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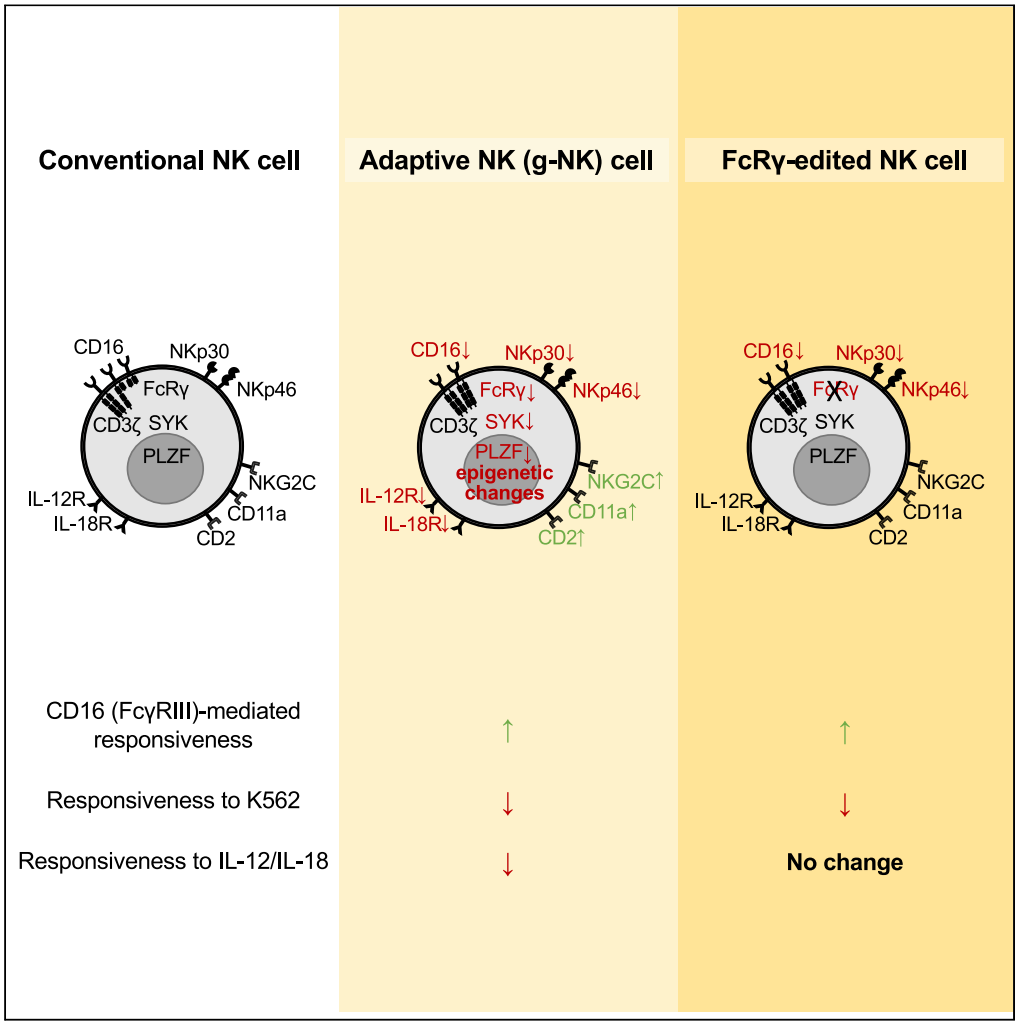

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HIGHLIGHTS

FcRγ deletion leads to increased cytokine production in response to CD16 stimulation

FcRγ deletion abolishes cell surface expression of NKp46 and NKp30

FcRγ deletion results in reduced responsiveness to K562 and Raji cells

PLZF deletion does not change responsiveness to CD16 and cytokine stimulation

Liu et al., iScience *23*, 101709
November 20, 2020 © 2020
https://doi.org/10.1016/
j.isci.2020.101709

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FcRγ Gene Editing Reprograms Conventional NK Cells to Display Key Features of Adaptive Human NK Cells

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SUMMARY

Adaptive human natural killer (NK) cells display significantly enhanced responsiveness to a broad-range of antibody-bound targets through the engagement of CD16 compared to conventional NK cells, yet direct reactivity against tumor targets is generally reduced. Adaptive NK cells also display a distinct phenotype and differential expression of numerous genes, including reduced expression of signaling adapter FcRγ and transcription factor PLZF. However, it is unclear whether differential expression of specific genes is responsible for the characteristics of adaptive NK cells. Using CRISPR-Cas9, we show deletion of FcRγ in conventional NK cells led to enhanced CD16 responsiveness, abolished cell surface expression of natural cytotoxicity receptors, NKp46 and NKp30, and dramatically reduced responsiveness to K562 and Raji tumor cells. However, deletion of PLZF had no notable effects. These results suggest multiple roles for FcRγ and identify its deficiency as an important factor responsible for the functional and phenotypic characteristics exhibited by adaptive NK cells.

INTRODUCTION

Natural killer (NK) cells constitute the third largest population of lymphocytes and play important roles in host defense against malignancy and viral infection (Vivier et al., 2011). Upon recognition of tumors or virus-infected target cells via activation receptors, NK cells can rapidly release cytotoxic granules and produce inflammatory cytokines. Unlike B and T lymphocytes that express gene-rearranged antigen-specific receptors, NK cells only express germline-encoded receptors and are considered part of the innate immune system. However, many recent studies have revealed adaptive immune features of NK cells in humans as well as in animal models (Cooper et al., 2009; Guma et al., 2004; Hammer and Romagnani, 2017; O'Leary et al., 2006; Paust et al., 2010; Reeves et al., 2015; Rolle and Brodin, 2016; Sun et al., 2009; Zhang et al., 2013).

In humans, two distinct but largely overlapping NK cell subsets have been identified that exhibit adaptive immune features, such as clonal-like expansion and long-term persistence (Beziat et al., 2013; Cichocki et al., 2018; Foley et al., 2012; Hammer et al., 2018; Lee et al., 2015; Muccio et al., 2018; Schlums et al., 2015; Zhang et al., 2013). These subsets were initially identified by either a deficiency in expression of the signaling adapter protein FcRγ or a high-level expression of the activation receptor NKG2C (Guma et al., 2004; Hwang et al., 2012). Epidemiological analyses indicate that the presence of these FcRγ-deficient NK cells (termed g-NK cells), as well as NKG2C^{+} NK cells that often co-express maturation marker CD57, is associated with previous infection by human cytomegalovirus (HCMV), a common herpesvirus that infects the majority of the human population (Guma et al., 2004; Zhang et al., 2013). Importantly, these adaptive NK cells display heightened potential for broad antiviral and antibacterial responses through enhanced function of CD16 (Costa-Garcia et al., 2015; Goodier et al., 2018; Hart et al., 2019; Lee et al., 2015; Schlums et al., 2015; Wu et al., 2013; Zhang et al., 2013; Zhou et al., 2015), a Fc receptor that can bind to the Fc portion of multiple subclasses of IgG (Bruhns et al., 2009). For instance, it has been shown that, compared to conventional NK cells, adaptive NK cells produce greater amounts of pro-inflammatory cytokines in response to cells infected with viruses (e.g., HCMV and HSV-1), as well as to the virus itself, in the presence of virus-specific antibodies. In contrast, adaptive NK cells generally show poor responsiveness to tumor targets, unless target-specific antibodies are provided (Beziat et al., 2012; Hwang et al., 2012; Liu et al., 2016). Moreover, adaptive NK cells show defective expression of natural cytotoxicity receptors (NCRs), NKp46 and NKp30, both of which are commonly expressed by conventional NK cells (Guma

