The IL-15-AKT-XBP1s signaling pathway contributes to effector functions and survival in human NK cells

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Interleukin 15 (IL-15) is one of the most important cytokines that regulate the biology of natural killer (NK) cells. Here we identified a signaling pathway—involving the serine-threonine kinase AKT and the transcription factor XBP1s—which regulates unfolded protein response genes—that was activated in response to IL-15 in human NK cells. IL-15 induced the phosphorylation of AKT, which led to the deubiquitination, increased stability and nuclear accumulation of XBP1s protein. XBP1s bound to and recruited the transcription factor T-BET to the gene encoding granzyme B, leading to increased transcription. XBP1s positively regulated the cytolytic activity of NK cells against leukemia cells and was also required for IL-15-mediated NK cell survival through an anti-apoptotic mechanism. Thus, the newly identified IL-15–AKT–XBP1s signaling pathway contributes to enhanced effector functions and survival of human NK cells.

Unspliced XBP1 (X-box binding protein 1) mRNA, known as XBP1u, encodes an unstable cytoplasmic protein with no transactivation domains. As a result of unconventional splicing mediated by the serine-threonine protein kinase and endoribonuclease IRE1α, mature XBP1 mRNA is converted to XBP1s4. The protein encoded by XBP1s can act as a transcription factor5, XBP1s has multiple roles in regulating the immune response. It regulates major histocompatibility complex II gene transcription in HeLa and COS cells, as well as the differentiation of plasma cells, eosinophils and CD8+ T cells6,7. XBP1s also modulates anti-tumor immunity by disrupting dendritic cell homeostasis. We investigated the expression of XBP1s in primary human natural killer (NK) cells purified from the blood of healthy donors in response to interleukin 2 (IL-2), IL-12 or IL-15 for 24 h prior to analysis by flow cytometry or immunoblotting. IL-15 induced the expression of XBP1s protein, whereas IL-2 and IL-12 showed reduced effects compared with IL-15 (Fig. 1a,b). Although IL-2 and IL-15 share the cognate receptors IL-2Rβ and IL-2Rγ, on NK cells, induction of XBP1s by IL-15 was significantly higher than that triggered by similar concentrations of IL-2 (Fig. 1b and Supplementary Fig. 1a). This suggests that the IL-15Rα chain expressed on NK cells may play a critical role in inducing XBP1s.

In addition, the expression of transcripts for XBP1s target genes, including ERDF4 and SEC61A1 (ref. 5), was significantly increased in IL-15-treated primary human NK cells compared with that of untreated, IL-2-treated or IL-12-treated cells (Fig. 1c).

We next investigated the effects of XBP1s overexpression on NK cell function. Primary human NK cells transfected with pCDH lentivirus carrying a wild-type XBP1s gene (pCDH-XBP1s) and co-cultured with K562, MOML13 or U937 leukemia cell lines had a higher percentage of CD107a+ NK cells than that of NK cells transfected with the lentivirus carrying an empty pCDH vector (pCDH-EV) (Fig. 1d). Upon co-culture with MOML13 target cells, the percentage of CD107a+ cells in primary human NK cells transduced with pLKO.1 lentivirus carrying XBP1s-specific short hairpin RNAs (shRNAs) (XBP1-knockdown (XBP1-KD)) was significantly decreased (an approximately 35% reduction) compared with cells transduced with pLKO.1 lentivirus carrying scrambled shRNAs (scramble-KD) (Fig. 1e). In addition, primary human NK cell degranulation against multiple myeloma MM.1S cells was observed in IL-15-treated but not in untreated primary human NK cells (Fig. 1f). When co-cultured with MM.1S multiple myeloma cells, the percentage of CD107a+ NK cells expressing XBP1s was approximately fourfold greater than that of CD107a+ NK cells lacking XBP1s (Fig. 1f). Moreover, the expression of XBP1s protein was significantly higher in CD107a+ than in CD107a- primary human NK cells co-cultured with MM.1S cells (Supplementary Fig. 1b), indicating that expression of XBP1s correlates with NK cell cytotoxicity against tumor cells. Collectively, our results suggest that IL-15 induces XBP1s protein expression and that the expression level of the transcriptional factor directly correlates with cytotoxic activity in human NK cells.

