killer cells (NK), dendritic cells (DC), CD4 and CD8 T cells, B cells, other T cells and other nonclassified cell populations (other). Next, we identified populations of cells that were altered by BETi treatment (Fig. 5B, dashed circles). We found that in the control groups, HSPC were the most frequent cell type followed by monocytes and NK cells (Fig. 5C). Treatment with BETi at day 0 of this culture increased the frequency of the myeloid (mono) populations while reducing HSPC and NK cell frequencies. We found that BETi-treatment increased both CD14 and CD16-like myeloid cells as well as lympho-myeloid primed progenitor cells (LMPP; Fig. 5D). This data indicated that BET proteins not only promote the development NK lineage cells, but restrict differentiation into myeloid lineage cells.

◀**Fig. 4**. Epigenetic regulators control NK cell lineage fate commitment from bone marrow hematopoietic stem cells. (**A**) Schema of ex vivo culture to promote NK cell fate from human bone marrow CD34$^+$ cells ($n = 4$–5 donors per condition). (**B**) Bar graphs showing expression of NK lineage marker genes at day 0 and day 21 of culture by quantitative PCR. Error bars indicate mean ± sem. $^{**}P \leq 0.01$, Wilcoxon-Mann-Whitney. (**C**) Bar graphs showing cell viability at day 21 of culture after harvest. (**D**) Bar graph showing overall cell number (cell count) of each culture condition at day 21. Error bars indicate mean ± sem. $^{**}P \leq 0.01$,$^{***}P \leq 0.0001$, Unpaired t-test. (**E**, **F**) Bar graphs of gene expression of (**E**) *NCAM1* and (**F**) *KLRK1* determined by qPCR at day 21 of culture. Error bars indicate mean ± sem. $^{***}P \leq 0.0001$, $^{**}P \leq 0.01$,$^{*}P \leq 0.05$, Unpaired t-test. (**G**, **H**) Volcano plot of Log2 fold-change and significance of differentially expressed genes from (**G**) Givinostat or (**H**) EZH2i compared to control determined by RNA-seq of differentiated NK cells from CD34$^+$ cells at day 21($n = 4$ per group). Blue dots represent significantly downregulated genes, and red dots represent significantly upregulated genes. Select individual genes are labeled. (**I**) Significantly enriched pathways in EZH2i-treated samples compared to control using GSEA. Number of genes (size), enrichment and normalized enrichment scores (ES, NES), and nominal and FDR-corrected p values shown (NOM p-val, FDR q-val).