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https://doi.org/10.1016/j.isci.2020.101709

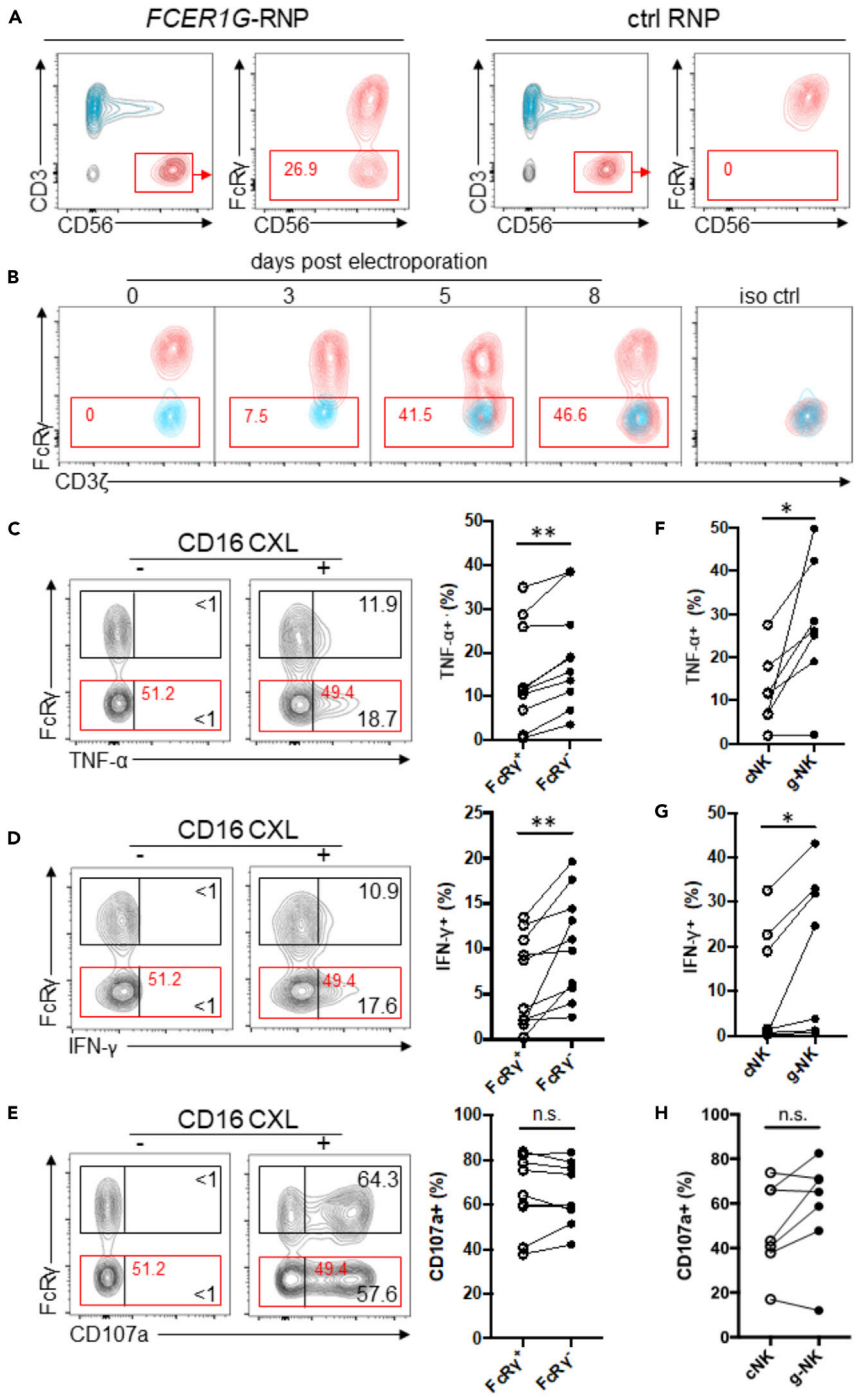

Figure 1. Deletion of FcRγ in NK Cells Leads to Enhanced Cytokine Production in Response to CD16 Cross-linking

(A) Contour plots show a mixture of NK ($CD3^-CD56^+$), T ($CD3^+CD56^-$ and $CD3^+CD56^+$), and non-NK/non-T ($CD3^-CD56^-$) cells after expansion of PBMC samples enriched for NK cells. NK cells (gated, red) were further analyzed for FcRγ expression following nucleofection with *FCER1G*-targeting Cas9/gRNA ribonucleoprotein (*FCER1G*-RNP) or non-targeting Cas9/gRNA ribonucleoprotein (ctrl RNP) after 10 days of *in vitro* culture. Data are representative of 5 independent experiments performed on NK cells from 10 donors.

(B) Time course analysis of FcRγ depletion in FCERIG-RNP-treated NK cells (red), with $CD3^+CD56^-$ T cells (blue) overlaid for comparison. The staining pattern obtained using a matched isotype control antibody (iso ctrl) is shown.

(C–E) Contour plots show flow cytometric analysis of (C) TNF-α, (D) IFN-γ production, and (E) cell surface CD107a expression by $FcR\gamma^+$ and $FcR\gamma^-$ NK subsets from a representative donor without (–) or with (+) CD16 cross-linking (CXL).

Figure 1. *Continued*

Numbers represent the relative percentages of FcRγ$^+$ or FcRγ$^-$ subset that produced indicated cytokine or displayed CD107a. Line graphs show the relative percentages of FcRγ$^+$ or FcRγ$^-$ subset that produced indicated cytokine or displayed CD107a from several donors in response to CD16 CXL (n = 10). Circles connected by a line designate data obtained from the same donor sample.

(F–H) Line graphs show the relative percentages of conventional NK (cNK) cells and g-NK cells that produced (F) TNF-α, (G) IFN-γ or (H) displayed CD107a following CD16 CXL (n = 7). Wilcoxon signed-rank test; *p < 0.05, **p < 0.01, n.s., not significant.

et al., 2004; Hwang et al., 2012; Moretta et al., 2001). However, the molecular basis responsible for the altered functional and phenotypic characteristics of adaptive NK cells is unclear.

