H2-K1 protects murine *MLL-AF9* leukemia stem cells from natural killer cell-mediated immune surveillance

Acute myeloid leukemia (AML) is characterized by an accumulation of myeloid blasts in the bone marrow and poor survival. The high rate of relapse is attributed to an inability of current therapies to eradicate chemotherapy-resistant leukemic stem cells (LSC), which is a self-renewing population responsible for disease initiation, progression, and relapse.[1] For leukemia to develop, LSC need to evade tumor immune surveillance mechanisms within the bone marrow niche.[2] While immune checkpoint inhibitors that activate T cells have demonstrated unprecedented clinical success across various solid tumors, there is an emerging recognition of the anti-leukemic potential of innate immune cells, including natural killer (NK) cells and macrophages. However, AML development is associated with dysfunctional NK cells and macrophages, but the mechanistic basis for this remains poorly understood.[3] In particular, NK cells have been associated with tumor immune surveillance, and strategies that bolster endogenous NK cells have therapeutic potential in myeloid malignancies.[4] NK cells are regulated by inhibitory and activating receptors and kill virus-infected cells and tumor cells by degranulation and apoptosis. A main group of inhibitory receptors are the killer-cell immunoglobulin-like receptors (KIR) in humans and the Ly49 receptors in mice, which both function by binding MHC class I molecules on target cells. In AML, hematopoietic stem cell transplantation with KIR-mismatching of the donor reduces the risk for relapse.[4] Moreover, ligands for activating NKG2D receptors are often downregulated on LSC, as a strategy to elude NK cells.[2] Identifying the mechanisms by which AML cells escape immune surveillance may translate into new therapeutic strategies aimed at reinstating effective cancer immune surveillance in patients.

We recently performed an *in vivo* CRISPR dropout screen targeting cell surface proteins using murine LSC driven by the *MLL-AF9* fusion gene. Among the top *in vivo* dependencies within the bone marrow niche was *H2-k1*, a classical MHC class-I molecule.[5] Here, by following the protocol approved by the animal care and use ethical committee of Lund/Malmö, Sweden, we found that *H2-k1* protects *MLL-AF9* leukemia-initiating cells from NK cell-mediated immune surveillance by altering NK-cell cytotoxicity and maturation. These findings highlight H2-K1 as a key molecule mediating immune evasion of LSC in the *MLL-AF9* mouse model.

The development of drugs that boost the immune system in AML has therapeutic potential but is often hampered by an impaired immunity in patients. Specifically, NK-cell function is compromised and also macrophages and T cells are suppressed.[6,7] In a recent *in vivo* CRISPR dropout screen targeting 961 genes encoding cell surface receptors in *MLL-AF9* leukemia-initiating cells, the MHC class-I molecule *H2-k1* scored among the top leukemia dependencies, but this finding was not further investigated (*Online Supplementary Figure S1A*).[5] To examine how *H2-k1*, the murine ortholog of human HLA-A, regulates the survival of c-Kit$^+$ leukemia cells, enriched for LSC, we first measured H2-K1 expression in serially propagated murine *MLL-AF9* leukemia cells. H2-K1 expression was markedly elevated in c-Kit$^+$ leukemia cells compared to their healthy bone marrow counterparts (Figure 1A). This significant upregulation suggests that H2-K1 could play a role in AML development. Similarly, in AML patients, *HLA-A* was upregulated on leukemic cells compared to normal hematopoietic stem cells (*Online Supplementary Figure S1B*). Given the role of MHC class I molecules in suppressing innate immune cells, we speculated that upregulation of H2-K1/HLA-A in AML might facilitate immune evasion. To investigate the role of H2-K1 in *MLL-AF9* leukemia cells, two single guide RNA (sgRNA) targeting *H2-k1* were expressed in the Cas9$^+$c-Kit$^+$ *MLL-AF9* leukemia cells using lentiviral vectors.[8] Next-generation sequencing of transduced cells confirmed a high editing efficacy in the *H2-k1* locus, which translated into reduced H2-K1 expression (Figure 1B, C). Whereas *H2-k1* disruption did not significantly affect the growth and survival of the *MLL-AF9* leukemia cells *ex vivo* (Figure 1D), following injection into sublethally irradiated recipient mice, a strong depletion of the leukemia cells was observed in both bone marrow and spleen (Figure 1E, F). Consistent with this finding, transplantation of sorted *H2-k1* sgRNA-expressing leukemia cells into mice resulted in increased survival compared to controls (Figure 1G).

