Identification of human NK cells that are deficient for signaling adaptor FcRγ and specialized for antibody-dependent immune functions

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Abstract

NK cells respond to tumor and virus-infected cells directly through several activation receptors, including natural cytotoxicity receptors, or indirectly through the activating Fc receptor CD16 for antibody-coated cells. Triggering of NK-cell effector functions through these receptors depends on physically associated transmembrane signaling adaptors, such as FcRγ (also known as FcεRIγ) and CD3ζ, both of which have been traditionally believed to be expressed by all mature NK cells. However, we have identified a distinct subset of human NK cells that are deficient for FcRγ expression but express normal levels of CD3ζ. FcRγ-deficient NK cells were readily detectable in about one-third of the healthy individuals examined. The deficiency was confined to the CD56dim population and was due to low FcRγ mRNA. FcRγ-deficient NK cells displayed dramatically reduced expression of the natural cytotoxicity receptors NKp46 and NKp30 but still expressed substantial levels of CD16. Compared to FcRγ-expressing NK cells, FcRγ-deficient NK cells showed poor direct reactivity toward tumor targets as measured by cytokine production and degranulation. Unexpectedly, however, FcRγ-deficient NK cells exhibited significantly more robust responsiveness upon stimulation through CD16, particularly for cytokine production, compared to FcRγ-expressing NK cells. Thus, our study reveals FcRγ-deficient NK cells as a novel subset of human NK cells that have remarkably potent responses toward antibody-coated targets. These findings also illustrate a differential contribution of FcRγ and CD3ζ for the expression and functional activity of their associated receptors.

Keywords: antibody, CD16, cytokine, FcRγ, NCR

Introduction

Upon recognition of tumor targets or virus-infected cells, NK cells can release cytotoxic granules and rapidly produce cytokines (1–6). Human NK cells utilize several activation receptors including the natural cytotoxicity receptors (NCRs) NKp46 and NKp30, which are considered to be the major receptors for recognition of tumor cells (7–9). Because of a lack of gene-rearrangement machinery, NK cells express only a handful of germline-encoded activation receptors, which limits the ability of NK cells to directly recognize a broad range of targets. However, the range of potential targets for NK cells is effectively expanded through expression of the activating Fc receptor CD16, which recognizes IgG bound to antigens expressed on a variety of targets, such as virus-infected cells.

CD16 and several other key activation receptors on NK cells require physically associated transmembrane signaling adapters for signal transduction (2, 3, 10). For instance, the signaling adapters FcRγ and CD3ζ, both of which can associate with CD16, NKp46 and NKp30 as either disulfide-linked homodimers or heterodimers (2, 8, 11, 12), transmit biochemical signals through immunoreceptor tyrosine-based activation motifs (ITAMs). Importantly, these adaptors can also play critical roles in the cell-surface expression of associated activation receptors, such as CD16 (2, 3, 10, 11, 13–16). For example, in vitro studies have shown that CD16 co-transfected with either FcRγ or CD3ζ, but not transfected alone, can lead to cell-surface CD16 expression (13–16). Using these heterologous systems, it has also
been shown that upon CD16 cross-linking, FcRγ and CD3ζ can transduce certain biochemical signals (17–19). In NK cells, signal transduction following CD16 cross-linking has been extensively studied by investigating phosphorylation of signaling molecules or Ca²⁺ influx (20–34). However, the relative contributions of FcRγ and CD3ζ to the expression or functional activity of associated receptors remains unclear.

It has been believed that all mature NK cells constitutively express FcRγ and CD3ζ (2). However, NK cells do not uniformly express the receptors that associate with FcRγ and CD3ζ. For example, it has been reported that some individuals have a subset of NK cells that expresses low levels of Nkp46 (7, 8, 12, 35–37), which is considered to be one of the most specific markers for NK cells across species (6, 8, 38). Interestingly, these cells show low expression of Nkp30 as well (12) and are therefore referred to as NCR dull (2). The low expression of NCRs is directly correlated with poor reactivity toward tumor targets, providing a molecular basis for the NCR dull phenotype and functionality remain unclear.