BETi altered NK cell phenotype during lineage commitment

Next, we focused on NK cells in the dataset by selecting cells that had expression of the NK cell lineage marker gene *NCAM1* that encodes CD56 (Fig. 6A). This resulted in 4,488 cells in the control (1,952 control 1, and 2,536 control 2) and 1,584 and 1,068 cells in the BETi AZD5153 and CPI-203 samples, respectively. We re-clustered the selected NK lineage cells for further analysis (Fig. 6B). This identified 19 distinct clusters of cells (Fig. 6C). We determined the upregulated genes that defined each cluster and identified four major classes of cells that were grouped by similar gene expression programs (Fig. 6D & Supplemental Data 8). The first class were a group of cells that were comprised of clusters 3, 8, 9 and 19 that expressed myeloid and precursor stem-like genes (e.g. *ELANE*, *LYZ*). The other three classes had gene expression features of NK-like cell populations. One group of cells that were comprised of clusters 0, 7 and 14 had increased expression of *IL1R1* but low levels of NK cell functional markers (*GZMB*, *KLRK1* (NKG2D), *FCGR3A* (CD16), IL1R1 + cells). The other two classes had high expression of *GZMB* and *KLRK1*, but differed with CD16 gene expression (*FCGR3A*). Clusters 1, 4, 5, 12 and 17 had low CD16 gene expression (CD16- NK), and cells in clusters 2, 6, 10,11, 12, 15, 16, had high expression of *FCGR3A* that encodes CD16 (Mature NK/T; Fig. 6D & Supplemental Data 8). There was an increased frequency of cells residing in clusters 2, 4, 5 and 12 and reduced numbers of cells in cluster 0, 7, and 14 for samples treated with BETi in clusters with at least 1% of total cell frequency (Fig. 6E). This was associated with increased frequencies of CD16- NK cell populations and decreased IL1R1 NK cells when treated with BETi. Within the CD16- NK cells, cluster 4 had increased expression of *BGLAP*, which encodes the bone hormone osteocalcin and *TNFSF10*, which encodes TRAIL. Cluster 5 had increased markers of NK cell activation and cell division (*MKI67*, *CENPF*; Fig. 6D). Both of these clusters had higher frequencies of cells in the BETi-treated samples. Expression of specific genes confirmed that CD56 gene expression (*NCAM1*) was lowest in the precursor cells and highest in the NK cell populations (Fig. 6F). The IL1R1 NK cells had lower expression of *GZMB* and *KLRK1*. Both CD16- and CD16 + expressing cells had expression of *GZMB* and *KLRK1*, but the CD16- cells had high levels of *XCL2* expression (Fig. 6F). XCL2 are chemokines that recruit other immune cells[51] and IL-1R1 has been shown to have high expression in immature NK cells[52]. Thus, while BETi promoted overall increased myeloid differentiation in the total population of cells, when focusing on NK cell populations, BETi promoted alterations in NK cell gene expression patterns compared with control treatment. This was also observed when performing differential gene expression analysis of BETi-treated samples compared to control for the *NCAM1*-expressing cell subsets (Supplemental Data 9 & 10). Among the top upregulated genes for the BETi-treated samples were *XCL1* and *XCL2*, while *IL1R1* was among the top downregulated gene (Supplemental Fig. 5A). Analysis of enriched KEGG pathways of the upregulated genes in the BETi-treated NK cells compared to control identified enriched pathways associated with NK cell cytotoxicity and antigen processing and presentation (Supplemental Fig. 5B). The analysis of NK cells expressing *NCAM1* (CD56) revealed distinct clusters with varying gene expression profiles, showing that BETi treatment increased frequencies of mature NK cell populations and reduced populations of IL1R1-expression cells, with significant changes in gene expression and enriched pathways related to NK cell cytotoxicity.

Discussion

Our study demonstrated that BETi significantly modulate both NK cell function and differentiation. BET inhibition not only reduced innate cytokine-driven and target cell-triggered proinflammatory cytokine secretion, but also promoted the maturation of NK cells and favored differentiation of NK cells with enhanced gene expression of genes important for cytotoxic function and lower gene expression of cell exhaustion markers. This dual effect of BETi on NK cells has profound implications for both cancer immunotherapy and the treatment of inflammatory and autoimmune diseases. This also highlights the critical importance of the BET proteins in the regulation of NK cell development and function.

Prior studies have identified a significant role of epigenetic regulation of NK cell inflammatory function[16,53–57]. This has been particularly studied in the context of generation of NK cells with a memory or adaptive phenotype, where epigenetic regulation imprints prior stimulation of NK cells to alter future responses to secondary stimulation[16,17,23,58]. Studies have shown that activation of NK cells with human cytomegalovirus could reprogram NK cells with increased inflammatory and effector cell responses[17]. Other studies have shown that stimulation with a cocktail of cytokines could program NK cells with enhanced effectiveness against tumor cells[24,59]. One study also identified that the BETi AZD5153 had an anti-inflammatory effect on NK cells, and that

