Ezh2 regulates differentiation and function of natural killer cells through histone methyltransferase activity

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Changes of histone modification status at critical lineage-specifying gene loci in multipotent precursors can influence cell fate commitment. The contribution of these epigenetic mechanisms to natural killer (NK) cell lineage determination from common lymphoid precursors is not understood. Here we investigate the impact of histone methylation repressive marks (H3 Lys27 trimethylation; H3K27^{me3}) on early NK cell differentiation. We demonstrate that selective loss of the histone-lysine *N*-methyltransferase Ezh2 (enhancer of zeste homolog 2) or inhibition of its enzymatic activity with small molecules unexpectedly increased generation of the IL-15 receptor (IL-15R) CD122^{+} NK precursors and mature NK progeny from both mouse and human hematopoietic stem and progenitor cells. Mechanistic studies revealed that enhanced NK cell expansion and cytotoxicity against tumor cells were associated with up-regulation of CD122 and the C-type lectin receptor NKG2D. Moreover, NKG2D deficiency diminished the positive effects of Ezh2 inhibitors on NK cell commitment. Identification of the contribution of Ezh2 to NK lineage specification and function reveals an epigenetic-based mechanism that regulates NK cell development and provides insight into the clinical application of Ezh2 inhibitors in NK-based cancer immunotherapies.

epigenetic regulation | NKG2D | hematopoietic stem and progenitor cells | histone modification | innate immunity

Natural killer (NK) cells play a critical role in immune surveillance against infection and transformation (1–4) and express germ-line–encoded receptors that interact with "stressed" or "missing-self" ligands on target cells upon cellular stress (5). Altered NK cell numbers or function have a profound impact on overall immune status and often correlate with cancer prognosis (6, 7). NK cell-based immunotherapy against both hematopoietic and solid tumors (8, 9) is under active clinical study and has shown reduced relapse and improved prognosis in many aggressive cancers (10, 11). Increased understanding of NK cell biology is required to improve the efficacy of these therapeutic approaches.

NK cells in bone marrow (BM) develop from NK precursors (NKp) from common lymphoid progenitors (CLP) (12). Functionally mature NK cells must undergo an education process that requires signals from germ-line–encoded and cytokine receptors, often leading to regulation of multiple transcription factors (TFs) (13). Evidence from IL-15 or IL-15 receptor (R) knockout mice has underscored the critical role of the IL-15R signaling pathway in NK cell development (14), which regulates the transcriptional activity of Id2, Tox, and Ets-1 for generation of NK cell precursors; E4bp4, T-bet, and Eomes for development of immature NK cells from NKp; and Helios, Runx3, and Blimp1 for NK cell maturation (15). Although the action of a single TF in regulation of NK cell development is well understood, the genetic and epigenetic regulatory networks that coordinate the action of multiple TFs in NK cell-fate determination and function remain largely unexplored.

Cell commitment from a multipotent precursor requires activation of lineage-specifying regulatory genes and repression of genes leading to alternative fates. This process depends on lineage-specific TFs and epigenetic regulators that coordinately determine genome-wide expression patterns in precursors. It is hypothesized that the simultaneous presence of bivalent methylation of histone H3, active modification (H3K4^{me3}) and suppressive modification (H3K27^{me3}), at regulatory elements keeps lineage-specific gene expression poised to switch on or off during lineage commitment (16). Removal of H3K27^{me3} leads to transcriptional activation.

The H3K27 methyltransferase Ezh2 (enhancer of zeste homolog 2) is a crucial regulator of cell-fate determination and plays an essential role in many biological processes and immune regulation (17). Ezh2 may regulate early B-cell development (18) and

Significance

How NK cell development diverges from T/B cell commitment at the common lymphoid progenitor stage is poorly understood. Histone modification near critical gene loci often influences lineage determination. Ezh2 is a histone methyltransferase frequently associated with gene repression. Here we observed that Ezh2-null hematopoietic stem and progenitor cells (HSPCs) or HSPCs treated with Ezh2 inhibitors gave rise to increased NK precursors and mature progeny that display enhanced cytotoxicity against tumor cells. The latter effects were associated with up-regulation of IL-15R (CD122) and the NKG2D-activating receptor. These findings may provide insight into the contribution of epigenetic regulation to the genesis of NK cells and suggest that Ezh2 inhibitors may inhibit tumor growth directly and indirectly through mobilization of NK cells.

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differentiative plasticity of CD4$^+$ Th1 and Th2 cells, as well as maintenance of Treg cell identity (19). Whether Ezh2 expression influences lineage commitment of lymphocyte subsets from their common progenitors is unclear. Here, we have investigated its contribution to NK cell lineage commitment and function to dissect regulatory mechanisms of NK cell development. We found that inactivation of *Ezh2* or inhibition of Ezh2 enzymatic activity through conditional knockout mice and small molecule inhibitors, respectively, enhanced NK cell lineage commitment and promoted increased NK cell survival and NKG2D-mediated cytotoxicity.