Individual NK cells can also differ in their ability to respond to stimulation through activation receptors. NK cells appear to acquire full capacity to respond to activating stimuli through a process termed licensing (also known as education), which requires the interaction between inhibitory receptors and their cognate MHC class I ligands (39–41). Consistent with this model, ‘licensed’ NK cells that express inhibitory receptors for self-MHC generally respond to activating stimuli more robustly than ‘unlicensed’ NK cells that lack such receptors (39, 41–44). However, whether licensing impacts NK cell responsiveness overall or impacts responses to only certain stimuli remains unclear, particularly in humans (44). In fact, controversial observations regarding the role of licensing in human NK cell responsiveness to CD16 stimulation have been reported (41, 44, 45). The reason for this discrepancy is currently unknown.

Through the analysis of human NK cell responsiveness to tumor target and CD16 stimulation, we identified a previously unknown population of NK cells that are deficient for the signaling adaptor FcRγ. These FcRγ-deficient NK cells were readily detected in peripheral blood samples of about one-third of healthy individuals. Phenotypic and functional analyses revealed that FcRγ-deficient NK cells express low levels of both NKP46 and NKP30 and exhibit poor reactivity toward tumor targets, providing a molecular basis for the NCR dull phenotype. In contrast, FcRγ-deficient NK cells respond more robustly than FcRγ-expressing NK cells when stimulated through CD16. Thus, our study also reveals FcRγ-deficient NK cells as an effector population that is specialized for antibody-dependent reactivity rather than the direct recognition of tumor cells.

**Methods**

**Human subjects and blood samples**

PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation of samples obtained from discarded, de-identified leukocyte reduction filters (American Red Cross), or blood donations from healthy volunteers with informed consent, approved by the Michigan State University Biomedical and Health Institutional Review Board.

**Phenotypic analysis of NK cells**

PBMCs were stained for flow cytometric analysis using fluorochrome-conjugated antibodies as previously described (44). Antibodies to detect the following proteins were from Beckman Coulter (CA, USA) [CD56 (N901), NKP44 (CD336; Z231)], BD Biosciences (CA, USA) [CD3 (UCHT1), CD16 (3G8), KIR2DL2/3 (CD158b; CH-L), KIR3DL1 (CD158e; DX9), NKP46 (CD335; 9E2), NKP30 (CD337; p30-15), NKG2D (CD314; 1D11), DNAM-1 (CD226; DX11), CD25 (M-A251), IFN-γ (B27), TNF-α (Mab11), CD107a (H4A3), Granzyme A (CB9), Granzyme B (GB11), Biologend (CA, USA) [CD14 (HC14), CD19 (HB19), 2B4 (CD244; C1.7), NTB-A (CD352; NT-7), CRACC (CD319; 162.1), CD69 (FN50)], and eBiosciences (CA, USA) [KIR2DL1 (CD158a; HP-MA4), Perforin (dG9)]. For detection of signaling adaptors, cells were fixed and permeabilized, then stained with FcεRI subunit (FcεRI) antibody (Millipore, CA, USA) that was conjugated with either Alexa-488 or Pacific blue, or mAbs against CD3ζ (6B10.2, Biologend).

**Cytokine production and degranulation assays**

PBMCs were stimulated for 4–6 h with plate-bound anti-CD16 (3G8) or target cells (K562 or 721.221) at a ratio of 10:1 (E:T). Antibody-dependent functional activity was determined by prelabeling P815 target cells with rabbit antitumor lymphocyte polyclonal Ab, or SCC4 squamous carcinoma cells with anti-EGFR mAb (Cetuximab). All cytokine assays were performed in the presence of Brefeldin A. For degranulation assays, PBMCs were stimulated in the presence of anti-CD107a and monensin for 4 h, followed by surface and intracellular staining for flow cytometry, as previously described (46).

**Immunoblot analysis**

Detection of FcRγ and CD3ζ in cell lysates was by immunoblot analysis using anti-FcεRI subunit (FcεRI) or anti-CD3ζ primary antibodies followed by antirabbit or antimouse secondary antibody, respectively, with visualization on Li-cor’s Odyssey (NE, USA).

**NK-cell sorting and limiting dilution**

The surface markers of PBMCs were stained prior to sorting to exclude non-NK cells and enrich for FcγRI-deficient (g–NK) and conventional NK cells. The resulting samples were plated by limiting dilution in NK cloning medium [RPMI1640 (Invitrogen, NY, USA) supplemented with 5% pooled human AB serum (Cellgro, VA, USA), 10% fetal bovine serum (HyClone, UT, USA), 1 µg/ml PHA (Roche, IN, USA), 100 U ml⁻¹ IL-2, and 10 ng ml⁻¹ IL-15 (Peprotech, NJ, USA)] along with PHA-stimulated (1 µg ml⁻¹ for 1 h) allogeneic PBMCs and RPMI 8866 cells that had been treated with mitomycin C (Sigma-Aldrich, MO, USA) for 2 h at 37°C. Feeder cells were added at 25,000 per well every 10–14 days for up to 2 months.