Recent gene expression profiling and epigenetic analyses have revealed differences in the expression of approximately 400 genes and widespread epigenetic changes between conventional and adaptive NK cells (Lee et al., 2015; Schlums et al., 2015). Flow cytometric analyses have confirmed altered expression of many genes at the protein level, including FcRγ and PLZF. FcRγ is an immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling adapter protein and is known to have physical association with CD16, along with CD3ζ, as homodimers or heterodimers. While CD3ζ contains 3 ITAMs, FcRγ contains only 1 ITAM. FcRγ and numerous other intracellular proteins are also deficient in adaptive g-NK cells, including transcription factors (e.g., PLZF and HELIOS) and signal transduction proteins (e.g., SYK and EAT-2). Through correlation studies, it has been suggested that deficiencies in SYK may contribute to some of the altered characteristics of adaptive NK cells (Lee et al., 2015). It was reported that epigenetic remodeling of the IFNG locus (Luetke-Eversloh et al., 2014), as well as elevated expression of the transcription regulator, ARID5B, correlates with enhanced IFN-γ production by NKG2C$^+$ NK cells (Cichocki et al., 2018). Moreover, because of PLZF's potential involvement in chromatin structure modification and regulation of several genes differentially expressed in adaptive NK and conventional NK cells (Gleimer et al., 2012; Mathew et al., 2012), PLZF deficiency has been suggested to be an important factor contributing to the observed adaptive properties (Schlums et al., 2015). However, given the numerous epigenetic and gene expression differences in adaptive NK cells, it has been challenging to define the molecular mechanism(s) responsible for the enhanced CD16 responsiveness and reduced tumor responsiveness of adaptive NK cells.

In this study, using CRISPR/Cas9-mediated gene editing, we sought to determine the roles of FcRγ and PLZF deficiencies in the functional response and phenotypic characteristics of primary human NK cells. Our data show that deletion of FcRγ resulted in enhanced CD16 responsiveness but reduced NCR expression and tumor responsiveness. However, deletion of PLZF had no notable effects. These data provide evidence that deficiency of FcRγ is an important factor responsible for the altered functional and phenotypic characteristics exhibited by adaptive NK cells.

RESULTS AND DISCUSSION

CRISPR/Cas9-mediated Deletion of FcRγ in Human NK Cells

Although NK cells express a limited repertoire of germline-encoded activation receptors, they are able to recognize and respond to a broad-range of targets in the presence of target-specific antibodies through the expression of CD16 (Bruhns et al., 2009; Costa-Garcia et al., 2015; Hwang et al., 2012; Lanier, 2008; Zhang et al., 2013). To determine the role of FcRγ in the function of CD16, we initially sought to silence FcRγ expression in human NK cells using a siRNA-mediated knockdown approach. However, this strategy resulted in only transient and partial (approximately 50%) reduction of FcRγ protein level with no notable change in functional responsiveness of NK cells (data not shown). As an alternative approach to achieve more stable and more complete depletion of FcRγ, we explored CRISPR/Cas9-mediated gene editing. To obtain sufficient quantities of NK cells for gene editing and subsequent functional analysis, NK cells were enriched from peripheral blood mononuclear cell (PBMC) samples specifically chosen for their lack of detectable FcRγ-deficient (hereafter termed g-NK) cells, and these enriched samples were expanded *in vitro* for 10–14 days. Expanded cells were subjected to nucleofection of *FCER1G* (encoding FcRγ)-targeting Cas9/gRNA ribonucleoprotein (*FCER1G*-RNP). After subsequent culturing, flow cytometric analysis revealed the appearance of a distinct subset of CD3$^-$CD56$^+$ NK cells deficient for FcRγ expression (Figure 1A). This effect was specific to *FCER1G*-RNP, as there was no change in FcRγ expression in cells treated with non-targeting control RNP (ctrl RNP). Moreover, the expression of CD3ζ, another CD16-associated signaling adapter protein that is homologous to FcRγ in function and sequence and encoded by a

gene located close to *FCER1G* on chromosome 1 (Eiseman and Bolen, 1992), was unaffected by *FCER1G*-RNP treatment (Figure 1B). Kinetic analysis showed a partial reduction of FcRγ expression detectable 3 days post-nucleofection, but approximately 1 week was needed to reveal a distinct population having complete depletion (Figure 1B). Since it is estimated that CRISPR/Cas9-mediated gene editing occurs within 12 hr post-nucleofection (Brinkman et al., 2018; Kim et al., 2014), our data suggest that pre-existing *FCER1G* transcripts and FcRγ proteins have relatively long lifespans. Therefore, subsequent analyses of *FCER1G* gene-edited cells were performed at least 10 days post-nucleofection. Under this condition, we were able to obtain on average approximately 60% efficacy in the production of FcRγ$^-$ NK cells.

Deletion of FcRγ Increases Cytokine Production in Response to CD16 Stimulation

To evaluate whether FcRγ deletion affects the functional responsiveness to CD16 stimulation, we examined cytokine production by the gene-edited FcRγ-null NK cells and NK cells expressing normal levels of FcRγ (hereafter termed FcRγ$^-$ subset and FcRγ$^+$ subset, respectively) following incubation with immobilized anti-CD16 mAb. Flow cytometric analysis showed that the FcRγ$^-$ subset produced significantly greater amounts of TNF-α compared to the corresponding FcRγ$^+$ subset in the same culture ($n = 10$, $p < 0.01$; Figure 1C). In addition to TNF-α, CD16 stimulation resulted in significantly more IFN-γ production by FcRγ$^-$ subset ($n = 10$, $p < 0.01$; Figure 1D). Of these donors, 7 were HCMV seronegative donor samples which also exhibited statistically significant increases in TNF-α and IFN-γ production ($p < 0.05$). However, there was no consistent difference in the degranulation response between FcRγ$^-$ and FcRγ$^+$ subsets, as measured by cell surface expression of CD107a (Figure 1E). These data demonstrate that FcRγ deletion in NK cells results in increased cytokine production but does not alter degranulation activity in response to CD16 stimulation, suggesting that FcRγ may function to modulate CD16-mediated cytokine production.

Given that CD16 is known to associate with either FcRγ-FcRγ or CD3ζ-CD3ζ homodimer or FcRγ-CD3ζ heterodimer in human NK cells (Lanier, 2008), exclusive association with CD3ζ-CD3ζ homodimer may yield biochemical signaling that is more effective for cytokine production. Thus, when both adapters are co-expressed, FcRγ may negatively regulate the CD16-CD3ζ signaling pathway by competing for association with CD16 and/or interaction with downstream signaling molecules. The lack of any detectable difference in CD107a expression between FcRγ$^-$ and FcRγ$^+$ subsets could be explained by a lower activation threshold for degranulation (Bryceson et al., 2009).