Results

Increased NK Lineage Cells in Ezh2-Deficient Mice. To investigate the contribution of Ezh2 to regulation of de novo lymphocyte development, we crossed *Ezh2*$^{fl/fl}$ mice with transgenic Vav-Cre mice to delete Ezh2 from hematopoietic stem and progenitor cells (HSPCs) and downstream progeny (Fig. S1*A*). Consistent with previous studies (18, 19), substantially reduced frequency and numbers of T and B cells in spleens from Vav1-Cre, *Ezh2*$^{fl/fl}$ (hereafter *Ezh2*$^{-/-}$) mice were observed compared with control *Ezh2*$^{+/+}$ mice (hereafter "WT") (Fig. 1 *A* and *B*). In contrast, the numbers and frequency of NK cells [T-cell receptor beta (TCRβ)$^-$ natural cytotoxicity-triggering receptor (NKp46$^+$)] (20) increased more than twofold in spleen, liver, and BM of *Ezh2*$^{-/-}$ mice (Fig. 1 *A* and *C*), suggesting that loss of Ezh2 may be associated with improved NK cell development.

The occurrence of NKp cells during NK cell development reflects the fate decision of the NK cell lineage, but not T or B cells from CLPs (Fig. S1*A*). Analysis of *Ezh2* mRNA expression in HSPC, CLP, NKp, and mature NK cells isolated from WT C57BL/6 mice showed substantial down-regulation of Ezh2 upon NK cell maturation (Fig. S1*B*). Robust Ezh2 expression in NKp cells suggests possible involvement of Ezh2 in regulation of NK cell lineage commitment.

Analysis of NKp (Lin$^-$ CD122$^+$) cells revealed substantially increased numbers and frequency in BM and spleens from *Ezh2*$^{-/-}$ compared with WT mice (Fig. 1*D*). Moreover, Ezh2 deficiency greatly enhanced CD122 expression at the single-cell level (Fig. 1*E*), suggesting that Ezh2 deletion from HSPCs and downstream progeny enhanced NK cell commitment, possibly accounting for increased NK cell numbers as shown above. Although Ezh2 deletion also led to phenotypic changes in NK cells (Fig. S2), our analysis focused on the impact of Ezh2 expression on early NK cell fate.

Increased NKp cell numbers in *Ezh2*$^{-/-}$ mice may reflect enhanced proliferation, diminished apoptosis, or both. Ezh2 deletion did not affect proliferation of NK subsets at various developmental stages (Fig. S3 *A*–*C*). However, *Ezh2*$^{-/-}$ NKp cells and committed NK subsets expressed significantly lower levels of Annexin V (Fig. S3*D*). We failed to observe a survival defect before the NKp stage in the absence of Ezh2 (Fig. S3*D*). Taken together, these results suggest that Ezh2 loss enhances NK cell lineage commitment and further development, at least in part, by promoting NKp cell survival.

Ezh2-Deficiency Enhances NK Lineage Commitment Intrinsically. Loss of Ezh2 perturbs many aspects of hematopoiesis (Fig. 1 *A* and *B*), which may indirectly influence NK cell development. To determine whether the effects on NK development reflect a cell-intrinsic role of Ezh2, we used a stromal cell-free culture system that supports NK cell development from HSPCs (21). We transduced FACS-sorted Lin$^-$Sca-1$^+$c-Kit$^+$ BM HSPCs from *Ezh2*$^{fl/fl}$ mice with retrovirus-expressing YFP-Cre or YFP only, followed by induction of NK cell development in vitro (Fig. 2*A*). Analysis of NK cell development showed that Ezh2 deletion in YFP$^+$ cells because of Cre-mediated recombination induced ~12% of cells to express CD122 and NKp46 at day 5, earlier than expected for normal NKp induction, as shown for YFP$^-$ and control cells expressing YFP alone (<2.5%) (Fig. 2*B*). Earlier NK lineage commitment subsequently led to production of ~fivefold more DX5$^+$ mature NK cells at day 9 (Fig. 2*B*). Thus, these results recapitulated the in vivo phenotype of