To test whether H2-K1 protects against cancer immune surveillance, we next depleted macrophages or NK cells in mice prior to transplantation of *MLL-AF9* leukemia cells. Selective depletion of macrophages using clodronate liposomes did not alter leukemia burden of either *H2-k1* knockdown or control cells (*Online Supplementary Figure S1C-E*). This finding suggests that macrophages do not significantly contribute to H2-K1-mediated immune surveillance of leukemia cells in this model. In contrast, depletion of NK cells in mice using an anti-NK1.1 antibody accelerated leukemia progression, indicating that NK cells protect against leukemia development (Figure 2A, B). Notably, depletion of NK cells rescued the antileukemic effect of *H2-k1* knockdown (Figure 2C and *Online Supplementary Figure S2A, B*). This observation suggests that *H2-k1* facilitates immune evasion of *MLL-AF9* leukemia cells by inhibiting NK cells.

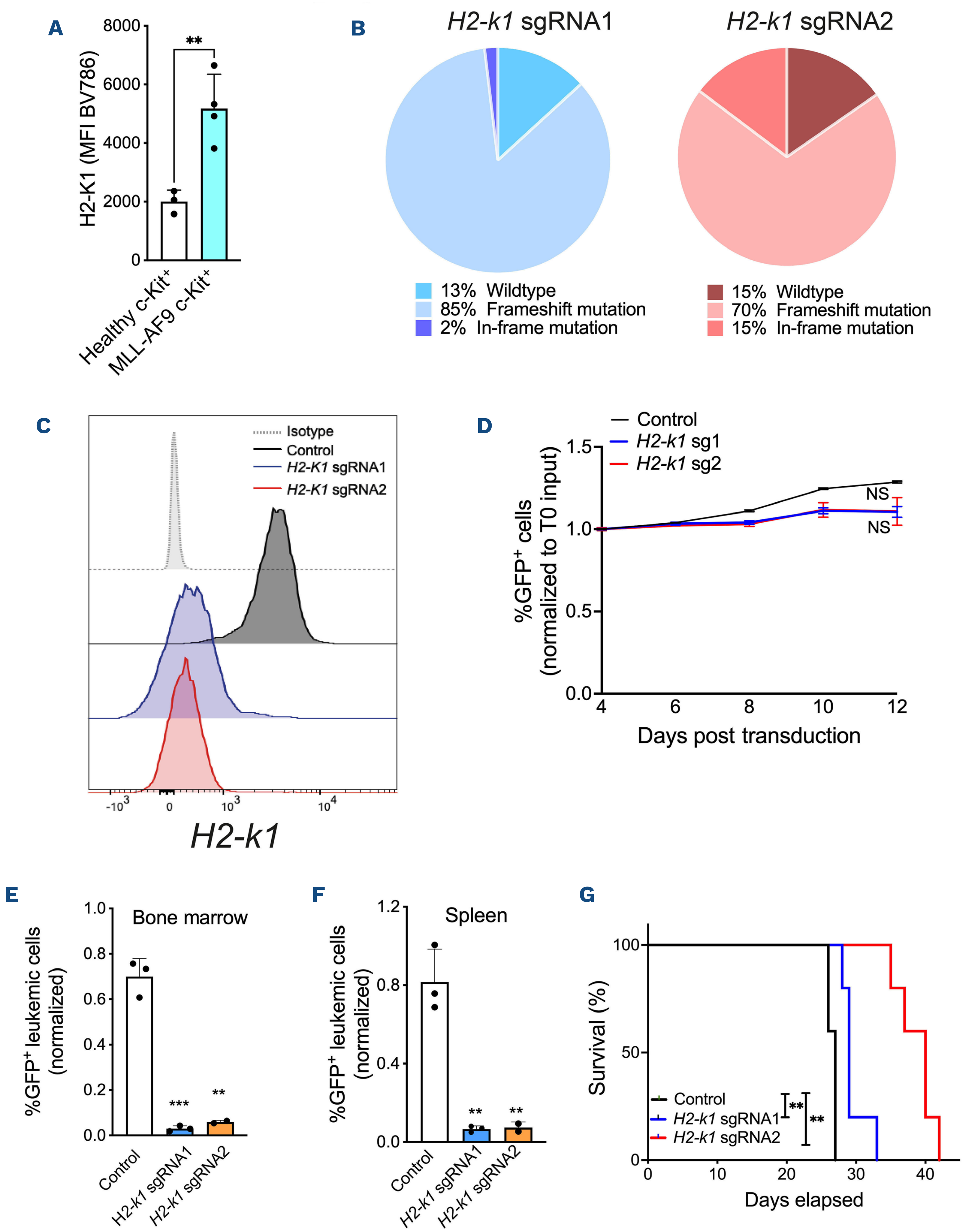

Figure 1. *H2-k1* is critical for the survival of *MLL-AF9* leukemia cells *in vivo*. (A) Flow cytometric analysis of H2-K1 expression in c-Kit+ *MLL-AF9* leukemic bone marrow cells and corresponding healthy cells. (B-G) dsRed+c-Kit+ *MLL-AF9* leukemia cells were transduced with *H2-k1* sgRNA or a non-targeting control. (B) Gene editing within the *H2-k1* locus was quantified by deep sequencing in sorted GFP+ cells 3 days post transduction. (C) Representative histogram of H2-K1 expression measured by flow cytometry within GFP+ leukemia cells 5 days post transduction. (D) *Ex vivo* competitive proliferation assay measured by the percentage of GFP+ leukemia cells in culture over time, normalized to the input percentage at day 2 (T0). (E and F) Transduced c-Kit+ leukemia cells were transplanted into sublethally irradiated recipients. Percentage of GFP+ cells within the *MLL-AF9* leukemia cells in the bone marrow (E) and spleen (F) of mice 13 days post transplantation. The percentage of GFP+ cells at day 13 was normalized to the input percentage of GFP+ cells 2 days post transduction (T0). (G) Kaplan-Meier survival analysis of mice transplanted with sorted GFP+ leukemia cells 2 days post transduction (N=5 mice per group; log-rank test). Data are presented as mean±standard deviation. N=3 unless otherwise stated. Significance was measured by non-parametric Student *t* test, **$P<0.01$; ***$P<0.001$. MFI: mean fluorescence intensity; NS: not significant.