To investigate how XBP1s regulates NK cell function, we analyzed the expression of genes encoding molecules related to NK cell effector functions, including GZMB (granzyme B), IFNG (interferon-γ), and PRF1 (perforin). Expression of GZMB and IFNG mRNA, but not that of PRF1 mRNA, was higher in pCDH-XBP1s-transduced primary human NK cells compared with pCDH-EV-transduced control NK cells (Fig. 2a), along with increased expression of GZMB.

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protein (Fig. 2b,c). Overexpression of the unspliced form of XBP1s, XBP1u—which can be processed into XBP1s through IRE1α-mediated mRNA splicing—in primary human NK cells by transduction with pCDH lentivirus carrying a wild-type XBP1u gene (pCDH-XBP1u) also increased the expression of GZMB compared with pCDH-EV NK cells (Fig. 2b,c). Moreover, primary human NK cells treated with thapsigargin, a chemical drug that induces endoplasmic reticulum stress and IRE1α catalytic activity, increased XBP1s protein and GZMB mRNA and protein compared with NK cells without thapsigargin treatment, in the absence or presence of IL-15 (Supplementary Fig. 2a–c). In addition, downregulation of GZMB protein expression was observed in primary human NK cells transfected with XBP1s-specific small interfering RNAs (siRNAs) compared with cells transfected with scrambled control siRNAs (Supplementary Fig. 2d,e). We also observed decreased expression of both GZMB and IFNG, but not of PRF1, in primary human NK cells with XBP1-KD using shRNA, compared with scramble-KD control NK cells (Fig. 2d). Inhibition of XBP1 mRNA splicing in primary human NK cells with 4μ8C, an inhibitor of IRE1α-mediated mRNA splicing, resulted in decreased expression of XBP1s protein and suppression of IL-15-induced GZMB protein and mRNA compared with cells not treated with 4μ8C (Supplementary Fig. 2f–h). The expression of XBP1s protein was positively correlated with the mRNA expression of GZMB and IFNG, all of which were induced by treatment with IL-15 at multiple time points in primary human NK cells (Fig. 2e). Thus, XBP1s positively regulates the expression of GZMB and interferon-γ in NK cells.

We next investigated the cellular localization of XBP1s. Immunoblot analysis of the cytoplasmic and nuclear protein fractions in primary human NK cells treated with IL-15 for 24 h indicated that XBP1s exists almost exclusively in the nucleus following induction by IL-15 (Fig. 3a), consistent with its role in regulating transcription. Next, we overexpressed T-BET and FLAG-XBP1u or FLAG-XBP1s in 293T cells. Co-immunoprecipitation followed...
by immunoblot using antibodies to FLAG or T-BET indicated that overexpressed FLAG-XBP1s interacted with T-BET (Fig. 3b,c and Supplementary Fig. 3a), a transcriptional regulator important for NK cell function. T-BET was previously assumed to associate with the GZMB promoter, although no specific binding sites have been identified15,16. Of note, in 293T cells FLAG-XBP1s did not interact with endogenous STAT5, an important transcriptional factor downstream of IL-15 signaling (Fig. 3c). Using confocal imaging with antibodies that identify both XBP1u and XBP1s, and a T-BET antibody, we observed the co-localization of T-BET and XBP1 in the nuclei of primary human NK cells (Fig. 3d) and in the human NK cell lymphoma cell line NK-92 (Supplementary Fig. 3b). Confocal microscopy indicated that T-BET has an almost exclusively nuclear localization in NK cells (Fig. 3d), which was validated by staining with an alternative antibody to T-BET in both primary NK cells and NK-92 cells (Supplementary Fig. 3c).

Next, we tested whether XBP1s interacted with its canonical binding motifs (G/C)ACGT15,16 located within the GZMB proximal promoter (Fig. 3e). Chromatin immunoprecipitation (ChIP) indicated that XBP1s and T-BET bound to the same proximal region of the GZMB promoter in primary human NK cells treated with IL-15, but there was little or no binding in untreated cells (Fig. 3f). In contrast, T-BET did not bind to the GZMB promoter in primary human NK cells treated with 4µgC (which do not express XBP1s; Supplementary Fig. 2g) in the presence of IL-15, compared with control cells treated with only IL-15 (Supplementary Fig. 3d). Using a luciferase assay to evaluate GZMB promoter activity, we observed that 293T cells transfected with XBP1s had much higher GZMB promoter activity than that of the cells transfected with EV (Fig. 3g). Of note, we also observed STAT5 binding to the GZMB promoter in primary human NK cells by ChIP assays (Fig. 3h), consistent with previous reports17. STAT5 positively regulates GZMB induction by XBP1s, we knocked down the expression of STAT5A or STAT5B in 293T cells compared with control 293T cells (175%) (Supplementary Fig. 4a). The induction of the GZMB promoter reporter by XBP1s overexpression was not inhibited by either STAT5A-KD (279%) or STAT5B-KD (184%) in 293T cells compared with control 293T cells (175%) (Supplementary Fig. 4b), indicating that induction of GZMB promoter activity by XBP1s and T-BET in 293T cells does not require STAT5. Together, our data suggest that XBP1s interacts with T-BET but not STAT5 and regulates the transcriptional activity of GZMB via promoter binding.