We have previously demonstrated that g-NK cells in PBMC produced greater quantities of cytokines compared to the corresponding conventional NK cells upon CD16 cross-linking, whereas degranulation responses between these groups of cells were similar (Hwang et al., 2012). Given that the current study tested *in vitro* expanded NK cells to examine the effects of FcRγ deletion, we also tested CD16 responsiveness of g-NK and conventional NK cells after expansion in the same culture conditions as the gene-edited cells. Consistent with observations of fresh NK cell populations, *in vitro* expanded g-NK cells produced significantly greater amounts of both TNF-α and IFN-γ compared to the corresponding conventional NK cells that had been expanded in the same culture conditions ($n = 7$, $p < 0.05$; Figures 1F and 1G), whereas no significant difference in degranulation was observed (Figure 1H). When these data were compared with gene-edited FcRγ$^-$ and FcRγ$^+$ subsets, a similar trend in cytokine production between g-NK and conventional NK cells was observed, although g-NK cells in certain donors appeared to produce dramatically higher levels of cytokine (Figures 1C, 1D, 1F, and 1G). In light of the genetic heterogeneity among donors, as well as differential expression of numerous genes between g-NK and conventional NK cells, determining the specific contribution of FcRγ loss to the enhanced CD16-mediated cytokine production of g-NK cells is challenging with currently available technology. However, given the notable positive impact of CRISPR/Cas9-mediated FcRγ deletion in the absence of other gene expression differences (Figures 1C and 1D), our data suggest that FcRγ-deficiency is an important factor responsible for the enhanced CD16-mediated cytokine production by g-NK cells.

Deletion of FcRγ Decreases Functional Responsiveness to Tumor Target Cells

In contrast to enhanced CD16 responsiveness, g-NK cells respond relatively poorly to the prototypical NK cell-sensitive leukemic cell line K562, as evidenced by a lower degranulation response and lower cytokine production compared to conventional NK cells (Hwang et al., 2012). Similarly, NKG2C$^+$ NK cells were also shown to respond poorly to this tumor target relative to conventional NKG2C$^-$ NK cells (Beziat et al., 2012; Liu et al., 2016). We evaluated the potential impact of FcRγ deletion on tumor responsiveness by examining FcRγ$^-$ and FcRγ$^+$ subsets for degranulation and cytokine production following incubation with K562 tumor cells. Compared to the FcRγ$^+$ subset, the FcRγ$^-$ subset showed dramatic reduction in both degranulation

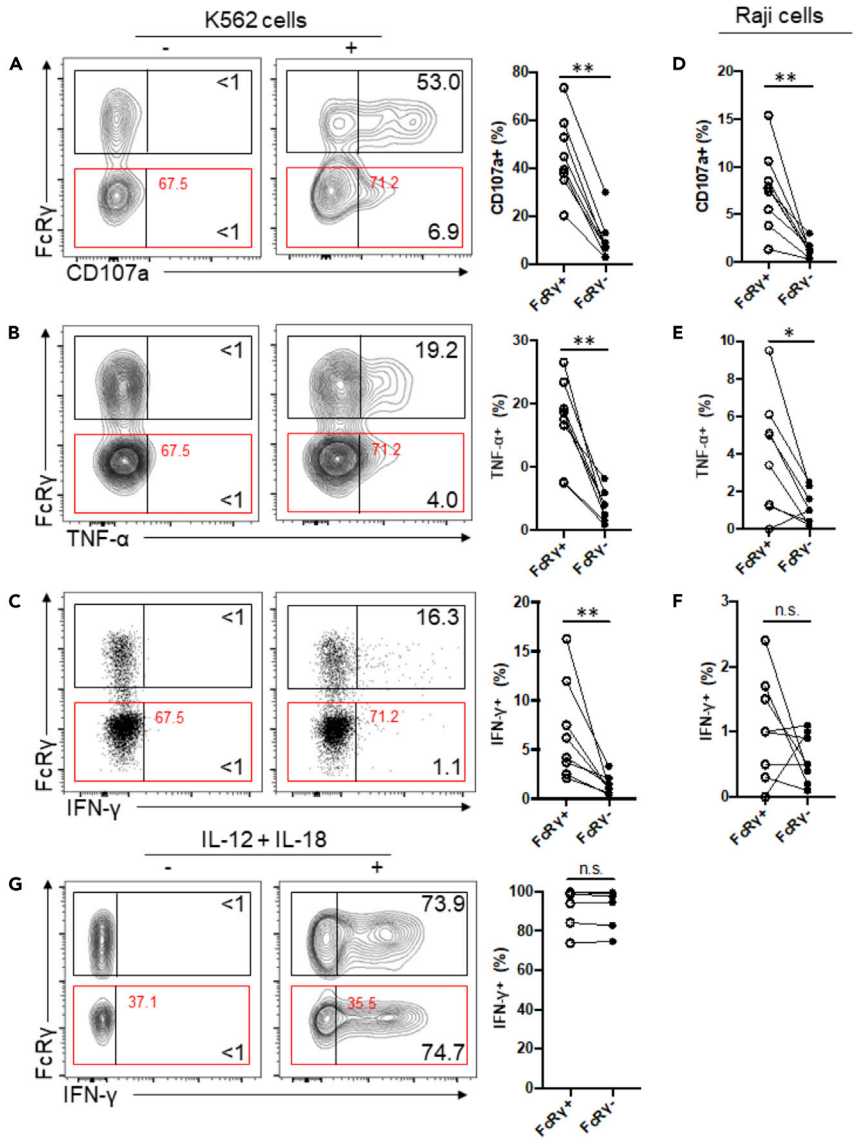

Figure 2. Deletion of FcRγ Yields Reduced NK Cell Responsiveness to K562 and Raji Tumor Cells

(A) Contour plots show flow cytometric analysis of CD107a expression by FcRγ$^+$ and FcRγ$^-$ NK subsets from a representative donor following incubation in the absence (–) or presence (+) of K562 tumor cells. Numbers represent the relative percentages of FcRγ$^+$ or FcRγ$^-$ subset that displayed CD107a. Line graph shows the relative percentages of FcRγ$^+$ or FcRγ$^-$ subsets that displayed CD107a from several donors in response to K562 stimulation (n = 8). Circles connected by a line designate data obtained from the same donor sample.

(B and C) Contour and dot plots show the percentages of FcRγ$^+$ and FcRγ$^-$ subsets that produced (B) TNF-α or (C) IFN-γ in response to K562 stimulation.

(D–F) Line graphs show the relative percentages of FcRγ$^+$ and FcRγ$^-$ subsets that displayed (D) CD107a or (E) produced TNF-α or (F) IFN-γ in response to Raji stimulation. (G) Contour plots and line graphs show the percentages of FcRγ$^+$ and FcRγ$^-$ subsets that produced IFN-γ in response to stimulation with IL-12 plus IL-18. Wilcoxon signed-rank test; *$p < 0.05$, **$p < 0.01$, n.s., not significant.

activity and cytokine production (Figures 2A–2C). Similar results were obtained following incubation with Raji tumor cells; FcRγ$^-$ subset showed a reduced degranulation response compared to the FcRγ$^+$ subset (Figure 2D), as well as a reduced cytokine response (Figures 2E and 2F). Taken together, these results indicate that FcRγ plays an important role in functional response of conventional NK cells to both K562 and Raji tumor cells. Thus, FcRγ deficiency is likely a major factor responsible for the reduced responsiveness of g-NK cells to tumor cells.