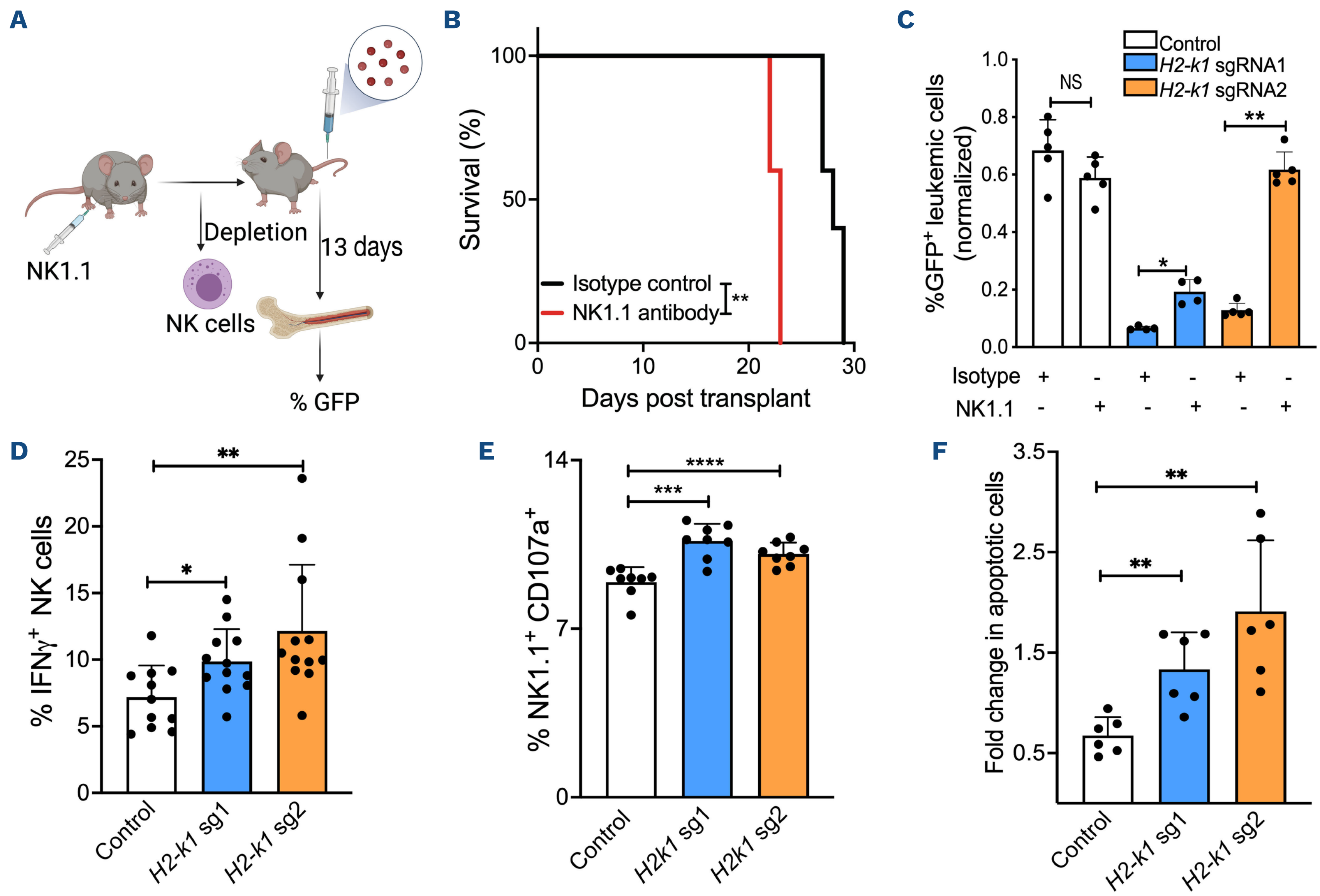

Figure 2. Natural killer cell-mediated immune surveillance is restored by *H2-k1* disruption in leukemic cells. (A) Schematic representation of the experimental design for depletion of natural killer (NK) cells using an NK1.1 antibody prior to transplantation of *MLL-AF9* leukemia cells. GFP^{+} cells represent leukemia cells transduced with *H2-k1* sgRNA or control. The illustration was generated using Biorender. (B) Kaplan-Meier survival analysis of mice with or without depletion of NK cells prior to transplantation of *MLL-AF9* leukemia cells (N=5 mice per group; log-rank test). (C) Percentage of GFP^{+} cells within *MLL-AF9* leukemia cells in the bone marrow following isotype or anti-NK1.1 antibody treatment was normalized to the input percentage of GFP^{+} cells 2 days post transduction (T0) (N=5 mice per group). (D) Percentage of Interferon-γ secreting cells, and (E) CD107a-expressing cells within NK1.1^{+} NK cells, isolated from spleen of healthy mice, and co-cultured with leukemia cells transduced with *H2-k1* sgRNA or control (N=4). (F) Fold change in percentage of apoptotic (Annexin V^{+}) leukemic cells co-cultured with or without NK cells (N=6). Data are represented as mean±standard deviation. Significance was measured by non-parametric Student *t* test or multiple *t* test. **P*<0.1; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