XBP1 encodes a survival molecule that protects cells from stress-induced death19,20, and IL-15 is a critical cytokine for NK cell survival21,22. Knockdown of XBP1 by transfection with XBP1-specific
siRNAs resulted in increased expression of cleaved caspase-3 in primary human NK cells, compared with cells transfected with scrambled siRNAs (Fig. 4a), indicating increased apoptosis in XBP1-KD NK cells. In contrast, primary human NK cells transduced with pCDH-XBP1u or pCDH-XBP1s showed decreased expression of cleaved caspase-3 compared with pCDH-EV control NK cells (Fig. 4b). Moreover, in IL-15-activated primary human NK cells in which XBP1 splicing was inhibited by treatment with 4μgC, cleaved caspase-3 levels were higher and survival was lower compared with similarly activated cells without 4μgC treatment (Fig. 4c–f). Taken together, these data indicate that XBP1s modulates IL-15-induced survival in human NK cells.

Next we investigated the molecular mechanism by which IL-15 regulated the expression of XBP1s protein. IL-15 stimulation of primary human NK cells did not increase the amount of XBP1s mRNA compared with unstimulated NK cells, as evaluated by XBP1 splicing assays and quantitative PCR (qPCR); however, XBP1s protein accumulated in the nucleus of IL-15-treated primary human NK cells within 2 h of stimulation (Fig. 5a,b). IL-15 stimulation also induced the phosphorylation of the serine-threonine kinase protein kinase B (PKB; also known as AKT) in NK cells mainly in the cytoplasm (Fig. 5b), as previously reported23. Blockade of AKT phosphorylation with the AKT inhibitor AKTi-1/2 (ref. 24) in IL-15-stimulated primary human NK cells resulted in decreased expression of XBP1s protein compared with cells treated with IL-15 in the absence of AKTi-1/2 (Fig. 5c and Supplementary Fig. 5a). Moreover, the expression of GZMB mRNA and GZMB protein was significantly downregulated in primary NK cells transduced with AKT1-specific shRNA compared with scrambled shRNA (Supplementary Fig. 5b,c). In addition, the expression of GZMB mRNA was significantly upregulated and GZMB protein was moderately upregulated in primary NK cells transduced with a constitutively active form of AKT (pCDH-myrAKTΔ4–129, encoding a 14-amino acid Src myristoylation signal peptide fused to the N terminus of AKTΔ4–129 (ref. 25)) compared with cells transduced with control pCDH-EV (Supplementary Fig. 5d,e). Treatment with AKTi-1/2 also reduced
the level of XBP1s protein in IL-15-stimulated NK cells in the presence of cycloheximide (CHX), which blocks de novo protein synthesis and thus prevents any confounding effects of increased protein translation (Fig. 5d and Supplementary Fig. 6a). These data indicate that AKT plays a role in increasing the IL-15-induced protein levels, but not mRNA expression, of XBP1s in primary human NK cells.

To further test whether AKT is required for maintaining the level of XBP1s protein, we co-transfected various concentrations of pECE-myrAKTΔ4–129 or pECE-EV with pCDH-FLAG-XBP1s or pCDH-EV into 293T cells. Immunoprecipitation experiments indicated that the level of FLAG-XBP1s protein was markedly increased in a dose-dependent manner by myrAKTΔ4–129 overexpression (Fig. 5e and Supplementary Fig. 6b), while XBP1s mRNA was not induced by overexpression of myrAKTΔ4–129 (Fig. 5f), consistent with the idea that IL-15-induced XBP1s upregulation is independent of XBP1s transcription. In addition, co-transduction of pCDH-XBP1s and pECE-myrAKTΔ4–129 enhanced the activity of the GZMB promoter in 293T cells compared with transfection of pCDH-XBP1s alone (Supplementary Fig. 6c). These data indicate that AKT activation is required for maintaining the level of XBP1s protein.