In addition to altered responsiveness to CD16 and tumor stimulation, g-NK cells display altered responsiveness to stimulation with IL-12 and IL-18, i.e., although conventional NK cells can produce copious amounts of IFN-γ following incubation with these cytokines, IFN-γ production by g-NK cells is dramatically reduced (Schlums et al., 2015). However, both FcRγ$^+$ and FcRγ$^-$ subsets showed comparable IFN-γ production with respect to percentage and median fluorescence intensity in response to stimulation with IL-12 and IL-18 by generating ample amounts of IFN-γ (n = 6; Figure 2G), indicating that FcRγ deletion does not affect IL-12 and IL-18 driven cytokine production. Thus, the impaired IL-12/18-mediated responsiveness of g-NK cells is likely controlled by a mechanism that is independent of FcRγ deficiency.

Role of FcRγ in the Expression of CD16, NKp46, and NKp30

It has been shown that co-expression of CD16 with either FcRγ or CD3ζ in heterologous expression systems can support cell surface expression of CD16 (Blazquez-Moreno et al., 2017; Lanier et al., 1991). To evaluate the specific contribution of FcRγ to cell surface expression of CD16 on primary NK cells, we compared CD16 expression on FcRγ$^-$ and FcRγ$^+$ subsets. Flow cytometric analysis showed that CD16 expression is lower on the FcRγ$^-$ subset compared to the FcRγ$^+$ subset for all donors tested (Figure 3A). Based on the median fluorescence intensities, CD16 expression was reduced on average by 53% in the FcRγ$^-$ subset (Figure S1A), indicating that normal CD3ζ expression in the FcRγ$^-$ subset (Figure 1B) cannot compensate for the absence of FcRγ and does not support normal levels of CD16 expression. This, coupled with the previous observation of lower CD16 expression on g-NK cells relative to conventional NK cells (Hwang et al., 2012), supports the conclusion that FcRγ deficiency is also responsible for reduced expression of CD16 on g-NK cells. Of note, NK cells in FcRγ knockout mice do not express any detectable cell surface CD16 (Takai et al., 1994), suggesting a differential role of FcRγ in humans and mice. Importantly, the enhanced CD16-mediated cytokine production of the FcRγ$^-$ subset (Figures 1C and 1D) was not due to higher CD16 expression. In the absence of FcRγ bearing 1 ITAM, the potential exclusive association between CD16 and homodimer of CD3ζ bearing 3 ITAMs may induce higher cytokine production. This possibility warrants further investigation.

As FcRγ is also known to be associated with NKp46 and NKp30 (Sivori et al., 2019), we examined cell surface expression of these activation receptors on FcRγ$^+$ and FcRγ$^-$ subsets. The FcRγ$^-$ subset showed a dramatic reduction in NKp46 expression (87% reduction on average), compared to FcRγ$^+$ subset (Figures 3B and S1B). Moreover, the FcRγ$^-$ subset also showed a dramatic reduction in NKp30 expression (92% reduction) (Figures 3C and S1C). These results indicate that FcRγ plays a crucial role in supporting cell surface expression of both NKp46 and NKp30. Given that both NKp46 and NKp30 were shown to associate with CD3ζ as well (Pende et al., 1999; Vitale et al., 1998), it is likely that CD3ζ alone in the FcRγ$^-$ subset cannot compensate for the absence of FcRγ and can only support a minimal level of expression of these receptors. Nonetheless, our results provide strong evidence that FcRγ deficiency is responsible for reduced expression of these receptors by g-NK cells, as well as by NKG2C$^+$ NK cells (Guma et al., 2004; Hwang et al., 2012; Schlums et al., 2015). Since NKp46 and NKp30 are considered major tumor recognition receptors, the abolished expression of these receptors likely resulted in the reduced responsiveness of FcRγ$^-$ subset to K562 and Raji tumor cells. Consistent with this possibility, both K562 and Raji cells were shown to express B7-H6, a ligand for NKp30 (Brandt et al., 2009). Taken together, our results suggest that FcRγ deficiency in g-NK cells is a major factor contributing to their reduced responsiveness toward tumor target cells through limiting the cell surface expression of NKp30 and NKp46.

In contrast to CD16, NKp46, and NKp30, we observed that the expression of other markers, including NKG2A, CD2, CD11a, NKG2C, CD57, ILT2, and Bcl-2, all of which are differentially expressed between g-NK and conventional NK cells (Hwang et al., 2012; Lee et al., 2015; Zhang et al., 2013), were unaffected by FcRγ deletion (Figure 3D), suggesting that altered expression of these molecules in g-NK cells is controlled by other mechanisms.

Deletion of PLZF Does Not Alter Responsiveness to CD16 or IL-12/18 Stimulation

In addition to FcRγ deficiency, altered expression of other genes may also contribute to the altered functional responsiveness exhibited by adaptive NK cells. Among such alterations, PLZF deficiency has been suggested to be an important factor contributing to the observed adaptive properties (Schlums et al., 2015). To explore the role of PLZF in NK cells, we sought to delete *ZBTB16* (encoding PLZF) from conventional NK cells by gene editing. NK cells enriched from PBMC samples that did not have detectable PLZF-deficient NK cells were expanded and then subjected to nucleofection of *ZBTB16*-targeting Cas9/gRNA ribonucleoprotein (*ZBTB16*-RNP). Flow cytometry analysis showed that a distinct NK cell subset deficient