To assess whether H2-K1 in c-Kit^{+} leukemia cells directly alters NK-cell activity, we performed co-culture experiments *ex vivo*. Consistent with higher cytotoxic activity, disruption of *H2-k1* in the leukemia cells resulted in increased IFN-γ and CD107a expression in NK cells (Figure 2D, E). This was accompanied by increased apoptosis of the leukemia cells (Figure 2F). H2-K1 exhibits high-affinity interactions with both the Ly49C and Ly49I receptors.[9] Additionally, H2-K1 also binds to the Ly49A receptor, albeit with lower affinity.[10] While the expression of Ly49 C/I/A in NK cells varied across the M1 to M3 maturation stages, no marked changes were observed following the *in vivo* exposure to *MLL-AF9* leukemia cells, regardless of *H2-k1* disruption (*Online Supplementary Figure S2C, D*). Taken together, these findings demonstrate that H2-K1 inhibits NK cells in the *MLL-AF9* mouse model, and suggest that *ex vivo* and *in vivo H2-k1* disruption sensitizes leukemia cells to killing by NK cells.

Leukemia development has been linked to the altered maturation of NK cells both in patients and in murine models. To investigate whether the progression of *MLL-AF9* leukemia impacts NK-cell development and maturation, we analyzed NK-cell populations during the course of disease development. We observed that the progression of *MLL-AF9* leukemia cells coincided with an increase of M1 (CD27^{+}CD11b^{-}) NK cells and a reduction in the more cytotoxic M2 (CD27^{+}CD11b^{+}) and M3 (CD27^{-}CD11b^{+}) NK cells in the mouse bone marrow (Figure 3A and *Online Supplementary Figure S3A*).[11] These findings suggest that the expansion of *MLL-AF9* leukemia cells *in vivo* leads to a skewing towards immature, less cytotoxic NK-cell populations, corroborating previous studies that reported a differentiation block of NK cells following leukemia onset.[12-14]