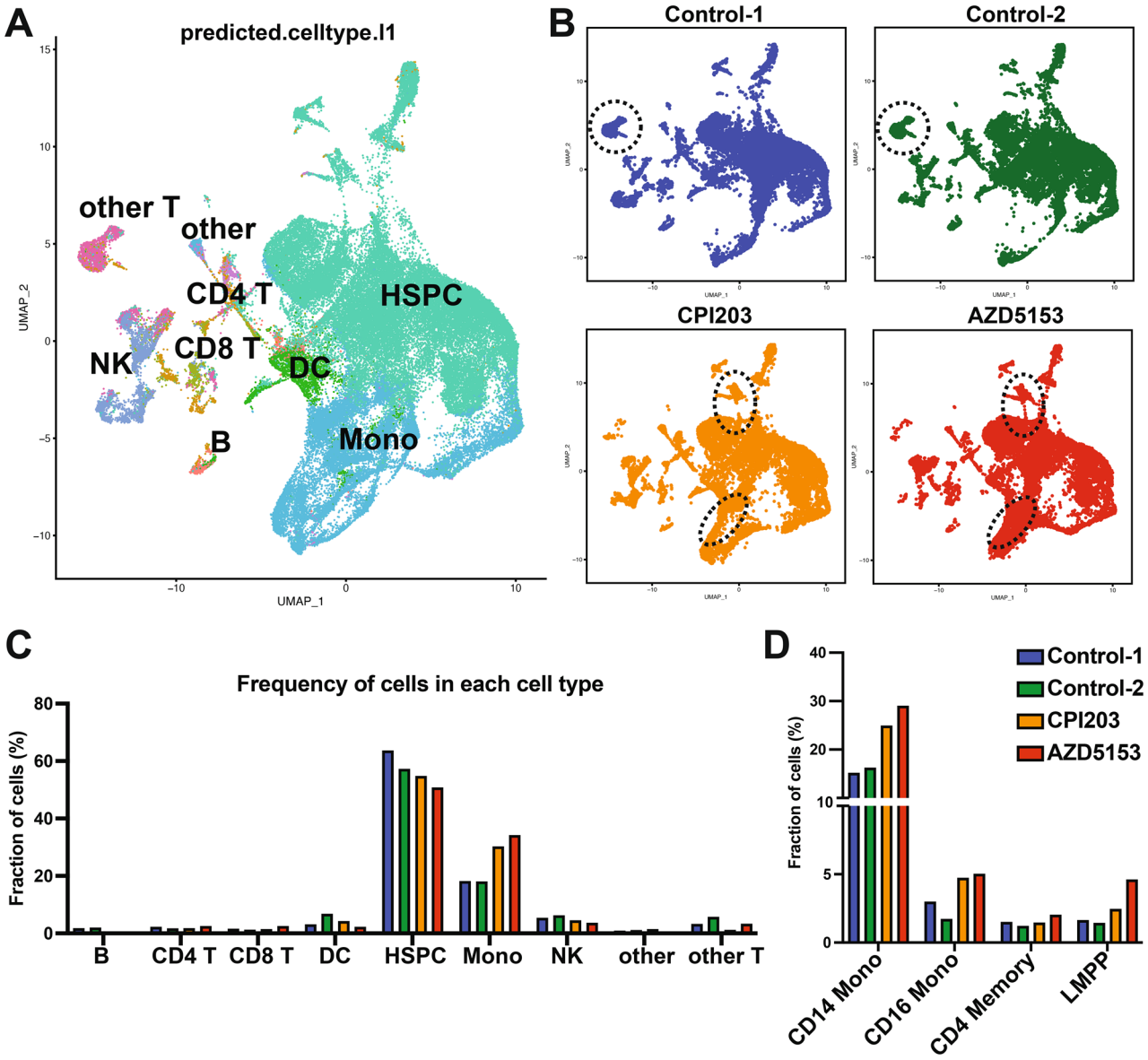

Fig. 5. Single-cell RNA-seq of NK cell lineage commitment from bone marrow progenitors. (**A**) UMAP plots showing groupings of 65,105 cells based on scRNA-seq from day 21 cultured cells with control or BETi conditions. Each condition contains equal numbers of cells pooled from two independent donors. Azimuth reference atlas (Bone marrow reference) was utilized to assign cell types. (**B**) UMAP plots showing individual cells from each control (Control-1 or Control-2) or BETi (CPI203 or AZD5153) conditions. Dashed circles highlight regions of cells unique to sample condition. (**C**, **D**) Bar graphs showing cell frequency in each of the Azimuth-predicted cell types by sample group.

the BET proteins BRD4 and BRD2 have different roles in regulated NK cell function[48]. Using a high-throughput small-molecule drug screen with drugs that targeted epigenetic regulators, we identified multiple drugs which targeted the BET family of proteins and resulted in a marked reduction in the secretion of proinflammatory cytokines such as IFN-γ and IL-6. Thus, BETi could be a tool for controlling and regulating NK cell-mediated inflammation. We utilized NK-92 cells in our drug screen due to the suitability for high-throughput screening, and validated effects of inhibitors on primary NK cells. However, differences in effects of BETi on NK cells could be impacted by individual genetic, sex, age and other clinical or demographic variables. In this case, future studies could determine the impact of these factors on BETi efficacy or identify biomarkers of NK cell responses.