**mRNA quantification**

Total RNA extracts were used to prepare cDNA for amplification by gene-specific primers in SYBR green PCR Mastermix (for enriched NK cells) or in the presence of gene-specific Taqman probes (for cultured NK cells) (Applied Biosystems,
CA, USA). Amplification and detection were performed using an Applied Biosystems StepOnePlus Instrument and software. Relative quantities were calculated after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were for SYBR green assays: FcRγ sense, 5'/GGACCAGTCTCCAGGCAAGA-3', and antisense, 5'/GATACAGGATGTGATGCC-3'; GAPDH sense, 5'/GAAAGGTGAAGGTCGG-3', and antisense, 5'/GAAGATGTTGATGGGATTTC-3. Taqman gene expression assays for FcRγ (Hs00175408_m1), CD3ζ (Hs00609515_m1) and GAPDH (Hs03929097_g1) were from Applied Biosystems.

Statistics
Statistical analyses were performed using the paired Student's t-test. Differences were considered statistically significant when $P < 0.05$.

Results
Identification of FcRγ-deficient NK cells
As an attempt to identify factors influencing NK-cell functional activity, PBMCs from a large panel of donor samples obtained from leukocyte reduction filters were analyzed for NK-cell IFN-γ production against K562 tumor cells or antibody-coated target cells. This analysis revealed that NK cells from several donors did not show a correlative response pattern; for instance, NK cells from donors #25 and #28 responded poorly to the human erythroleukemic cell line, K562, but responded markedly well to antibody-coated P815 target cells (Fig. 1A). Donor NK cells that had responded well to antibody-coated P815 cells also responded robustly to immobilized anti-CD16 mAb (Fig. 1B), confirming the direct involvement of CD16. Thus, within certain donors, the responsiveness of NK cells to stimulation through CD16 versus tumor-recognition receptors can be dramatically different.

To explore potential mechanisms underlying the differential responsiveness, we examined the expression of FcRγ and CD3ζ, both of which are associated with CD16, as well as with NKp46 and NKp30, two NCRs that are important receptors for recognition of tumor cells (7, 9). Flow cytometric analysis following intracellular staining showed that in the majority of the donors, essentially all NK cells expressed both FcRγ and CD3ζ (Fig. 1C, donors #26 and #27), consistent with current understanding (2). However, in some donors, including those donors with high CD16 responsiveness and poor tumor reactivity, such as donors #25 and #28, we found a distinct subset of NK cells that were deficient for FcRγ, whereas all NK cells expressed CD3ζ (Fig. 1C). Human NK cells can be divided into two distinct populations; that is, the major CD3ζ-CD56bright population (comprising about 90–95% of the NK cell pool) and a minor CD3ζ-CD56dim population (1, 5). The FcRγ-deficient NK cells were restricted to the CD56dim population in all donors examined, and the CD56bright population expressed intermediate levels of both adaptor chains (Fig 1C).

The presence of FcRγ-deficient NK cells was initially observed during analyses of PBMCs isolated from de-identified leukocyte reduction filters, where 21 out of 80 samples contained readily detectable numbers of FcRγ-deficient NK cells, that is, more than 3% of the CD56dim NK cells. To confirm that these FcRγ-deficient cells are present in freshly isolated samples, we also analyzed PBMCs from 42 recruited healthy donors. Among these healthy individuals, 18 had FcRγ-deficient NK cells that comprised more than 3% of their CD56dim NK cell pool. Together, appreciable numbers (frequencies ranging from 3% to as much as 85% of the CD56dim NK cell pool) of FcRγ-deficient NK cells were present in about one-third (39 out of 122 donors) of the healthy individuals tested (Fig. 1D).