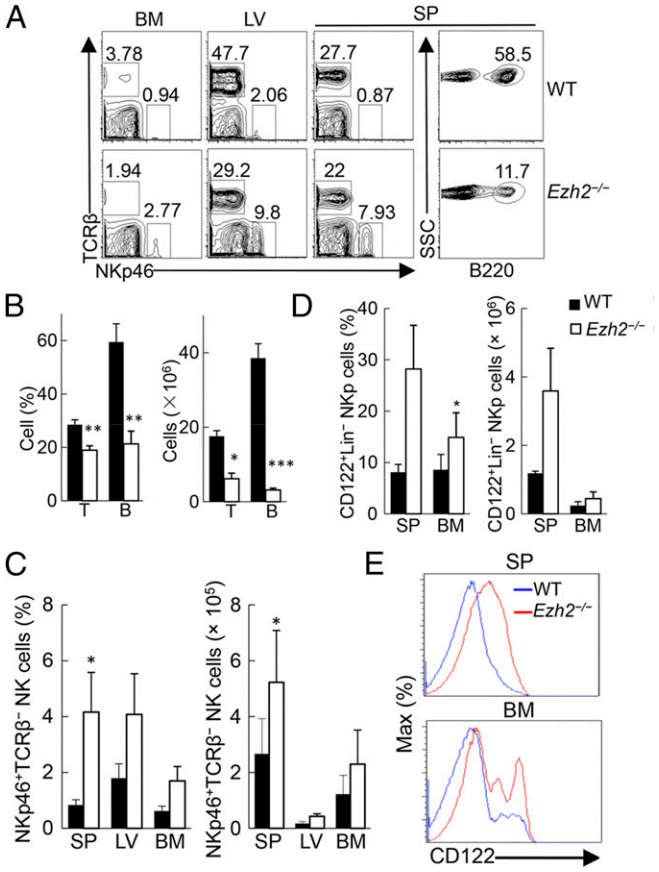

Fig. 1. Increased NK lineage cells in Ezh2-deficient mice. (*A*) Flow cytometry of cells from each organ of WT and *Ezh2*$^{-/-}$ mice. Gated numbers indicate percent T cells (TCRβ$^+$NKp46$^-$), NK cells (TCRβ$^-$NKp46$^+$) and B cells (B220$^+$). Frequency and numbers of splenic T and B cells (*B*) and NK cells (*C*) from the indicated organs in *A*. (*D*) Frequency and numbers of NKp cells (Lin$^-$CD122$^+$) in spleen and BM of WT and *Ezh2*$^{-/-}$ mice. (*E*) CD122 expression on Lin$^-$ cells from spleen and BM of WT and *Ezh2*$^{-/-}$ mice. $n = 3$–4 mice per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (error bars, mean ± SEM). Data are representative of at least three independent experiments. LV, liver; SP, spleen.

Ezh2$^{-/-}$ mice, supporting an intrinsic role of Ezh2 loss in regulation of NK cell development.

Selective Inhibition of Ezh2 Activity Promotes NK Cell Development. Polycomb repressive complexes (PRCs), including PRC1 and PRC2, are composed of polycomb group proteins that contribute to epigenetic silencing by modifying histones and other proteins (22). Ezh2 lies within the PRC1/2 complex and confers gene silencing by trimethylating histone H3 at lysine 27. To directly analyze the contribution of Ezh2 methyltransferase activity to regulation of NK cell development, we used small-molecule inhibitors UNC1999 and EPZ005687 (23, 24) to examine the effect of inhibitors on mouse NK cell development by treatment of HSPCs at the start of cellular differentiation (Fig. 3*A*). Both inhibitors decreased H3K27^{me3} levels at 5 μM (Fig. 3*B*). Surprisingly, induction of CD122$^+$NKp46$^+$ cells was observed at day 3 in inhibitor-treated cultures compared with controls (Fig. 3*C*) and this accelerated NKp induction led to ~twofold more mature NK cells (NK1.1$^+$DX5$^+$) after 6 d of cell differentiation (Fig. 3*C*).

Human NK cells produced in vitro from hematopoietic progenitor cell antigen CD34$^+$ HSPCs using a similar cell culture system as murine NK cells, albeit at longer incubation time (Fig. 3*D*) (25), were used to evaluate the effects of inhibitors on human NK cell development using concentrations to reduce H3K27^{me3} levels (2.5 μM)

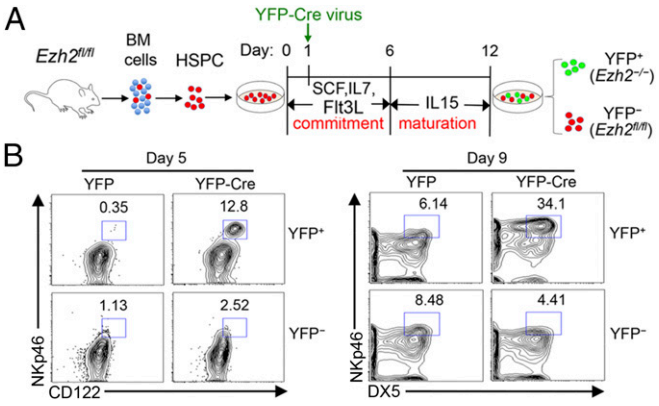

Fig. 2. *Ezh2* deletion promotes NK cell development in vitro. (*A*) Schematic of in vitro mouse NK cell differentiation using a two-step cell culture system described in *Materials and Methods*. $Ezh2^{fl/fl}$ HSPCs were infected with retrovirus expressing YFP-Cre to delete Ezh2 or control virus expressing YFP only. (*B*) Flow cytometry of developing NK cells in *A*. Gated numbers indicate percent $CD3^-NKp46^+CD122^+$ cells at day 5 (*Left*) or $CD3^-NKp46^+DX5^+$ cells at day 9 (*Right*). YFP^+: Ezh2-deleted; YFP^-: Ezh2-intact control. Data represent three independent experiments.

(Fig. 3*E*). After 2 wk of differentiation, we observed an increased frequency of cells expressing adhesion molecule CD56 and NKp46, and further expansion after 3 wk of culture following addition of Ezh2 inhibitors compared with controls (Fig. 3*F*). Increased human NK cell production was more evident when UNC1999 was used.

Taken together, these data suggest that selective inhibition of Ezh2 methyltransferase activity promotes both mouse and human NK cell development in vitro. The impact of Ezh2 deficiency on NK cell development, therefore, likely results, in large part, from loss of its enzymatic activity.