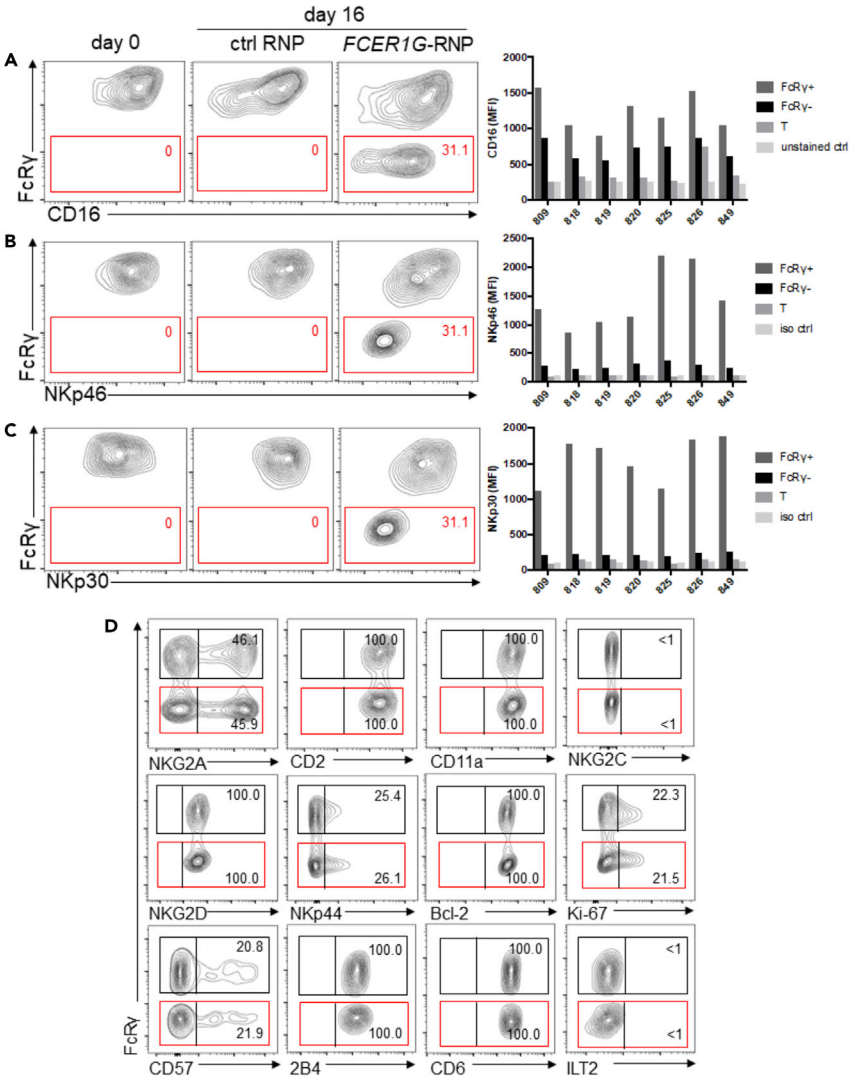

Figure 3. Loss of FcRγ Leads to Reduced Cell Surface Expression of CD16, NKp46, and NKp30

(A–C) Contour plots show (A) CD16, (B) NKp46, and (C) NKp30 expression with respect to FcRγ status in NK cells from one representative donor. Bar graphs summarize (A) CD16, (B) NKp46, and (C) NKp30 median fluorescence intensity (MFI) of FcRγ$^+$, FcRγ$^-$ NK subsets, T cells, and staining controls from 7 donors.

(D) Contour plots show expression of indicated markers with respect to FcRγ status in NK cells. Data are representative of at least three donors.

in expression of PLZF appeared approximately 1 week post-nucleofection (Figure 4A). The cytokine production assay demonstrated that following incubation with immobilized anti-CD16 mAb, PLZF$^-$ NK cells produced similar levels of IFN-γ and TNF-α as PLZF$^+$ NK cells (n = 7; Figures 4B and 4C). Degranulation responses were also comparable (Figure 4D). In addition, stimulation by IL-12 and IL-18 resulted in equivalently high levels of IFN-γ by both PLZF$^-$ and PLZF$^+$ NK cells (data not shown). We also observed no difference in the expression of components for IL-12 or IL-18 receptors (Figure S2). Finally, we observed that FcRγ, CD16, NKp46, and NKp30 were all normally expressed by PLZF$^-$ NK cells (data not shown). Taken together, unlike the deletion of FcRγ, the available data do not demonstrate any notable functional or phenotypic changes resulting from the deletion of PLZF.

Concluding Remarks

Collectively, our results demonstrate that CRISPR/Cas9-mediated deletion of FcRγ in human NK cells led to enhanced CD16 responsiveness but reduced tumor responsiveness in association with reduced expression

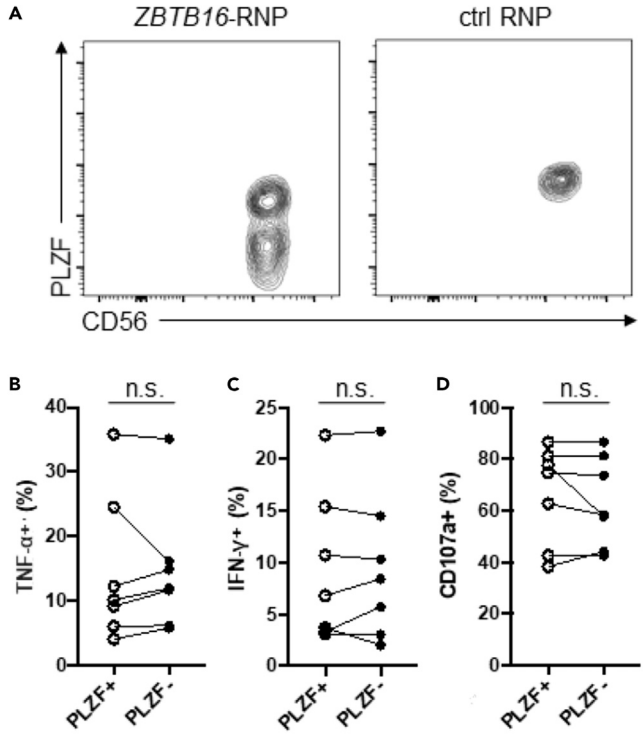

Figure 4. Deletion of PLZF in NK Cells Does Not Affect Responsiveness to CD16

(A) Contour plots show the expression of PLZF in NK cells following nucleofection with *ZBTB16*-targeting Cas9/gRNA ribonucleoprotein (*ZBTB16*-RNP). Data are representative of 4 independent experiments performed on NK cells from 7 donors.

(B–D) Line graphs show the relative percentages of $PLZF^+$ and $PLZF^-$ subsets that produced (B) TNF-α, (C) IFN-γ or (D) displayed CD107a from several donors in response to CD16 CXL (n = 7). Circles connected by a line designate data obtained from the same donor. n.s., not significant.

of NCRs. However, deletion of PLZF did not elicit notable changes. Importantly, our results also provide strong evidence that FcRγ deficiency is an important factor responsible for both enhanced CD16-mediated cytokine production and reduced tumor responsiveness exhibited by adaptive human NK cells. However, it should be noted that our data do not exclude the possibility that factors other than FcRγ deficiency may further contribute to the altered functional responsiveness of adaptive NK cells. Future studies utilizing the CRISPR-Cas9 gene editing approach will be helpful in identifying such factors, thereby deepening our understanding of the mechanism(s) contributing to adaptive NK cell functionality and illuminating potential development for clinical application.

Limitations of the Study

In the gene knock-out experiments, NK cells were cultured *in vitro* before gene editing to expand the number of available cells and then again after editing to completely deplete any pre-existing target proteins before analysis. It is likely that during *in vitro* culture, expression of many genes, including those involved in functional responses of NK cells, may be altered. Therefore, it is possible that in addition to the disrupted expression of the target genes being edited in this study, other genes with altered expression may have contributed to the phenotypic and functional characteristics exhibited by the gene-edited NK cells.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sungjin Kim (sjikim@ucdavis.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate datasets or code.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101709.

ACKNOWLEDGMENTS

We thank Jaewon Lee, Rachel Baek, and Tracey Rourke for technical assistance. W.L was partially supported by the China Scholarship Council. This work was supported by the NIH grant (AI110894) to S.K.

AUTHOR CONTRIBUTIONS

W.L. conceived the study, performed experiments, analyzed data, and wrote the manuscript; J.M.S. analyzed data and wrote the manuscript; E.L., H.C., and P.H.P performed experiments; S.K. conceived the study, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

S.K. is a consultant, stock owner, and advisory board member of Indapta Therapeutics, Inc. The other authors declare no competing interests.