To examine the subset composition of FcRγ-deficient NK cells, we surveyed the expression of killer cell immunoglobulin-like receptors (KIRs) KIR2DL1, KIR2DL2/3 and/or KIR3DL1, three well-characterized KIRs expressed primarily within the CD56dim NK cell population (47). These KIRs are inhibitory receptors for MHC I and are expressed by partially overlapping subsets of NK cells. Although CD56dim NK cells expressing FcRγ (hereafter referred to as conventional NK cells) did not show predominant expression of particular KIRs, among the FcRγ-deficient NK-cell-positive donors that we analyzed further, approximately half showed skewed expression of certain KIRs on their FcRγ-deficient NK cells (Fig. 2A and data not shown). For instance, in donor #209, the vast majority (>95%) of the FcRγ-deficient NK cells expressed KIR2DL2/3, but not KIR2DL1 or KIR3DL1. However, the remaining half (of the FcRγ-deficient NK-cell-positive donors) did not display such predominant KIR expression on the FcRγ-deficient NK cells; e.g. donor #211 had abundant FcRγ-deficient NK cells, but these cells had no predominant expression of KIR2DL1, KIR2DL2/3 or KIR3DL1 (Fig. 2A). Taken together, the KIR subset composition of FcRγ-deficient NK cells can be different from that of conventional NK cells within individual donors. Furthermore, FcRγ-deficient NK-cell composition with respect to KIR expression can be quite different between donors.

The exact extent of the reduction in FcRγ expression within FcRγ-deficient NK cells was difficult to determine, as we used a polyclonal antibody reagent to detect FcRγ protein, and several control polyclonal antibodies yielded variable levels of background staining. Therefore, to further address this issue, we analyzed the expression of mRNA from FcRγ-deficient NK cells that were enriched from freshly isolated PBMCs to about 70% purity by cell sorting based on expression of KIRs that correlated well with FcRγ deficiency: two donors with KIR2DL2/3, and one donor with KIR3DL1 (Fig. 2A and data not shown). We found that the amount of mRNA for FcRγ, but not CD3ζ, was reduced in enriched FcRγ-deficient NK-cell preparations compared to enriched conventional NK cells from the same donors (Fig. 2B). To further increase the purity of FcRγ-deficient NK cells beyond 70%, we cultured the sorted cells (e.g. KIR2DL2/3 NK cells) under limiting dilution conditions. Conventional NK cells (e.g. KIR2DL2/3) were also sorted and cultured in parallel. Through this process, we were able to obtain many different FcRγ-deficient, as well as conventional, NK-cell clones (data not shown), indicating that the FcRγ-deficient phenotype is stable during the in vitro culture period. Analysis of these clones showed that mRNA for FcRγ was dramatically reduced in FcRγ-deficient clones compared to conventional NK-cell clones. By contrast, the expression of mRNA for CD3ζ among these clones was comparable (Fig. 2C). Finally, immunoblot analysis showed that the FcRγ-deficient NK-cell clones did not express detectable levels of FcRγ protein, unlike conventional NK-cell clones.
that expressed high levels of FcRγ, whereas CD3ζ protein levels were comparable (Fig. 2D). Taken together, the reduction of FcRγ expression is likely due to low levels of mRNA, suggesting that the deficiency of FcRγ expression in these NK cells (hereafter referred to as g−NK cells) results from impaired transcription, or differential mRNA stability or processing, rather than post-translational events. Collectively, these results reveal that this distinct subset of human NK cells, characterized by a deficiency in FcRγ mRNA and protein expression with normal CD3ζ expression, is present in a significant proportion of the human population.

Distinct phenotype of g−NK cells
To determine whether deficiency of FcRγ may impact the expression of activation receptors with which it associates (2, 6, 8), we evaluated g−NK and conventional NK cells for their expression of the NCR NKp46. Compared to conventional NK cells, the expression of NKp46 was dramatically reduced in g−NK cells in all donors examined [median fluorescence intensity (MFI): 914 ± 110 for conventional NK versus 250 ± 21 for g−NK cells; n = 11], although we also noted examples of conventional NK cells that expressed FcRγ but displayed relatively low levels of NKp46 in a few donors (Fig. 3A). Furthermore, g−NK cells displayed much lower expression of another NCR, NKp30, in all donors examined (MFI: 631 ± 89 versus 186 ± 4, n = 11) (Fig. 3A). These data suggest that FcRγ is required for the expression of both NKp46 and NKp30. By contrast, the analysis of CD16 showed less dramatic reduction; the expression level of CD16 on g−NK cells was approximately 60% of that on conventional NK cells (MFI: 1362 ± 263 versus 844 ± 227, n = 13), and there was substantial variation between donors for unknown reasons (Fig. 3B). These data suggest that FcRγ contributes to, but is not essential for, CD16 expression. Taken together, FcRγ deficiency appears to dramatically affect the cell-surface expression of NKp46 and NKp30, but it has only a limited effect on CD16 expression.