Over time, during 4h of incubation, a decrease was observed in FLAG-XBP1s protein in 293T cells transfected with pECE-EV but not in cells transfected with pECE-myrAKTΔ4–129 following CHX treatment (Fig. 5g and Supplementary Fig. 6d), indicating that signaling downstream of AKT protects XBP1s from degradation. Blockade of proteosomal degradation with the cell-permeable proteasome and the calpain inhibitor MG132 recovered FLAG-XBP1s protein in pECE-EV-transfected but not pECE-myrAKTΔ4–129-transfected 293T cells treated with CHX (Fig. 5g and Supplementary Fig. 6d), indicating that overexpression of myrAKTΔ4–129 enhanced the protein stability of XBP1s in 293T cells. Ubiquitination of FLAG-XBP1s was reduced following transfection of pECE-myrAKTΔ4–129 compared with pECE-EV in 293T cells (Fig. 5h), while ubiquitination of XBP1s was substantially increased in IL-15-stimulated primary human NK cells treated with AKT1-1/2 compared with untreated cells (Fig. 5i). Our data suggest that AKT controls XBP1s stability, possibly through a mechanism involving the ubiquitination of XBP1s.

Our studies describe a pathway that links IL-15 signaling with intracellular mechanisms that contribute to NK cell cytotoxicity and survival. We show that IL-15 stabilizes XBP1s protein through...
phosphorylation of AKT, allowing its downstream interaction with T-BET. T-BET activity correlates with GZMB expression. However, there are no direct T-BET binding sites on the GZMB promoter. On the basis of the presence of an XBP1s binding motif within the GZMB promoter, we showed that XBP1s mediates the interaction between T-BET and the GZMB promoter, as suggested by the ability of XBP1s to bind the promoter of GZMB and to interact with T-BET.

We showed that AKT is involved in the regulation of XBP1s downstream of IL-15. Phosphatidylinositol-3-kinase (PI3K)–AKT signaling is stimulated by IL-2 and IL-15, with AKT phosphorylation being more sensitive to IL-15 than to IL-2, in agreement with our data that IL-15 was the most potent cytokine (among IL-2, IL-12 and IL-15) in increasing XBP1s protein expression and function. AKT is known to regulate both protein ubiquitination and protein deubiquitination, with distinct mechanisms for each process. Ubiquitination and degradation of PTEN, a natural inhibitor of the PI3K–AKT pathway, in tumor cells requires stabilization of the E3 ligase MKRN1 via AKT-mediated phosphorylation. However, AKT also induces protein stabilization by activating USP-14, a ubiquitin-specific protease that handles the turnover of short-lived proteins via deubiquitination. We showed here that AKT signaling caused the deubiquitination and promoted the accumulation of XBP1s. Consistent with our data, deficiency in either PI3K or subunits p110γ or p110δ, which regulate AKT activity, has been reported to disrupt NK cell maturation, development