Received: December 23, 2019
Revised: June 3, 2020
Accepted: October 16, 2020
Published: November 20, 2020

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Supplemental Information

FcRγ Gene Editing Reprograms Conventional NK Cells to Display Key Features of Adaptive Human NK Cells

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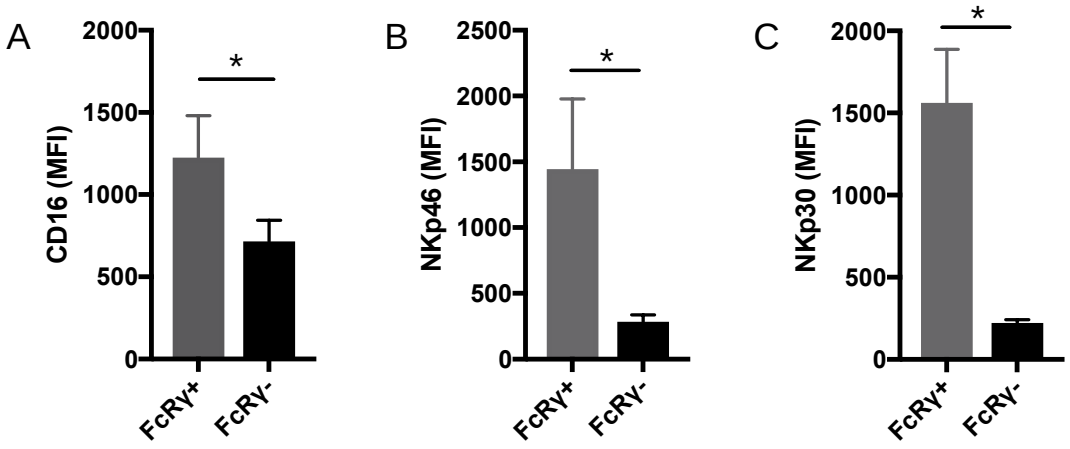

Figure S1. Loss of FcRγ leads to reduced cell-surface expression of CD16, NKp46 and NKp30, Related to Figure 3. Bar graphs show CD16 (A), NKp46 (B) and NKp30 (C) median fluorescence intensity (MFI) of FcRγ+ and FcRγ- NK subsets from 7 donors. *p<0.01.

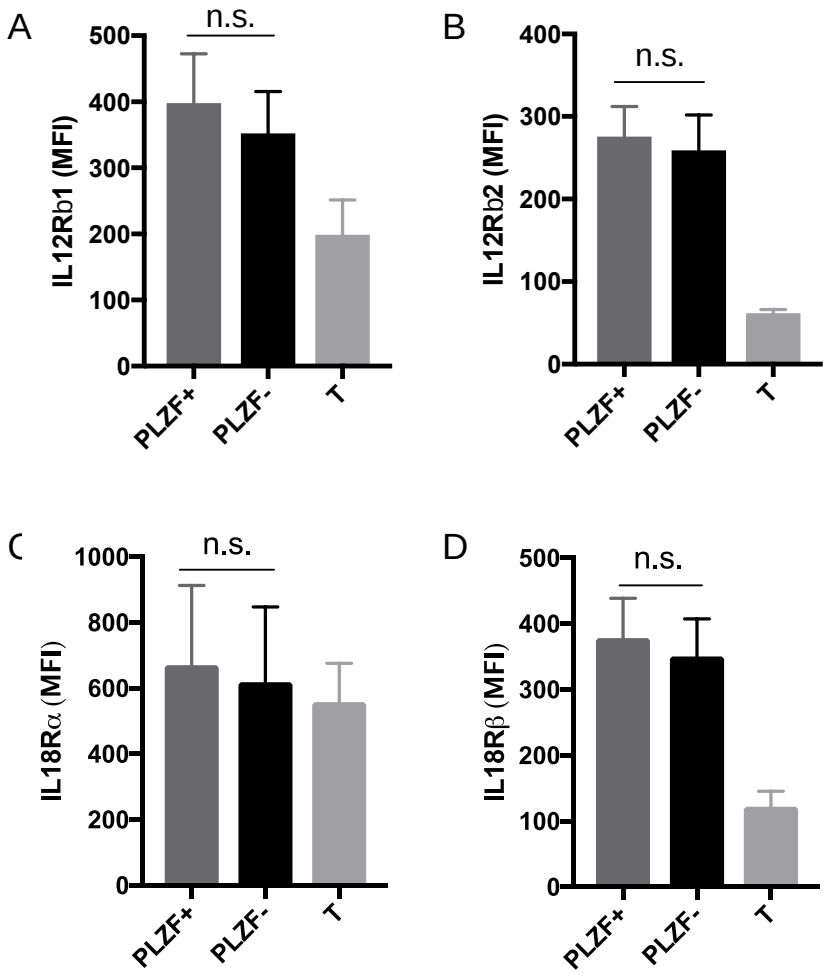

Figure S2. Loss of PLZF does not change the expression of receptors for IL-18 or IL-12, Related to Figure 4.
Bar graphs show median fluorescence intensity (MFI) of indicated markers on FcRγ+, FcRγ- NK subsets and T cells from 6 donors. n.s.: not significant.

STAR★Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Fc epsilon R1gamma - AF488	MBL International	Cat# M191-A48; RRID:AB_11160966
anti-CD247 (TCRζ, CD3ζ) - PE	BioLegend	Cat# 644106; RRID:AB_2565721
Anti-CD3 - AF700	BD Biosciences	Cat# 557943; RRID:AB_396952
Anti-CD14 - APC/Cyanine7	BioLegend	Cat# 301820; RRID:AB_493695
Anti-CD19 - APC/Cyanine7	BioLegend	Cat# 302218; RRID:AB_314248
Anti-CD56 - PE/Cyanine7	Beckman Coulter	Cat#A51078; RRID:N/A
Anti-CD107a - BV786	BD Biosciences	Cat# 563869; RRID:AB_2738458
Anti-IFN-gamma - APC	BioLegend	Cat# 502512; RRID:AB_315237
Anti-TNF-alpha - Pacific Blue	BioLegend	Cat# 502920; RRID:AB_528965
Ultra-LEAF Purified anti-human CD16 Antibody	BioLegend	Cat# 302050; RRID:AB_2561481
Anti-CD16 - Pacific Blue	BioLegend	Cat# 302032; RRID:AB_2104003
Anti-NKp30 - APC	BioLegend	Cat# 325210; RRID:AB_2149449
Anti-NKp46 - APC	BioLegend	Cat# 137608; RRID:AB_10612758
Mouse IgG1, κ Isotype ctrl Antibody - APC	BioLegend	Cat# 400121; RRID:AB_326443
Anti-NKG2C - PE	R and D Systems	Cat# FAB138P; RRID:AB_2132983
Anti-NKG2A - APC	Miltenyi Biotec	Cat# 130-114-089; RRID:AB_2726447
Anti-CD2 - APC	BioLegend	Cat# 300214; RRID:AB_10895925
Anti-CD11a - PE	BD Biosciences	Cat# 555380; RRID:AB_395781
Anti-CCR5 - APC	BD Biosciences	Cat# 560748; RRID:AB_1937308
Anti-PLZF - AF647	BD Biosciences	Cat# 563490; RRID:AB_2738238
Bacterial and Virus Strains		
Biological Samples		
Healthy donor whole blood	Gulf Coast Regional Blood Centern	N/A