Excessive inflammation is a common feature in various autoimmune and inflammatory conditions. I-BET was one of the first BETi discovered and was shown to reduce inflammation during a mouse model of bacterial sepsis[60,61]. There are now numerous BETi that exhibit anti-inflammatory and immunomodulatory functions with most targeting all four BET proteins with various binding affinities. Other preclinical studies have shown that the anti-inflammatory properties of BETi improve arthritis[62], psoriasis[63], lupus[64], and colitis[45]. Moreover, BETi have been shown to have utility in central nervous system injuries through reducing inflammation[43,65].

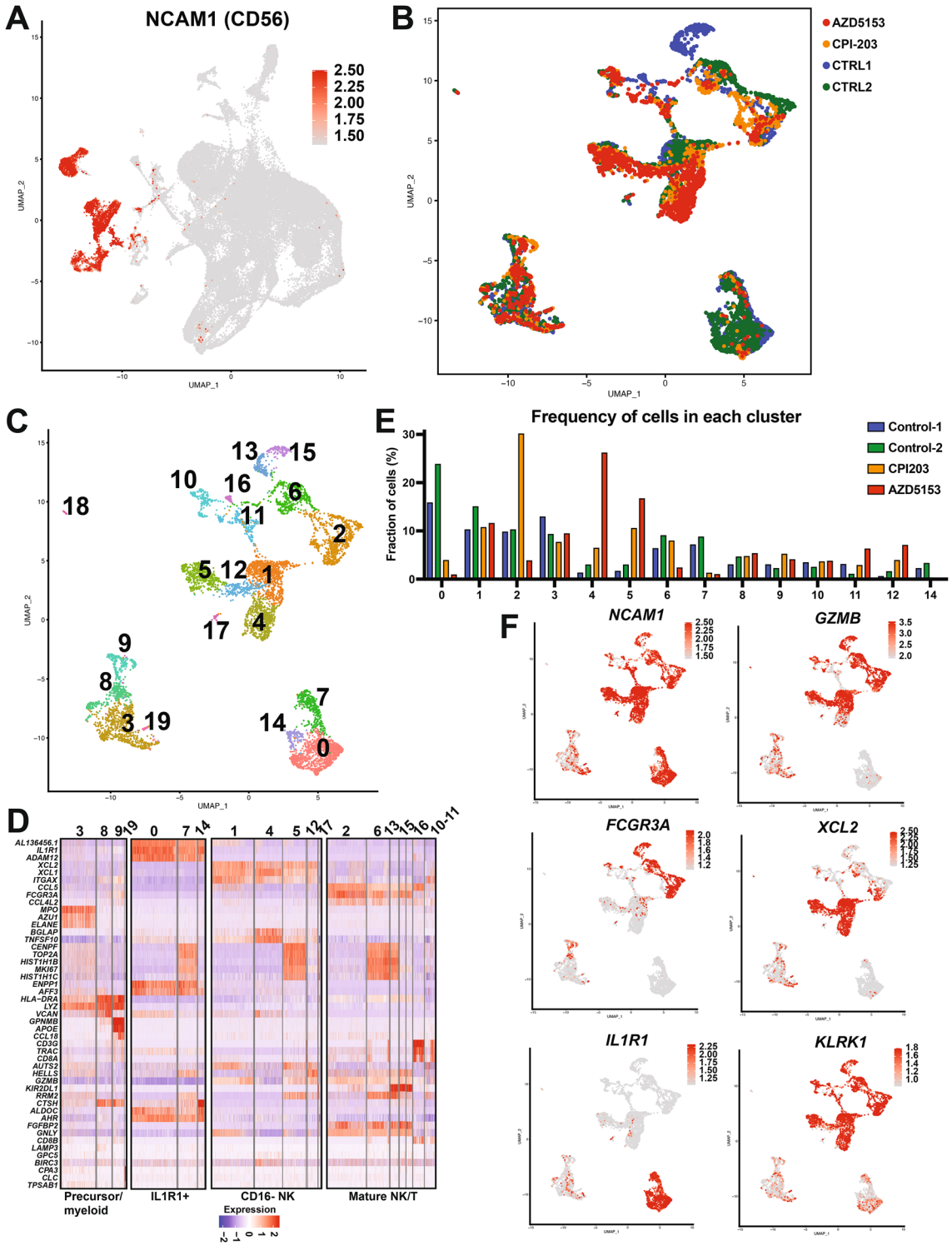

Fig. 6. Diversity of NK-lineage cells produced from bone marrow precursors. (**A**) UMAP plot showing expression level of the mature NK cell lineage marker CD56 (*NCAM1*) that was used to sub-select cells for downstream analysis. (**B**) UMAP plots showing individual cells from each control (Control-1 or Control-2) or BETi (CPI203 or AZD5153) conditions of re-clustered *NCAM1*-postive only cells. (**C**) Graph-based clustering used to identify unique clusters of cells based on transcriptome expression. 20 distinct clusters were identified. (**D**) Heatmap showing top genes upregulated in each cluster of cells and identification of four major groups of clusters (precursor/myeloid, IL-1R1 + high-expressing cells, CD16- NK cells and mature NK/T cells). (**E**) Bar graphs showing cell frequency in each cluster by sample group. Only clusters with at least 1% of cells shown (Cluster1-14). (**F**) Feature plots of UMAPs showing expression levels of NK cell marker genes.

The observed reduction in inflammation in these conditions is not entirely dependent on NK cells, and the BETi function through anti-inflammatory mechanisms in multiple immune and non-immune cell types. In our flow-cytometry experiments, we utilized PBMCs for BETi treatment to maintain physiological cell-cell interactions, and indirect effects on NK cells from other immune cells cannot be ruled out. However, we validated the effect of BETi using transcriptome analysis on isolated NK cells supporting a direct cell-intrinsic role of BETi on NK cells. Future studies should be focused on how BETi impacts pleiotropic effects on different immune cells and how this could impact immune cell communication. Most of the BETi target all four BET protein members, thus dissection of the role of specific BET proteins in these cell populations in regulating inflammation is still required. Moreover, human clinical trials of various BETi have only explored BETi in the context of oncology for targeting tumor growth, and moving the preclinical findings for the treatment inflammatory conditions to human clinical trials to determine efficacy is still needed. Our findings here align with prior studies by confirming the anti-inflammatory effects of BETi on NK cells, suggesting their potential to alleviate symptoms in diseases characterized by chronic inflammation. Interestingly, analysis of the transcriptomes of NK cells treated with BETi, identified reduced expression of immune exhaustion markers (PD1, LAG3, TIGIT) and increased expression of NKG2D. Other studies have shown that BETi could increase NK cell activation against tumor cells[66,67]. Thus, while BETi could reduce proinflammatory cytokine levels, NK cell cytotoxic and survival programs could be augmented. More studies are needed to determine the effects of BETi on NK-mediated tumor control. One limitation of our study is that we studied the effects of BETi on immune cells after a single exposure after 48 h. Future studies that determine the impact of repeated BETi dose, and the duration of BETi-mediated immune suppression will be required to establish the utility of BETi for treatment of chronic inflammatory conditions.