**Fig. 5 | AKT mediates stability of XBP1s.** a. XBP1 splicing assays by PCR (top) and quantification of XBP1s by qPCR in NK cells (bottom). Bar graphs display mean±s.e.m. of n=3 donors. b. Immunoblotting of cytoplasmic and nuclear protein fractions of NK cells treated with IL-15 (100 units ml⁻¹) for the indicated time period. c. NK cells were pretreated with or without AKTi-1/2 (10 µM) for 30 min, followed by stimulation with IL-15 (100 units ml⁻¹) for 6 h prior to immunoblotting. n=3 donors. d. NK cells were pretreated with IL-15 (100 units ml⁻¹) alone or plus AKTi-1/2 for 1 h, then cultured with or without CHX (10 µg ml⁻¹) for 30 min prior to immunoblotting. Data are summarized in Supplementary Fig. 6a. n=3 donors. e. 293T cells were transfected with the indicated amount of pECE-FLAG-tagged XBP1s vector or respective control vector (dash) and treated with or without CHX (10 µg ml⁻¹) for 15 min prior to immunoblotting. Data are summarized in Supplementary Fig. 6b. n=3 independent experiments. f. XBP1s mRNA expression was analyzed by qPCR in 293T cells co-transfected with pECE-FLAG-XBP1s for 24 h (endogenous XBP1s is very low). Bar graphs display mean±s.d. of n=3 independent experiments. NS, not significant by Student’s two-tailed paired t-test. g. 293T cells were co-transfected with pCDH-FLAG-XBP1s and pECE-myrAKT4–129 or pECE-EV for 4 h and then treated with CHX (10 µg ml⁻¹) for the indicated time periods with or without MG132 (10 µM). n=3 independent experiments. h. myrAKT4–129 or control (dash) was co-transfected with pECE-FLAG-XBP1s into 293T cells for 24 h with or without MG132 (10 µM) for 4 h. Protein ubiquitination (Ub) of XBP1s was analyzed by immunoprecipitation with anti-FLAG, followed by immunoblotting with densitometric quantification. i. NK cells were pretreated with or without AKTi-1/2 (10 µM) for 1 h followed by MG132 (10 µM) treatment for 3 h in the presence of IL-15 (100 units ml⁻¹). Ubiquitination of XBP1s was analyzed by immunoprecipitation with anti-ubiquitin combined with immunoblotting with anti-XBP1s. The experiment was repeated independently three times (b, h, i) with similar results. Gel (a) and blot images (b–i) were cropped, and the full scans are shown in the Supplementary Dataset.
and cytotoxicity\(^1\). Moreover, inhibition of PI(3)K suppresses GZMB expression in NK cells and decreases NK cell cytotoxicity against tumor cells\(^2\). These observations are consistent with our findings that IL-15 stimulation of NK cells caused an increase of AKT phosphorylation that correlated with decreased degradation of XBP1\(s\), which subsequently enhanced NK cell degranulation following co-culture with tumor cells. Moreover, the active form of AKT, myrAKT\(\Delta\) contributes to the survival of XBP1\(s\)-induced transcription of GZMB. Notably, myrAKT\(\Delta\) alone slightly induced GZMB transcription in 293T cells, but a synergistic effect on GZMB transcription was observed following co-transfection with XBP1\(s\).

IL-15 promotes NK cell survival\(^3\). However, the molecular basis of this mechanism remains poorly understood. Our current study supports a model in which XBP1\(s\) is located downstream of PI(3)-Akt, and AKT contributes to the protein stability of XBP1\(s\) in NK cells following treatment with IL-15, eventually controlling NK cell survival. Consistent with our data, XBP1\(s\) is known to rescue cells from pro-apoptotic processes induced by endoplasmic reticulum stress, oxidative stress or hypoxia\(^4\). The anti-apoptotic protein Bcl-2 is highly expressed in resting NK cells\(^5\) and contributes to NK cell survival in the resting state\(^6\). However, Bcl-2 appears to be redundant for survival of activated or proliferating NK cells\(^7\). Here we found that XBP1\(s\) was highly expressed in IL-15-activated NK cells, but its expression was relatively low in resting NK cells. Moreover, inhibition of XBP1\(s\) abolished the survival of IL-15-activated NK cells but had no effect on the survival of resting NK cells. Thus, we speculate that Bcl-2 is responsible for the survival of resting NK cells, while XBP1\(s\) is responsible for the survival of IL-15-activated NK cells. In conclusion, we showed that XBP1\(s\) acts as an essential transcriptional factor downstream of IL-15 and AKT signaling in controlling two important aspects of NK cell biology: effector functions and survival. The IL-15–AKT–XBP1\(s\) axis may offer a potential target to improve the therapeutic efficacy of ex vivo–expanded NK cells and/or chimeric-antigen-receptor-modified NK cells\(^8\) for the treatment of various cancers.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41590-018-0265-1.

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Author contributions
Y.W. performed experiments, designed research and wrote the manuscript; Y.Z., P.Y., W.D., Z.Z. and L.C. performed experiments; A.P.N. revised and proofread the manuscript; J.Z. performed statistical analyses; D.M.B., B.L.M-B., A.G.F. and M.A.C. designed research, reviewed the manuscript and/or acquired funding; J.Y. conceptualized the idea, designed research, wrote the manuscript, acquired funding and supervised the study.

Competing interests
The authors declare no competing interests.