In addition to FcRγ-associated receptors, we analyzed other NK-cell activation receptors, such as NKG2D, which is known to be associated with the signaling adaptor DAP10 (2, 6). In g−NK cells, NKG2D was expressed at normal levels compared to conventional NK cells (Fig. 3C). There were also no consistent differences between g−NK and conventional NK cells for other activation receptors including 2B4, DNAM-1, NTB-A and CRACC (Fig. 3C) (2, 3). Similar to conventional NK cells, the g−NK cells did not express markers (CD69, CD25 and NKp44) that are known to be up-regulated on activated NK cells (Fig. 3C). Finally, analysis of cytolytic effector molecules showed that granzyme B expression is slightly higher, whereas perforin and granzyme A expression is slightly lower in g−NK cells compared to conventional NK cells (Fig. 3D). Together, g−NK and conventional NK cells differ in the expression of FcRγ-associated receptors and cytolytic effector molecules, whereas there is no detectable difference in the expression of NK-cell activation receptors that are not associated with FcRγ and CD3ζ.

Functional responsiveness of g−NK cells
To evaluate the functional activity of g−NK cells against target cells, we examined NK-cell cytokine production following stimulation with K562 cells. Compared to conventional NK cells, g−NK cells produced significantly less IFN-γ (P < 0.001) in response to K562 stimulation (Fig. 4A). In addition, the degranulation response, measured by evaluating the cell-surface expression of CD107a as a surrogate marker, was poorer in g−NK cells than conventional NK cells (P < 0.01) (Fig. 4B). Similar results were obtained with an EBV-transformed cell line, 721.221, although the degranulation response did not reach statistical significance (Fig. 4C). However, we noted substantial variation between donors in response to these targets, especially for 721.221, presumably reflecting complex NK: target cell interactions, as well as genetic heterogeneity among donors. Nonetheless, these data indicate that the direct responsiveness of g−NK cells to
tumor or transformed cells is generally poor. These results are consistent with the impaired expression of both NKp46 and NKp30 and their importance for antitumor activity, as well as previous findings that NCR<sup>dull</sup> NK cells show poor reactivity to tumor targets (7).

To evaluate the responsiveness of g<sup>−</sup>NK cells to CD16 stimulation, we examined cytokine production following incubation with immobilized anti-CD16 mAb. Unexpectedly, g<sup>−</sup>NK cells produced significantly greater amounts of IFN-γ compared to conventional NK cells (P < 0.0001) (Fig. 5A), providing an explanation for our initial observations in donors #25 and #28 (Fig. 1A and 1B). In addition to IFN-γ production, CD16 cross-linking resulted in significantly more TNF-α production by g<sup>−</sup>NK cells (P < 0.0001) (Fig. 5B). Evaluation of the degranulation response showed that although CD107a expression trended higher in g<sup>−</sup>NK cells than in conventional NK cells, the difference was not statistically significant.

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**Fig. 2.** The FcRγ deficiency correlates with low mRNA levels. (A) Dot plots show expression of indicated KIRs by CD56<sup>dim</sup> CD3<sup>−</sup> CD14<sup>−</sup> CD19<sup>−</sup> NK cells with respect to FcRγ expression from two representative donors (n = 12). Numbers indicate the percentage of cells in each quadrant. (B and C) Relative quantities of FcRγ mRNA from enriched NK cells (B) and FcRγ and CD3ζ mRNA from NK-cell clones (C) were determined by RT–qPCR, normalized to GAPDH mRNA and expressed relative to total NK cells (B), or clone #2 (C). Samples included RNA from total NK, enriched conventional NK cells (NK) and enriched FcRγ-deficient NK cells (g<sup>−</sup>NK) as indicated in (B) or NK and g<sup>−</sup>NK cell clones in (C). Data shown are representative of three donors. (D) Immunoblot analysis of total cell lysates prepared from NK clones derived from conventional NK (lane 1) or g<sup>−</sup>NK (lanes 2 and 3) clones. Data are representative of two independent experiments.