Ezh2 Deletion Alters the Genetic Program in NKp Cells. To understand how Ezh2 loss enhances NK cell development, we performed microarray analysis of NKp cells produced from in vitro cultures of $Ezh2^{fl/fl}$ HSPCs infected with YFP-Cre retrovirus compared with control virus-infected cells (Fig. 2*A*). In Ezh2-depleted NKp cells, 532 genes were up-regulated and 302 were down-regulated (Fig. 4*A*). Two-way hierarchical cluster analysis revealed that $Ezh2^{-/-}$ NKp cells displayed different gene expression patterns from WT NKp cells (Fig. 4*B*). Functional analysis of 532 up-regulated genes revealed that Ezh2 deficiency was involved in pathways for hematopoietic lineage development and immune regulation (Table S1). Furthermore, 24 differentially-expressed genes overlapped with a cluster of genes identified within the GLAD4U database (26), which may play critical roles in NK cell development and function (Fig. 4*C*). These 24 genes were categorized into encoding receptors, enzymes, cytokines, and DNA binding proteins (Fig. 4*D*). Expression of the *Klrk1* (killer cell lectin-like receptor subfamily K, 1) gene, encoding the activating NKG2D receptor, was elevated ~eightfold after Ezh2 deletion. Genes encoding cytokine receptors IL2ra and IL7r, important in NK cell expansion and survival (27, 28), were also increased in Ezh2-deficient NKp cells. Moreover, the abundance of mRNAs encoding chemokine receptors (Cxcr3, Ccr7, Xcr1), costimulatory and activating receptors (Slamf7, Tnfrsf9), Toll-like receptors (Tlr3, Tlr8), TFs (Tox, Blimp1) (29), and cytotoxicity-related proteases (Gzma, Gzmb) were also elevated following deletion of *Ezh2* (Fig. 4*D*). Therefore, Ezh2 deletion in mouse NKp cells increased expression of a series of genes critical for NK cell development and function.

Deletion or Inhibition of Ezh2 Activity Up-Regulates NKG2D Expression. Among those genes up-regulated after Ezh2 deletion, *Klrk1* was restricted to the NK cell lineage. Committed NKp cells already express the NKG2D receptor (Figs. S1*A* and S4). However, except for the role of NKG2D in mediating NK cell activation, little is known about its contribution to NK cell-fate decision and development. This prompted us to investigate how NKG2D up-regulation contributes to NK cell development.

To confirm NKG2D up-regulation at the protein level, we determined that YFP-Cre–mediated deletion of Ezh2 in $Ezh2^{fl/fl}$ HSPCs greatly increased surface NKG2D expression on day 5 differentiated cells (Fig. 5*A*). We then investigated whether this effect depended on Ezh2 enzymatic activity using Ezh2-selective small-molecule inhibitors UNC1999 and EPZ005687. Consistent with observations noted in Fig. 3*C*, addition of inhibitors accelerated CD122 expression after 3 d of culture and was accompanied by significantly increased NKG2D expression (Fig. 5*B*). Enhanced induction of CD122 and NKG2D expression in HSPCs from $Rag_2^{-/-}$

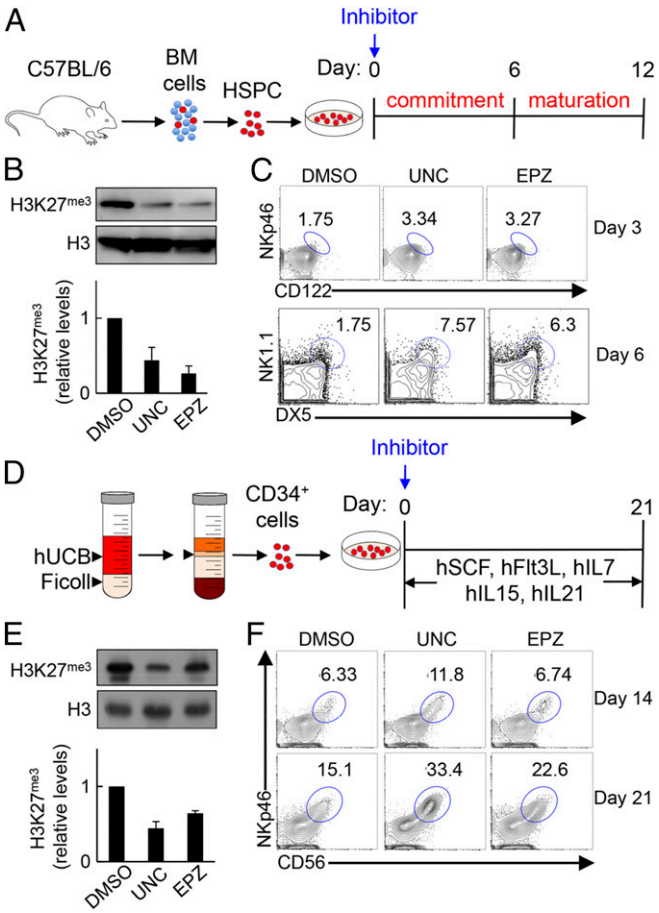

Fig. 3. Inhibition of Ezh2 activity enhances mouse and human NK cell development. (*A*) Schematic of in vitro mouse NK cell differentiation, as in Fig. 2*A*, followed by addition of Ezh2 inhibitors or DMSO (control). (*B*) Immunoblot analysis of $H3K27^{me3}$ and total H3 in mouse HSPCs at day 3 posttreatment with DMSO, UNC1999 (UNC), or EPZ005687 (EPZ) (5 μM). Densitometric levels of $H3K27^{me3}$ normalized to H3 levels are presented relative to those of DMSO-treated cells, set as 1 (*Lower*). (*C*) Flow cytometry of developing mouse NK cells in *A*. Gated numbers indicate $CD3^-NKp46^+CD122^+$ cells at day 3 (*Upper*) or $CD3^-NK1.1^+DX5^+$ cells at day 6 (*Lower*) in each group. (*D*) Schematic of in vitro human NK cell differentiation using a two-step cell culture system followed by addition of Ezh2 inhibitors or DMSO (control). (*E*) Immunoblot analysis of $H3K27^{me3}$ and total H3 in human HSPCs at day 21 posttreatment with DMSO or Ezh2 inhibitors (2.5 μM). Densitometric levels of $H3K27^{me3}$ normalized to H3 levels are presented relative to those of DMSO-treated cells, set as 1 (*Lower*). (*F*) Flow cytometry of developing human NK cells in *D*. Gated numbers indicate $CD3^-NKp46^+CD56^+$ cells at day 14 (*Upper*) or at day 21 (*Lower*) in each group. Data are representative of three independent experiments.