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Methods

Isolation of primary human NK cells. Leukocyte-enriched peripheral blood samples of human healthy donors were obtained from the American Red Cross. Primary NK cells were isolated using the MACsPrep NK Cell Isolation Kit (Miltenyi Biotec) and Erythrocyte Depletion Kit (Miltenyi Biotec). Enriched NK cells were approximately 99% pure, which was confirmed by flow cytometry using antibodies to CD3 and CD56 (BD Biosciences). Primary human NK cells were used for the experiments in this study unless otherwise indicated that the NK-92 cell line was used for specific experiments. The protocols for human specimen collection were approved by the institutional review boards of The Ohio State University.

Cell culture. The K562 and NK-92 cell lines were purchased from the American Type Culture Collection (ATCC). The U937, MM.1S and MOML13 cell lines were obtained from the laboratory of M.A.C. These cell lines and primary NK cells were cultured in RPMI 1640 medium containing t-glutamine (Sigma-Aldrich) and supplemented with 10% or 20% heat-inactivated FBS (Sigma-Aldrich). Enriched NK cells were cultured in RPMI 1640 medium containing t-glutamine (Sigma-Aldrich) and supplemented with 10% or 20% heat-inactivated FBS (Sigma-Aldrich). The 293T cell line, which was purchased from ATCC, was cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

Antibodies and other reagents. Antibodies to β-actin, lamin B, XRBP1 and T-BET (immunoblotting, immunoprecipitation and immunofluorescence) were purchased from Santa Cruz Biotechnology. Antibodies to p-AKT (S473), AKT, α-tubulin, FLAG, p-STAT3, STAT3, GZMB, T-BET (ChIP and immunofluorescence), ubiquitin (for immunoblot), GAPDH and C-CASP3 were purchased from Cell Signaling Technology (CST). The monoclonal antibody to ubiquitin for immunoprecipitation was purchased from MilliporeSigma. Antibody to HA ubiquitin from Sigma-Aldrich. Antibody to XB1P1s for immunoblot was purchased from BioLegend. Antibodies to hCD56-FITC (557699), hCD56-V450 (560360), hCD107a-APC (560664), hCD19-PE-AF647-APC-H7 (561343), hXBP1s (563382) and hGZMB (560123) were used to observe surface and intracellular flow cytometry were purchased from BD Biosciences. Chemical inhibitors (4 µM CHX), hCD56-FITC (557699), hCD56-V450 (560360), hCD107a-APC (560664), hCD19-PE-AF647-APC-H7 (561343), hXBP1s (563382) and hGZMB (560123) were used in cell treatment experiments were purchased from Selleck Chemicals. CHX was purchased from Sigma-Aldrich. The CellTrace CFSE Cell Proliferation Kit and the Far Red Cell Proliferation Kit were purchased from Thermo Fisher Scientific. The scrambled and XRBP1-specific endonuclease-prepared siRNAs (esiRNAs) were purchased from Sigma-Aldrich. The scrambled (SHC007), XB1P1-target (shXB1P no. 1, TRCN000019804; shXB1P no. 2, TRCN000019808), STAT3-target (TRCN0000232134), STAT5B-target (TRCN0000232140) and AKT1-target (TRCN0000039797) esiRNAs cloned in the pLKO.1-puro vector were purchased from Sigma-Aldrich. The selection marker puromycin in the pLKO.1-puro vector was replaced by green fluorescent protein (GFP) for sorting (FACS) of transduced cells. IL-2 (catalog no. 200-02), IL-12 (200-12) and IL-15 (200-15) were purchased from PeproTech.

Transient transfection and lentivirus infection. The 293T cells were seeded and incubated at 37°C in 5% CO₂ environment until they were 60–80% confluent. The cells were transfected with plasmids using Lipofectamine 3000 Reagent (Invitrogen) and gene-specific primers. The following primer sequences were used: forward primer, GGGCTCAAACACATACCTGC; reverse primer, GZMB forward primer, CTCGCCTTCTGAGAAGAGGAGG; XRBP1 reverse primer, CCATGGCGGATTTCCGAGG. The following PCR conditions were used: 98°C for 30 s, followed by 40 cycles of 98°C for 15 s, 62°C for 30 s and 72°C for 60 s.

Immunoblotting. Cells were suspended in lysis buffer on ice for 1 h. Equal amounts of protein (~20 µg) were resolved by 5–20% SDS–polyacrylamide gels (Bio-Rad) and then transferred onto a PVDF membrane (Thermo Fisher Scientific). The membrane was incubated with a primary antibody at 4°C for 16 h and a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature (1:1000). The immunobLOTS were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Densitometric analysis was performed to quantify intensity of gel bands.