We next explored whether the expression profile of activating receptors on NK cells was altered upon *MLL-AF9* leukemia development. Notably, leukemia progression was accompanied by decreased expression of the activating receptor NKG2D across the M1-M3 NK-cell populations (Figure 3B). Reduced expression of NKG2D ligands on LSC in AML patients has been associated with immune evasion and subsequent leukemia progression.[2] In murine

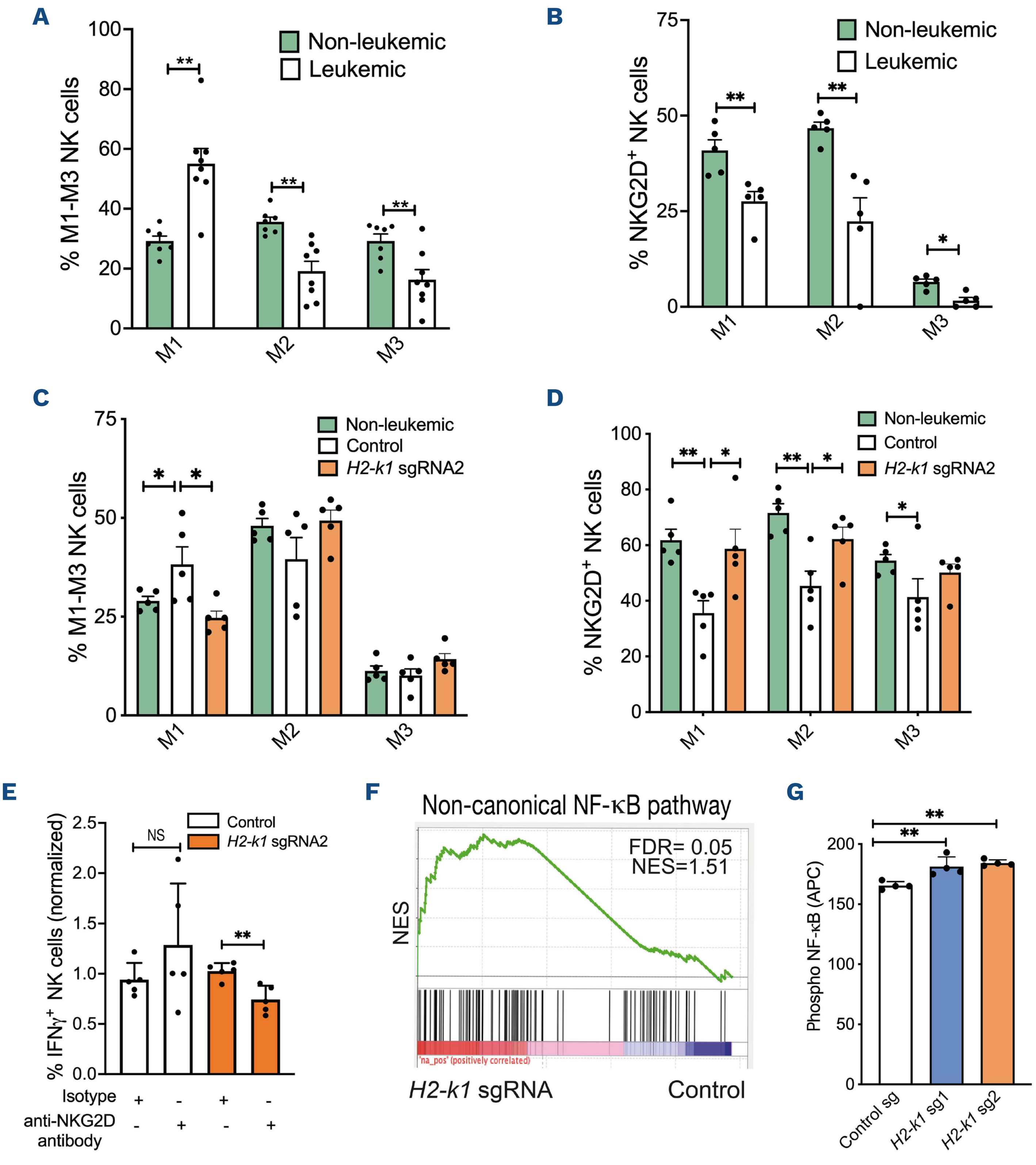

Figure 3. *H2-K1* disruption in leukemic cells affects natural killer cell maturation and activation. (A) Percentage of M1-M3 populations within mature natural killer (NK) cells (Lin$^-$CD122$^+$) in bone marrow (BM) of healthy and leukemic mice (N=7 mice per group). (B) Percentage of NKG2D$^+$ cells within M1-M3 NK cell populations in the BM of leukemic and healthy mice (N=7 mice per group). (C) Percentage of M1-M3 populations within mature NK cells (Lin$^-$CD122$^+$) in the BM of mice transplanted with sorted *H2-k1* sgRNA or control tranduced leukemic cells (N=5 mice per group). Mice not receiving leukemia cells, referred to as non-leukemic, were included as an additional control. (D) Percentage of NKG2D-expressing cells within M1-M3 NK cells in BM of mice transplanted with sorted *H2-k1* sgRNA or control-transduced leukemic cells (N=5 mice per group). Mice not receiving leukemia cells, referred to as non-leukemic, were included as an additional control. (E) Percentage of Interferon-γ secreting NK cells in co-culture with leukemia cells transduced with *H2-k1* sgRNA 2 or control. (F) Gene set enrichment analysis of the transcriptional signature in NK cells from healthy mice co-cultured with *H2-k1* sgRNA *versus* control transduced leukemic cells (N=4) overnight at an effector:target ratio of 1:1. (G) Mean fluorescence intensity (MFI) of phosphorylated (phospho) NF-κB on NK1.1$^+$ NK cells, isolated from spleen of healthy mice, and co-cultured with leukemic cells transduced with the *H2-k1* sgRNA or control (N=4) for 6 hours at an effector:target ratio of 1:1. Data are represented as mean±standard deviation. Significance was measured by non-parametric Student *t* test or multiple *t* test. **P*<0.1; ***P*<0.01.