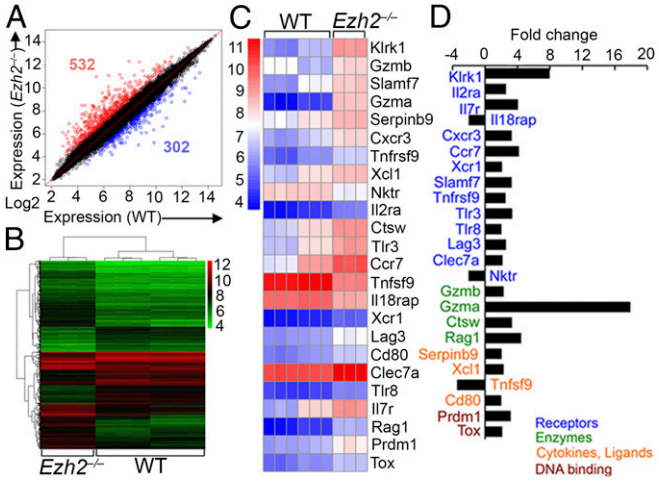

Fig. 4. Altered genetic program in NKp cells by Ezh2 deletion. (*A*) Microarray analysis of NKp cells ($CD122^+Lin^-$) differentiated from $Ezh2^{fl/fl}$ HSPCs infected with retrovirus expressing YFP-Cre ($Ezh2^{-/-}$) or control virus expressing YFP only (WT), as in Fig. 2*A*. Scatterplot of gene expression in sorted YFP^+ $Ezh2^{-/-}$ NKp cells (triplicates) relative to WT NKp cells ($n = 6$): 532 (red) genes up-regulated and 302 (blue) down-regulated in $Ezh2^{-/-}$ NKp cells (cut-off > twofold and $^*P < 0.05$). (*B*) Two-way hierarchical cluster heatmap of a total of 834 differentially expressed genes in each individual sample in *A*. (*C*) Heatmap displays 24 of the total 834 differentially expressed genes identified using the GLAD4U database, which highly correlate with regulation of NK cell development and function. Genes with the highest significance scores related to their functional importance are listed at the top. Scales (*B* and *C*) indicate values of the $\log_2$ robust multiarray signal intensity. (*D*) Twenty-four transcripts in *C* in $Ezh2^{-/-}$ NKp cells relative to their expression in WT NKp cells are listed.

mice was also observed (Fig. 5*C*). These data support the notion that increased NKp induction and NKG2D expression is restricted to NK cells and not influenced by deficiency in other lymphoid compartments (Fig. 1 *A* and *B*). Moreover, significant NKG2D up-regulation was observed in in vitro-differentiated human NK cells after treatment with inhibitors (Fig. 5*D*).

To determine whether chromatin marks at the *hKLRK1* locus following Ezh2 loss correlate with transcription, we performed ChIP-quantitative PCR (qPCR) analysis of in vitro-cultured human umbilical cord blood (hUCB) HSPCs treated with UNC1999 or DMSO. Four pairs of primers located sequentially along the proximal promoter, first intron, and exon 2 of *hKLRK1* to quantify $H3K27^{me3}$ in ChIP-enriched DNA by real-time PCR (Fig. 5*E*) showed a significant decrease in $H3K27^{me3}$ mark at the *KLRK1* promoter and gene body in UNC1999 treated cells compared with DMSO controls (Fig. 5*F*). Thus, decreased H3K27me3 deposition was associated with up-regulation of NKG2D expression (Fig. 5*D*).

Enhanced NK Cell Development by Inhibition of Ezh2 Activity Requires NKG2D Expression. Previous studies of conventional $Klrk1^{-/-}$ mice showed that absence of NKG2D adversely affected transition from immature to mature NK subsets, leading to reduced NK cell numbers (30). This ex vivo analysis using complete gene knockout mice may have overlooked the role of NKG2D during the early stages of NK cell differentiation. To determine whether NKG2D up-regulation mediated by Ezh2 inhibition contributed to NK cell development, we differentiated NK cells from B6 WT and $Klrk1^{-/-}$ HSPCs treated with Ezh2 inhibitors. Consistent with findings in Fig. 3*C*, increased $CD122^+NK1.1^+$ populations and elevated CD122 levels were detected at day 5 in culture in UNC1999-treated WT cells (Fig. S5*A*). $CD122^+NK1.1^+$ cells were diminished in $Klrk1^{-/-}$ cell cultures compared with WT in DMSO- and UNC1999-treated groups (Fig. S5*A*). The impact of NKG2D deficiency on NK cell development was greater after 9 d of in vitro differentiation and substantially increased NK cell differentiation was observed after treatment of WT HSPCs with Ezh2 inhibitors (Fig. 6), indicating that NKG2D deficiency blunts the effects of Ezh2 inhibitors. These results suggest that increased NK cell commitment and development following inhibition of Ezh2 requires expression of NKG2D as an intermediary.