Immunoprecipitation. Cell lysates were prepared with NP40 lysis buffer. For 293T cells, cell lysates were prepared 48 h after plasmid transfection unless otherwise indicated. A beads-antibody complex was prepared using appropriate primary antibody and Pierce Protein G Agarose (Thermo Fisher Scientific), followed by immunoprecipitation according to the manufacturer’s protocol and as we have previously reported41. The precipitated proteins were detected by immunoblotting.

ChIP. ChIP assays were carried out with a Magna ChiP A/G Chromatin Immunoprecipitation Kit (EMD Millipore). Briefly, an equal amount (10 µg) of antibody against XRBP1s antibody (BioLegend), rabbit anti-STAT5 antibody (CST), rabbit anti-T-BET antibody (CST) or normal immunoglobulin G (IgG; Santa Cruz Biotechnology) was used to precipitate the cross-linked DNA–protein complexes from 10×10⁶ NK cells. After reversal of cross-linking, the precipitated chromatin of the GZMB promoter region was detected by PCR using the following primers: forward primer, GGCCTCGACACAGCATACCTGCG; reverse primer, TGACCACATCATCACCCAG.

Luciferase reporter assay. Luciferase reporter assays were carried out with a Dual-Luciferase Reporter Assay System (Promega), following our published protocol45 with modifications. After 48 h of transfection, cells on a 24-well plate were lysed in 100 µl of 1X Passive Lysis Buffer (Promega). Lysates (20 µl) were transferred to a 96-well plate, 100 µl 1X Glo luciferase assay substrate (Promega) was added to each well, and firefly luciferase was collected from the transfected pGL3 plasmid using the GloMax 96 Microplate Luminometer (Promega). Renilla luciferase from the co-transfected pRL-TK plasmid (as a normalized control) was collected after injection with 100 µl 1X Stop Substrate (Promega).

Flow cytometry. Cells were labeled with monoclonal antibodies at room temperature for 15 min and washed with PBS containing 2% BSA prior to analysis using an LSRII flow cytometer (BD Biosciences) to detect surface expression of each antigen. NK cells were gated as CD56+CD3- lymphocytes. For analysis by intracellular flow cytometry, cells were permeabilized and fixed using a Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience).

Statistical analysis. For continuous, normally distributed data, two-sample t-tests or paired t-tests were used to compare two independent or two paired groups. A linear mixed model was used to compare three or more groups with a variance-covariance structure due to repeated measures from the same donors. A two-way analysis of variance (ANOVA) model was applied to the synergistic effect test between two factors. P values were adjusted for multiple comparisons using Holmi procedure. A P value of 0.05 or less was considered statistically significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All summary or representative data generated and supporting the findings of this study are available within the paper. Raw data that support the findings of this study are available upon reasonable request.

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46. Yu, J. et al. Pro- and anti-inflammatory cytokine signaling: reciprocal antagonism regulates interferon-γ production by human natural killer cells. Immunity 24, 575–590 (2006).
Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ [ ] n/a
[ ] Confirmed

☐ [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ [ ] An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ [ ] The statistical test(s) used AND whether they are one- or two-sided

☐ [ ] Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ [ ] A description of all covariates tested

☐ [ ] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ [ ] A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

☐ [ ] Give P values as exact values whenever suitable.

☐ [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ [ ] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

☐ [ ] Clearly defined error bars

☐ [ ] State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

No customized software was used; BD FACSDiva version 6 was used to collect flow cytometric data; Applied Biosystems StepOnePlus real-time PCR system and associated software were used to collect real-time PCR data; Promega GloMax® 96 Luminometer and associated software were used to collect luciferase data; microscope data were collected using Zeiss ZEN and associated software (blue edition). Western blot data were collected by FlourChem E system. Additional information about software was described in the manuscript or available upon request.