In addition to regulating mature NK cell phenotype and function, NK cell lineage fate commitment from multipotent precursors is also epigenetically regulated. NK cells develop from common lymphoid progenitors in the bone marrow. This requires activation of genes that promote NK cell fate and repressing gene programs for alternative cell fates. Alterations in the process can result in different numbers of mature NK cells produced in the peripheral blood and tissues, and differences in mature NK cell phenotypes. One study identified the role of the H3K27 methyltransferase EZH2 as a critical regulator of cell-fate determination of NK cells[31]. Specifically, the study demonstrated that inhibition of EZH2 promoted NK cell lineage commitment and increased mature NK cell survival and NKG2D-mediated cytotoxicitiy[31]. Genetic mutations in transcriptional regulators, infections or cancer have been shown to alter the phenotype and function of NK cells developing in the bone marrow[68,69]. Our single-cell RNA sequencing analysis of NK cell fate commitment from bone marrow hematopoietic progenitor cells revealed that BETi treatment promoted the differentiation of myeloid cell populations, but the differentiated NK cell populations were more mature with increased cytotoxicity markers upon treatment with BETi. These findings suggest that BET proteins are critical for suppressing the myeloid cell lineage program during NK cell fate commitment and important for maintaining immature populations of NK cells in the bone marrow. This suggests that systemic BETi-treatment could alter the number and phenotype of mature NK cells produced. There were several limitations to our approaches studying NK cell differentiation. First, was that while cells with NK-like phenotypes are produced in our ex vivo differentiation protocol, how the transitional developmental states reflect in vivo NK cell differentiation are unclear. Second, we only studied NK cell differentiation from $CD34^+$ bone marrow cells from a limited number of donors. Human $CD34^+$ cell variability could impact the resulting NK cell phenotypes. Third, while CD56 and NKG2D are NK cell lineage markers, they can be expressed on other immune subsets, so more detail surface receptor studies of the populations of cells from the culture could give insights on NK cell subtypes and function. Fourth, was the observed frequency of NK cells differentiated. The mature NK cells are a small fraction of the total cells. Thus, some of the effects observed could be due to BETi alterations of progenitor cell populations, or subpopulations along differentiation that drive differences, and not directly on mature NK cells. Future studies studying different cell subpopulations at different timepoints across the differentiation culture using large sample-sizes of donors, or studies using animal models of BETi on NK cell maturation, in vivo, could identify further mechanisms of how BETi are impacting NK cell differentiation from stem cell progenitors.