Chemicals, Peptides, and Recombinant Proteins		
RPMI 1640 medium, with L-glutamine and sodium bicarbonate	Sigma Aldrich	Cat# R8758-24X500ML
L-Glutamine (200 mM)	Thermo Scientific	Cat# 25030081
Penicillin-Streptomycin (10,000U/ml)	Gibco	Cat# 15-140-122
Phytohemagglutinin-L (PHA-L)	Sigma Aldrich	Cat# 11249738001
Human AB serum	Corning	Cat# 35060CI
Recombinant Human IL-2	Peprotech	Cat# 200-02
Sodium pyruvate (100mM)	Gibco	Cat# 11-360-070
MEM Non-Essential Amino Acid Solution (100X)	Gibco	Cat# 11-140-050
Fetal Bovine Serum (FBS), qualified, heat inactivated	Fisher Scientific	Cat# 16140089
Recombinant Human IL-12 p70 (CHO derived)	Peprotech	Cat# 200-12
Recombinant Human IL-18	MBL International	Cat# B001-5
DPBS, without calcium chloride and magnesium chloride	Sigma Aldrich	Cat# D8537
Lymphoprep	STEMCELL technologies	Cat# 07851
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	Cat# D4540
Brefeldin A Solution (1000X)	BioLegend	Cat# 420601
Formaldehyde solution	Sigma Aldrich	Cat# F8775
Bovine Calf Serum (BCS)	Sigma Aldrich	Cat# 12133C
Sodium azide (NaN3)	Sigma Aldrich	Cat# 71289
Saponin	Sigma Aldrich	Cat# 47036-250G-F
Critical Commercial Assays		
EasySep Human NK Cell Isolation Kit	STEMCELL technologies	Cat# 17955
LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation	Invitrogen	Cat# L34973
human natural killer cell Nucleofector Kit	Lonza	Cat# VPA-1005
Deposited Data		
Experimental Models: Cell Lines		
RPMI 8866 Cell Line human	Sigma aldrich	Cat# 95041316-CDNA-20UL
K562 ATCC® CCL-243	ATCC	N/A
Raji Cell Line human	Sigma aldrich	Cat# 5011429-CDNA-20UL
Experimental Models: Organisms/Strains		
Oligonucleotides		

ON-TARGETplus Human FCER1G siRNA (SMARTPool)	Horizon	Cat# L-011856-00-0005
FCERIG KO sgRNA 1: 5'-UCUAUCCCCUCAGCGGCCCU-3'	Synthego	Cat# N/A
FCERIG KO sgRNA 2: 5'-GCAGAGCUGAGGCUCUCCCA-3'	Synthego	Cat# N/A
chemically modified non-targeting (negative) control sgRNA	Synthego	Cat# N/A
ZBTB16 KO sgRNA 1: 5'-CAGAACCCUAGCCACCCCAC-3'	Synthego	Cat# N/A
ZBTB16 KO sgRNA 2: 5'-AGAACCCUAGCCACCCCACG-3'	Synthego	Cat# N/A
Recombinant DNA		
Software and Algorithms		
Prism 7	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Other		

Experimental Model and Subject Details

Human Blood Samples

All studies were approved by the Institutional Review Board of the University of California, Davis. Leukopacks from healthy donors were obtained from Gulf Coast Regional Blood Center (Houston, Texas). PBMCs were isolated using SepMate tubes (STEMCELL technologies) and Lymphoprep density gradient media by centrifugation per manufacturer's instructions. Isolated PBMCs were cryopreserved in FBS with 10% DMSO.

Cell Lines

Cell lines were maintained at 37°C in 5% CO_2 humidified incubators. RPMI 8866, K562 and Raji cells were grown in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin.

Method Details

NK Cell Enrichment and Expansion

NK cells were enriched from cryopreserved PBMCs by immunomagnetic negative selection using EasySep Human NK Cell Isolation Kit (STEMCELL technologies). The resulting samples were co-cultured with feeder cells (a mixture of allogeneic PBMCs and RPMI 8866 cells) that had been irradiated. These cells were cultured in NK cell culture media (RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 5% human AB serum, 500 U/ml IL-2, sodium pyruvate, 1X MEM non-essential amino acids) in the presence of 10 ug/ml Phytohemagglutinin-L for 10-14 days before nucleofection.

Nucleofection of Cas9/gRNA Ribonucleoprotein

To prevent FcRγ or PLZF expression, two target-specific modified sgRNAs were used together to maximize chances for successful knockouts. The online “Knockout Guide Design” tool featured by Synthego (https://design.synthego.com/#/) was used to select sgRNAs with highest activity and least off-target rate. sgRNAs and Cas9 2NLS nuclease were pre-assembled at a 9:1 ratio in nucleofector solution provided within the human natural killer cell Nucleofector Kit. 0.5 nmol ribonuclease complex was nucleofected into two million expanded cells by Nucleofector 2b Device (Lonza) using program “X1”. Pulsed cells were cultured in NK cell culture media. Sequences of sgRNAs are listed in the Key Resources Table.

In Vitro Stimulation of Cells

Cells were resuspended in 10% FBS supplemented RPMI 1640 before any functional assay. Functional assays using immobilized anti-CD16 mAb were performed by stimulating cells in 96-well ELISA plate coated with Ultra-LEAF Purified anti-human CD16 mAb. A maximum of 0.2 million cells were seeded into each well and were stimulated for 6 hours with the addition of Brefeldin A and anti-CD107a mAb at the beginning of this assay. Functional assays using K562 and Raji cells were performed at an effector: target ratio of 5:1 for a 6-hour co-culture period. Brefeldin A and anti-CD107a mAb were added at the beginning of the co-culture. Functional assays using IL-12 and IL-18 were conducted by adding a mixture of 20 ng/ml IL-12 and 100 ng/ml IL-18 into cell culture 16 hours prior to the addition of Brefeldin A. Cells were harvested after another 6 hours.

Flow Cytometry

Cells were stained for flow cytometry using fluorochrome-conjugated mAbs, and $CD56^{+}CD3^{-}CD14^{-}CD19^{-}$ cells were gated as previously described (Hwang et al., 2012). For detection of intracellular FcRγ, CD3ζ, PLZF, IFN-γ and TNF-α, cells were fixed and

permeabilized, then stained with fluorochrome-conjugated mAbs. Unstained or isotype-matched control antibody stained cells were used to determine background staining in corresponding channels. Data were collected on an LSR Fortessa flow cytometer (BD Biosciences) and were analyzed using FlowJo Software, version 10.6.1. Antibodies are listed in the Key Resources Table.

Quantification and Statistical Analysis

All statistical analyses were performed with Graphpad Prism 7. The Wilcoxon matched pairs signed rank test was used for all assays. Differences were considered significant when $p<0.05$.