Data analysis

No custom-made software was used for data analysis. Statistical analyses were performed using R3.4.0, SAS 9.3, Microsoft Excel 2016 or Graphpad Prism 7. The microscope images were analyzed using Zeiss ZEN software (blue edition). Flow cytometry data were analyzed using Flowjo 7.6 or 10.0. Immunoblot data analysis was performed using AlphaIView software (version 3.2.2.0).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All summary or representative data generated and supporting the findings of this study are available within the paper. Raw data that support the findings of this study are available upon request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Most of our experiments are in vitro experiments using donor primary cells. Sample sizes were chosen on the basis of our previous studies or publications, as well as the availability of donors. As a result, assuming a CV of 30%, with 3-9 donors, we were able to detect a group difference of 2-2.5 fold with at least 80% power and type I error controlled at 0.05, after adjusting for multiple comparisons. For simplicity, paired t test or Student’s t test was used for the power analysis.

Data exclusions
No data were excluded for all figures.

Replication
All experiments were reliably reproduced and results are represented as mean +/- SEM or +/- SD as appropriate, which is indicated in figure legends. Student’s t-tests or paired t-tests were used to compare two independent or two paired groups, respectively. Linear mixed model was used to compare three or more groups with repeated measures from the same donors. A two-way ANOVA model was used to test synergistic effects. P values were adjusted for multiple comparisons using Holm’s procedure. A P value of 0.05 or less was considered statistically significant, which is described in the methods section of the main text.

Randomization
Leukopaks used to isolate NK cells were de-identified and randomly picked up. For the experiments with a small sample size, randomization was not used.

Blinding
Considering appropriate handling and data acquisition, investigators were not blinded to the studies. Moreover, because samples were treated equally and data collection and/or analysis were mainly performed by computer-based methods (such as flow cytometric analysis), we believe the blinding was not necessary to our study.

Reporting for specific materials, systems and methods

Materials & experimental systems

Methods

n/a Involved in the study

Antibodies

Applications: Immunoblotting (IF, 1:1000 dilution); Immunofluorescence (1:200 dilution): https://datasheets.scbt.com/sc-1616.pdf
Validation

No customized antibodies were used. Validation data about the antibodies purchased from commercial vendors are available on the manufacturers’ website (see antibodies used for the link of each antibody) and/or datasheets sent to us. We used primary human cells or cell lines that are known to be positive for a specific antigen or target protein for validation of antibodies that were used for flow cytometry and immunoblotting.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

The K562 and NK-92 cell lines were purchased from ATCC. The U937, MM.1S, and MOLM-13 cell lines were obtained from the Caligiuri laboratory.

Authentication

Cell lines were not independently authenticated, beyond the identity provided from the supplier (e.g., ATCC).
Mycoplasma contamination

Stocks of all cell lines were tested for mycoplasma contamination prior to use in this study. All were negative.

Commonly misidentified lines

No cell lines used in this study are in the database of commonly misidentified cell lines.

Human research participants

Policy information about studies involving human research participants

Population characteristics

We purchased leukopaks from the American Red Cross or obtained samples from OSU Comprehensive Leukemia Tissue Banks. All samples are de-identified and no populations were excluded.

Recruitment

Recruitment is not relevant to this study. There are no clinical trials involved, and the majority of samples were purchased from the American Red Cross.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).

☑ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells were labeled with monoclonal antibodies at room temperature for 15 minutes and washed with PBS containing 2% BSA prior to analysis using an LSR II flow cytometer (BD Biosciences) to detect surface expression of each antigen. The NK cells were gated as CD56-FITC(+)CD3-APC-H7(-) lymphocytes. For CD107a assays, NK cells were co-cultured with tumor cells for 4 h in the presence of GolgiStop (BD), followed by staining with an anti-CD107a-APC or anti-CD107a-APC-H7 antibody. For the intracellular flow cytometric analysis, cells were permeabilized and fixed using a Foxp3/Transcription Factor Fixation/Permeabilization kit (eBioscience), followed by staining with anti-XBP1s-V450 or anti-GZMB-APC antibodies. Data were analyzed using FlowJo v10.

Instrument

Samples were analyzed on an LSR II (BD) or FACS Aria II unit (BD)

Software

Data were collected using FACSDiva (BD) and analyzed using FACSDiva (BD) or FlowJo v7.6 or v10. Graphing and statistical analysis were performed using Prism 7 (GraphPad).

Cell population abundance

When applicable, cell populations were sorted to >95% purity in all experiments, as determined by flow cytometry.

Gating strategy

Unless otherwise indicated, positive and negative gates were set using fluorophore-matched IgG controls. NK cells including those co-cultured with tumor cells were gated on CD3 negative and CD56 positive.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.