The ability of BETi to modulate NK cell function and promote maturation has significant translational implications. In the context of cancer immunotherapy, BETi could be used to control differentiation and function of NK cells, and may potentially improve their ability to target and eliminate tumor cells. We observed that when NK cells pretreated with stimulatory cytokines, BETi and then mixed with tumor cells, had reduced cytokine and cytotoxic marker levels by flow cytometry. This could indicate a context and time specific effect on NK cell function and phenotype. This will need to be confirmed using different dosing strategies and cytotoxic cellular assays to determine at what point BETi could enhance or inhibit NK cell functions. Furthermore, the anti-inflammatory properties of BETi could be leveraged to treat autoimmune and inflammatory diseases, providing a dual therapeutic benefit. Recent clinical trials have explored the combination of BET inhibitors with other immunomodulatory agents, highlighting the potential for synergistic effects. For example, the combination of BET inhibitors with checkpoint inhibitors has shown promise in preclinical models of cancer. Moreover, many immune checkpoint therapies result in inflammatory triggers that lead to organ and tissue damage. Thus, BETi could be explored to ameliorate these inflammatory comorbidities. Our findings suggest that careful modulation of BETi dosage and combination strategies could optimize the therapeutic potential of these immunotherapies.

Our study provides evidence for a role for BET proteins in NK cell differentiation and function. Future studies should focus on elucidating the precise molecular mechanisms by which BETi modulate NK cell function and differentiation. Including studies dissecting the contribution of each of the BET proteins to NK cell fate and function. This could include functional validation of the gene expression signatures that are altered with BETi in mature or developing NK cells. Additionally, in vivo studies are necessary to validate the therapeutic potential of BETi in clinical settings.

In conclusion, our study highlights the critical role of BET inhibitors in regulating NK cell function and differentiation. The dual effect of BETi on reducing proinflammatory cytokine secretion and promoting NK cell maturation opens new avenues for the development of targeted therapies for cancer and inflammatory diseases. These findings underscore the therapeutic potential of BET inhibitors in fine-tuning immune responses and improving patient outcomes.

Data availability

All RNA-seq and scRNA-seq data is available for download from NCBI SRA under accession number PRJNA1288009. All other data is available from the corresponding author upon request.

Code availability

No new code was generated in this analysis. Publicly available R packages were used to conduct analysis in RStudio, as described in Methods.

Received: 6 August 2025; Accepted: 18 December 2025
Published online: 03 January 2026

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Acknowledgements
We thank Dr Isabela Pedroza-Pacheco for helpful discussions, and Prof Masafumi Takaguchi for kindly providing the CD4.221 cell line. We acknowledge Daniel Louiselle and the GMC sequencing core facility for genomic assay assistance.

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Funding
This work was supported by funding from the NIH, NIAID, DAIDS: R01 AI 147778 (P.B. and T.B); Consortium for HIV/AIDS Vaccine Development (CHAVD) UM1 AI 144371 (P.B.); and Martin Delaney Collaboratory of AIDS Researchers for Eradication (CARE) UM1 AI 164567 (P.B.). P.B. is a Jenner Institute Investigator.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-33437-1.

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