c-Kit[+] leukemia cells, expression of the NKG2D ligands *Ulbp1*, *Raet1d* and *Rae1* was clearly detected (*Online Supplementary Figure S3B*). We next explored the impact of H2-K1 on the distribution of M1-M3 NK cells and their NKG2D expression levels. Although the total percentage of mature NK cells was not altered (*Online Supplementary Figure S3C*), knockdown of *H2-k1* partially reversed the leukemia-induced changes in M1-M3 subpopulations and restored normal NKG2D expression (Figure 3C, D). These findings indicate that H2-K1, through its interaction with its ligands on NK cells, plays a role in regulating NKG2D expression and NK-cell maturation without affecting NK-cell production. Notably, blocking NKG2D with an antibody suppressed the cytotoxicity of NK cells in co-culture with c-Kit[+] leukemia cells in an H2-K1-dependent manner (Figure 3E).

To further assess how H2-K1 regulates NK cells, we next performed RNA-sequencing of NK cells co-cultured with c-Kit[+] leukemia cells. Disruption of *H2-k1* in the *MLL-AF9* leukemia cells led to dysregulation of critical regulatory NK-cell genes, with marked enrichment of interleukin 2 (IL2) and IL6-induced JAK/STAT signaling and NF-κB activation (Figure 3F and *Online Supplementary Figure S3D-F*), associated with NK-cell maturation and activation.[15] These data align with our observations that the murine c-Kit[+] leukemia cells express NKG2D ligands and that NKG2D associates with DAP10 and DAP12 receptors on NK cells to activate NF-κB, enhancing NK-cell cytotoxicity and cytokine release. *Ex vivo* co-cultures confirmed that disruption of *H2-k1* in the *MLL-AF9* leukemic cells increased NF-κB activation in NK cells (Figure 3G), highlighting the role of H2-K1 in modulating signaling pathways in NK cells.

In summary, here we identified activation and maturation defects of NK cells in the *MLL-AF9* leukemia mouse model, contributing to immune evasion of the leukemia cells. We discovered that H2-K1 expression on leukemia cells within the bone marrow niche plays a pivotal role in suppressing NK-cell activity and their maturation process. The observation that deletion of *H2-k1* alone restored NK-cell mediated immune surveillance against murine LSC suggests that uncovering similar mechanisms in human AML could translate into new treatment opportunities.

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Contributions
MJ and SG conceived the study and designed the experiments. MR-Z, GT-D, KR, RR and ES helped in performing the experiments and interpreting the data. MJ and SG wrote the manuscript.

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Data-sharing statement
Data will be shared upon request.

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