**Fig. 3.** FcRγ-deficient NK (g<sup>−</sup>NK) cells display reduced expression of the NCRs NKp46 and NKp30. (A and B) Flow cytometric analysis of surface expression of NKp46 and NKp30 (A), or CD16 (B) on conventional NK (NK: dotted line) and g<sup>−</sup>NK cells (solid line) from four representative donor samples. Upper and lower numbers indicate median fluorescence intensities of conventional and g<sup>−</sup>NK cells, respectively. Shaded peaks are from control staining. (C and D) Histograms show representative samples from at least seven donors for the expression of indicated surface markers (C) or intracellular cytolytic effector molecules (D) on conventional and g<sup>−</sup>NK cells. Inset numbers are as in (A).
The less dramatic difference in CD107a expression may be explained by a lower activation threshold or lower transcriptional requirements for degranulation as compared to cytokine production (48). Nonetheless, g−NK cells exhibit significantly greater cytokine production upon CD16 cross-linking than conventional NK cells.

When stimulated by antibody-coated P815 cells, g−NK cells also produced both IFN-γ and TNF-α more abundantly than conventional NK cells (Fig. 6A). To test CD16 responsiveness in the context of human IgG Fc, PBMCs were stimulated with EGFR⁺ human squamous cell carcinoma (SCC4) coated with humanized anti-EGFR mAb. Under these conditions, g−NK cells again produced higher amounts of both IFN-γ and TNF-α than conventional NK cells (Fig. 6B and 6C). In the absence of antibody, neither g−NK nor conventional NK cells yielded any detectable cytokine production (Fig. 6B), indicating that this response was mediated through CD16 engagement. We also evaluated the NK-cell degranulation response following incubation with antibody-coated SCC4 cells and found that similar to CD16 stimulation, g−NK cells in several donors displayed increased responsiveness compared to conventional NK cells, but the difference did not reach statistical significance (Fig. 6D). Taken together, g−NK cells display a markedly enhanced ability to respond to antibody-coated targets, particularly for cytokine production.

Discussion

Here, we have identified a phenotypically and functionally distinct subset of NK cells, termed g−NK cells, which are characterized by a deficiency in FcRγ expression. Despite FcRγ deficiency, g−NK cells express CD3ζ at normal levels and display robust responsiveness to CD16 stimulation. It is intriguing that appreciable numbers of g−NK cells are present in the peripheral blood of only about one-third of the healthy individuals we examined. Because there is no absolute specific marker for human NK cells and it was difficult to exclude all non-NK cells (that might not express FcRγ) from the NK-cell gate, we used 3% as an arbitrary cut-off value to define the presence or absence of g−NK cells for this study. Therefore, the presence of g−NK cells in about one-third of healthy individuals could be an underestimate of the actual prevalence among people, as some individuals may have very low numbers of g−NK cells. For more accurate estimates, further studies using more specific NK-cell markers and larger numbers of donors will be needed. In addition,
the numbers of g−NK cells are highly variable depending on
the donor, and in some donors, they comprise a major subset
of NK cells. Taken together, our discovery of g−NK cells and
their characteristics reveal a novel means for diversification
of human NK cells based on the expression of signaling adaptor
molecules.

Our data demonstrate that g−NK cells express dramatically
reduced levels of FcRγ mRNA but normal levels of CD3ζ mRNA.
In addition to mRNA measurement, the FcRγ deficiency was
verified using polyclonal antibodies for intracellular staining
and immunoblot analysis. These observations along with the
fact that g−NK cells exist together with conventional NK cells
within the same individuals makes it unlikely that the FcRγ
deficiency in g−NK cells is due to allelic polymorphisms in
the FcRγ gene. Rather, the FcRγ deficiency could be due to
differential mRNA stability or processing, or an outcome of
impairment of transcription of the FcRγ gene, perhaps due
to epigenetic modifications such as hypermethylation or
differential expression of transcription factors controlling the
FcRγ gene expression. Importantly, the impaired expression
of NKP46 and NKP30 on g−NK cells was observed for NK-cell
clones (Supplementary Figure 1 is available at International
Immunology Online), as well as fresh NK-cell samples,
indicating that expression of these receptors may require the
association with FcRγ. In line with this possibility, a previous
report showed that siRNA-mediated suppression of FcRγ, but
not CD3ζ, expression leads to reduced expression of NKP46
(49). Together, it is intriguing to consider the possibility that
FcRγ deficiency may provide a molecular basis for the
phenotype and functionality of NCR dull NK cells reported in
numerous studies (7, 8, 12, 35–37). With regards to NKP44,
an NCR that is not associated with FcRγ, expression levels
on g−NK cells were not up-regulated following culture
in IL-2, unlike NKP44 up-regulation by conventional NK
cells (Supplementary Figure 2 is available at International
Immunology Online), suggesting that there may be additional
changes in g−NK cells that could influence overall NCR
expression.