We found that Ezh2 deficiency promoted NK cell survival at the NKp and post-NKp stages (Fig. S3*D*). Consistent with previous studies showing increased susceptibility of $Klrk1^{-/-}$ NK cells to apoptosis (30), we found that $Klrk1^{-/-}$ NKp cells expressed higher basal levels of Annexin V than WT NKp cells, and UNC1999 treatment slightly reduced Annexin V expression in WT NKp cells but further aggravated $Klrk1^{-/-}$ NKp cell apoptosis (Fig. S5*B*).

Preconditioning HSPCs with Ezh2 Inhibitors Enhances NKG2D-Dependent NK Cell Cytotoxicity. Adoptive immunotherapy based on transfer of autologous cell subsets to eradicate tumor cells is a current therapeutic approach to induce tumor regression in patients with metastatic cancer or hematopoietic tumors. Our findings of improved

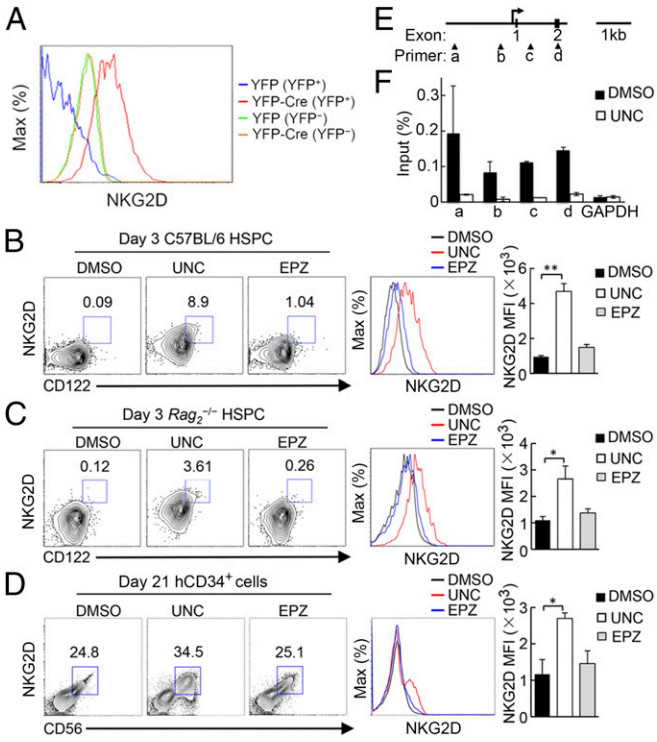

Fig. 5. Ezh2 deletion or inhibition of its activity up-regulates NKG2D expression. (*A*) Expression of NKG2D on in vitro differentiated mouse NKp cells at day 6 postculture of $Ezh2^{fl/fl}$ HSPCs following by infection with retrovirus expressing YFP-Cre or YFP only, as in Fig. 2*A*. YFP^+: Ezh2-deleted; YFP^-: Ezh2-intact control. Flow cytometry of developing mouse NK cells day 3 postculture of HSPCs from C57BL/6 mice (*B*) or $Rag_2^{-/-}$ mice (*C*), followed by treatment with DMSO or Ezh2 inhibitors, as in Fig. 3*A*. Gated numbers represent $CD3^-NKG2D^+CD122^+$ cells. Expression of NKG2D (*Center*) and quantitation of NKG2D MFI (*Right*) are shown. (*D*) Flow cytometry of developing human NK cells at day 21 postculture of $CD34^+$ cells, and treatment with the indicated reagents, as in Fig. 3*C*. Gated numbers represent $CD3^-NKG2D^+CD56^+$ cells. Expression of NKG2D (*Center*) and quantitation of NKG2D mean fluorescence intensity (MFI) (*Right*) are shown. Data represent three independent experiments. (*E*) Schematic diagram of the *hKLRK1* locus. Arrowheads, PCR primer pairs for ChIP analysis. (*F*) ChIP analysis of DNA precipitated with anti-$H3K27^{me3}$ or anti-IgG isotype control from in vitro differentiated NK cells at day 14 postculture of human $CD34^+$ cells and treatment with DMSO or UNC1999, followed by amplification with the indicated primers in *E* or GAPDH primer as negative control. No detectable or very low levels of signals with anti-IgG at all amplified regions. Percent of input DNA is shown in triplicates. $^*P < 0.05$ and $^{**}P < 0.01$ (error bars, mean ± SD).

NK cell commitment and development from HSPCs following Ezh2 inhibitor treatment open the possibility that NK cell output may be augmented by preconditioning patients' cells with Ezh2 inhibitors. Moreover, inhibition of Ezh2 increased expression of NKG2D, the most well-characterized NK activating receptor that senses stressed cells expressing up-regulated NKG2D ligands, including tumor cells (31). To test the feasibility of this approach, isolated HSPCs from donor CD45.1 mice were cultured in the presence of cytokine mixture and Ezh2 inhibitors for 2 d before washing and injection into sublethally irradiated (CD45.2) congenic $Rag_2^{-/-}\gamma_c^{-/-}$ hosts, devoid of T, B, and NK cells (Fig. 7*A*). Following 7 d of reconstitution, we observed increased expression of CD122, NKG2D on emerging CD45.1$^+$ NK cells, and more CD122$^+$NKG2D$^+$ NK cells in peripheral blood and spleen of hosts infused with UNC1999- or EPZ005687-pretreated HSPCs compared with DMSO control HSPCs (Fig. S6 *A* and *B*). To test the functional activity of newly generated NK cells, NK cells from spleens of hosts were enriched, expanded in vitro with IL-2 and subject to an NK killing assay against Yac-1 lymphoma cells, which are sensitive to NKG2D-mediated lysis (Fig. 7*A*). Both UNC1999- and EPZ005687-treated NK cells enhanced lytic activity against Yac-1 cells compared with DMSO-treated controls (Fig. 7*B* and Fig. S6*C*). Moreover, anti-NKG2D–mediated blockade impaired lytic activity in both control and Ezh2 inhibitor-treated cells (Fig. 7*B* and Fig. S6*C*), indicating that NK cell functional activity is NKG2D-dependent. We also observed that UNC1999- and EPZ005687-treated human NK cells differentiated from hUCB HSPCs were more potent in killing K562 target cells than DMSO-treated cells in an NKG2D-dependent manner (Fig. 7 *C* and *D*).

Taken together, the feasibility of de novo generation of NK cells with increased numbers and potency after inhibition of Ezh2 activity suggests new adoptive immunotherapeutic strategies to treat cancer using Ezh2 inhibitors, which are currently used only to directly target tumor cells.

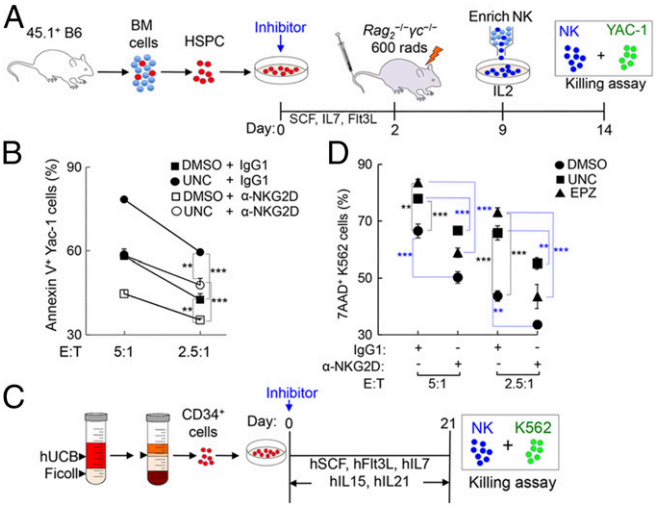

Fig. 7. NK cells differentiated from HSPCs treated with Ezh2 inhibitors exhibit increased cytotoxicity against tumor cell lines. (*A*) Schematic of in vitro NK cell differentiation from CD45.1$^+$ HSPCs treated with inhibitors, followed by adoptive transfer, donor NK cell enrichment, and analysis of in vitro cytotoxicity against Yac-1 target cells, as described in *Materials and Methods*. (*B*) Cytotoxicity of IL-2–expanded donor NK cells enriched in *A* against CFSE (carboxyfluorescein diacetate, succinimidyl ester)-labeled Yac-1 cells at the indicated effector-to-target (E:T) ratio in the presence of anti-NKG2D or isotype IgG1 control antibody, shown as percent Annexin V$^+$CFSE$^+$ cells. (*C*) Schematic of in vitro NK cell differentiation from hUCB CD34$^+$ cells treated with inhibitors, followed by cytotoxicity assay against K562 cells. (*D*) Cytotoxicity of developed NK cells at day 21, postculture in *C* against CFSE-labeled K562 cells at the indicated E:T ratio in the presence of anti-NKG2D or isotype IgG1 control antibody, shown as percent 7AAD$^+$CFSE$^+$ cells. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (error bars, mean ± SEM of triplicate wells). Results are representative of three experiments.

Discussion

Bivalent H3 methylation status at lineage-specifying gene loci may regulate cell fate determination from multipotent precursors. Deliberate alteration of the chromatin state during NK cell lineage commitment from HSPCs was assessed by manipulating Ezh2, an essential component of PRC2, which deposits histone mark H3K27^{me3}. Here we show that Ezh2 deficiency by gene knockout or small-molecule inhibition enhances generation of NK cells and improves NK-mediated cell lysis.

Ezh2 exerts cell-intrinsic effects on NK lineage development. Genetic deletion of Ezh2 or inhibition of its enzymatic activity by small molecules substantially increased expression of the IL-15R CD122 and NKG2D activating receptor, resulting in enhanced NK cell generation from HSPCs. IL-15 is an essential factor in regulation of NK cell survival and development. Mice that lack or fail to respond to IL-15 (e.g., *Il15*$^{-/-}$, *Il15ra*$^{-/-}$, and *Rag2*$^{-/-}\gamma C^{-/-}$) harbor no peripheral NK cells (32–34). Although the mechanism of Ezh2-mediated regulation of CD122 expression remains to be defined, increased and accelerated expression of CD122 upon Ezh2 inhibition may enhance IL-15 responsiveness of NKp cells and promote NKp survival and commitment.