Our data also indicate the expression of CD16 is less depend-
ent on association with FcRγ than is the expression of NCRs.
This is in line with previous data showing that co-expression
of CD3ζ can support the cell-surface expression of human
CD16 (13). Thus, less dramatic reduction of CD16 expression
is likely due to the ability of CD3ζ to better support the sur-
face expression of CD16 than that of the NCRs. However, the
observed reduction (~40% average) in CD16 expression on
g−NK cells suggests that CD3ζ expression alone is not suf-
ficient to support normal levels of CD16 expression in some

![Fig. 5.](image-url)
Fig. 6. g^NK cells produce cytokines abundantly in response to antibody-coated target cells. (A) Line graphs show percentages of conventional or g^NK cells that produced indicated cytokines following stimulation with antibody-coated P815 cells (IFN-γ, n = 13; TNF-α, n = 13). Dots connected by a line represent data observed from the same donor sample. (B) NK-cell responsiveness with SCC4 squamous carcinoma cells, unlabeled or prelabeled with anti-EGFR mAb (Cetuximab) from one representative donor. Inset numbers indicate the relative percentages of conventional or g^NK cells that responded. (C) Graphs show percentages of conventional or g^NK cells that produced indicated cytokines following stimulation with antibody-coated SCC4 cells (IFN-γ, n = 10; TNF-α, n = 10). (D) Percentages of conventional and g^NK cells that displayed CD107a following incubation with antibody-coated SCC4 cells (n = 10). *P < 0.01; **P < 0.001; ***P < 0.0001; ns, not significant.
of the donors. The reason for the variable CD16 expression in different donors remains unclear at this time. A potential explanation for the robust responsiveness of g−NK cells to CD16 stimulation, despite generally reduced CD16 expression and FcRγ deficiency, is that exclusive association of CD16 with CD3ζ in g−NK cells may lead to more robust or prolonged biochemical signaling, given that CD3ζ has three ITAMs, whereas FcRγ has only one ITAM (2). Since it was challenging to obtain a substantial number of g−NK cells, the comparative analysis between g−NK and conventional NK cells with regards to biochemical signaling differences is currently very limited. Thus, the exact mechanism for the enhanced responses to CD16 stimulation is yet to be determined.

Several important issues arise from our findings, including the origin of g−NK cells. Considering that g−NK cells are not detectable in all individuals, along with evidence suggesting that g−NK cells are derived from expansion of specific subsets in some donors, the generation of g−NK cells may be associated with an environmental factor, such as prior infection(s), which is under investigation. Whether or not CD16 polymorphisms influence the frequency or generation of g−NK cells requires further investigation. Also, the tissue distribution of g−NK cells remains to be determined. With respect to licensing, available data indicate that a large proportion of g−NK cells in certain donors do not express KIRs, which are known to be involved in licensing or education (41–44). Thus, despite their robust responsiveness to CD16 stimulation, the g−NK cells in these donors would be primarily classified as ‘unlicensed’, providing a possible explanation for controversial observations regarding the role of licensing in CD16 responsiveness (41, 44, 45). This warrants that future studies of the licensing impact on NK-cell function should consider the presence or absence of g−NK cells.

Robust responsiveness to CD16 stimulation with relatively poor responsiveness to tumor cells suggests that g−NK cells may represent an effector population that is specialized for antibody-mediated defense against infection rather than anti-tumor activity. Through the Fc receptor-mediated recognition of target cells that are bound by antibodies, g−NK cells are likely capable of mounting robust immune responses to infected cells, particularly during chronic or recurring infection, where reactive antibodies are readily available. Given that g−NK cells exist in a fraction of healthy individuals, the presence or absence, as well as quantity, of g−NK cells may contribute to heterogeneity of immune responses to infections and cancer among people. Finally, g−NK cells may be important for other antibody-mediated processes, such as immune complex diseases, or targeted antibody-based therapies for the treatment of cancer (50, 51), and whether g−NK cells contribute to these physiological conditions awaits investigation.

Supplementary data
Supplementary data are available at International Immunology Online.

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