Enhanced NK cell generation from Ezh2-deficient HSPCs may also depend in part on elevated NKG2D expression. Although the role of NKG2D in NK cell commitment remains unclear, the finding that inhibition of Ezh2 activity enhanced survival of both NKp and its progeny and that this positive effect was diminished by NKG2D deficiency may partially explain the contribution of NKG2D expression to early NK cell differentiation. Failure of Ezh2 inhibition to promote generation of *Klrk1*$^{-/-}$ NK cells from HSPCs further supports NKG2D as an important intermediary downstream of Ezh2-mediated regulation of NK cell commitment. De-coupling of the signaling events triggered by NKG2D and its adaptor DAP10 from those initiated by IL-15R–Jak3 renders NK cells unresponsive to IL-15, resulting in lower numbers of NK cells (35). Possibly, signals emanating from these two receptors, which are both enhanced by Ezh2 inhibition, synergistically regulate NK cell differentiation.

Our data show that Ezh2 inhibition not only enhances NK cell commitment but also improves mature NK cell function. Continuous down-regulation of Ezh2 after the NKp stage may ensure maintenance of a chromatin state adjacent to NK-specific genes, including *Klrk1* and genes encoding granzymes, which are crucial for mature NK cell effector activity. Stabilized NKG2D expression throughout NK cell maturation after its initial up-regulation before NKp generation is compatible with this hypothesis and also suggests that regulation of Ezh2 activity determines the quantity and quality of NK cells.

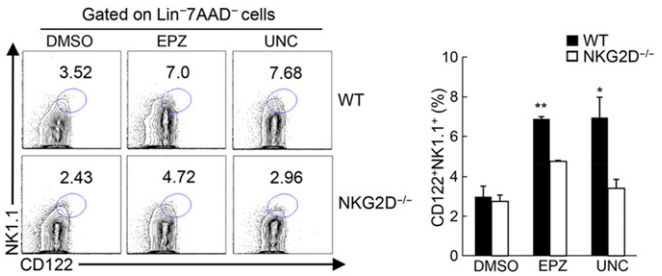

Fig. 6. Enhanced NK cell development by inhibition of Ezh2 activity requires NKG2D expression. Flow cytometry of developing NK cells at day 9 postculture of HSPCs from C57BL/6 or *Klrk1*$^{-/-}$ mice, and treatment with the indicated reagents. Gated numbers indicate CD3$^-$NK1.1$^+$CD122$^+$ NK cells. (*Right*) Frequency of CD3$^-$NK1.1$^+$CD122$^+$ mature NK cells is shown. $^*P < 0.05$ and $^{**}P < 0.01$ (error bars, mean ± SEM). Data are representative of at least three independent experiments.

Histone modifications correlate with transcriptional activity in normal tissues and are often altered in pathological conditions. Histone modifications are reversible, making them suitable targets for therapeutic intervention. Alterations in histone lysine methyltranferases are frequently associated with physiological and pathological processes, including cancer (36, 37) and autoimmune disease (38). Increased expression of Ezh2 or Ezh2 mutations that enhance methyltransferase activity are observed in cancer and often correlate with poor prognosis (36). Several small-molecule inhibitors of Ezh2 under preclinical development suggest in vivo activity in cancer (39). Our observations that Ezh2 inhibitors increase NK cell numbers and activity against tumor cell lines suggest an additional potential role of these inhibitors in mobilizing antitumor activity of NK cells. Thus, the therapeutic effects of Ezh2 inhibitors on tumor growth should also include analysis of their impact on immune responses, which may suggest new strategies that synergize with the use of Ezh2 inhibitors.

Epigenetic machinery, which lies at the interface between environmental stimulation and transcriptional regulation, actively shapes the gene-expression pattern of a cell and decides its functional outcome. Our findings provide new insight into the contribution of epigenetic modifiers to NK cell biology and suggest new therapeutic strategies in cancer immunotherapy.

Materials and Methods

Mice. C57BL/6J (B6) mice, *Ezh2*$^{fl/fl}$ mice, *Klrk1*$^{-/-}$ mice, *Rag2*$^{-/-}\gamma_c^{-/-}$ mice, Vav1-Cre mice (Jackson Laboratories) and B6SJL (CD45.1^{+}) mice (Taconic Farms) were housed in specific pathogen-free conditions and were used at 7–12 wk of age. Deletion of *lox*P-flanked *Ezh2* in hematopoietic cells was achieved by crossing *Ezh2*$^{fl/fl}$ mice with Vav1-Cre mice that express recombinase Cre under the proto-oncogene *Vav1* promoter. Animal handling and experimental procedures were performed in compliance with federal laws and institutional guidelines as approved by the Animal Care and Use Committee of Dana-Farber Cancer Institute.

Flow Cytometry, Cell Isolation, Retroviral Transduction, and in Vitro Culture. Details of flow cytometry analysis, cell isolation, retroviral transduction and in vitro culture are provided in *SI Materials and Methods*.

Adoptive Cell Transfer, NK Cell Enrichment, and in Vitro NK Cell Killing Assay. Details of adoptive cell transfer, NK cell enrichment and in vitro NK cell killing assay are provided in *SI Materials and Methods*.

Microarray, ChIP, qRT-PCR, and Immunoblot. Details of microarray, ChIP, qRT-PCR and immunoblot analysis are provided in *SI Materials and Methods*.

Statistical Analysis. Analyses were performed with a two-tailed, unpaired Student's *t* test with GraphPad Prism software. A P value < 0.05 was considered statistically significant. No exclusion of data points was used.

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