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The Human NK Cell Response to Yellow Fever Virus 17D Is Primarily Governed by NK Cell Differentiation Independently of NK Cell Education

Nicole Marquardt,* Martin A. Ivarsson,* Kim Blom,* Veronica D. Gonzalez,* Monika Braun,* Karolin Falconer,* Rasmus Gustafsson,† Anna Fogdell-Hahn,† Johan K. Sandberg,* and Jakob Michaełsson*

NK cells play an important role in the defense against viral infections. However, little is known about the regulation of NK cell responses during the first days of acute viral infections in humans. In this study, we used the live attenuated yellow fever virus (YFV) vaccine 17D as a human in vivo model to study the temporal dynamics and regulation of NK cell responses in an acute viral infection. YFV induced a robust NK cell response in vivo, with an early activation and peak in NK cell function at day 6, followed by a delayed peak in Ki67 expression, which was indicative of proliferation, at day 10. The in vivo NK cell response correlated positively with plasma type I/III IFN levels at day 6, as well as with the viral load. YFV induced an increased functional responsiveness to IL-12 and IL-18, as well as to K562 cells, indicating that the NK cells were primed in vivo. The NK cell responses were associated primarily with the stage of differentiation, because the magnitude of induced Ki67 and CD69 expression was distinctly higher in CD57+ NK cells. In contrast, NK cells expressing self- and nonself-HLA class I–binding inhibitory killer cell Ig-like receptors contributed, to a similar degree, to the response. Taken together, our results indicate that NK cells are primed by type I/III IFN in vivo early after YFV infection and that their response is governed primarily by the differentiation stage, independently of killer cell Ig-like receptor/HLA class I–mediated inhibition or education. The Journal of Immunology, 2015, 195: 3262–3272.
expression, as well as an increase in the number of KIRs expressed per NK cell. However, it is clear that CD56dim NK cells do not undergo a strict linear differentiation, because all possible combinations of KIR and NKG2A expression can be found among CD57+ and CD57− CD56dim NK cells. For example, some NK cells may acquire multiple KIRs but still express NKG2A and lack CD57 expression; conversely, some NK cells can lack the expression of KIRs and express both NKG2A and CD57. A model for a continuous process of NK cell differentiation with multiple cellular intermediates was proposed recently, whereby NKG2A+ CD57− NK cells lacking KIR expression represented the least differentiated subset, and NKG2A+ CD57+ NK cells expressing multiple KIRs represented the most differentiated subset (15).

In this study, we made use of the live attenuated yellow fever virus (YFV) vaccine strain 17D as an in vivo model of an acute viral infection in humans. This vaccine gives rise to a modest, but detectable, viral load, T and B cell responses, and innate immune responses (14, 18–22). Previous studies indicated that NK cells can be activated by the YFV vaccine (14, 19, 23–26), although most of those studies lacked markers to specifically analyze NK cells (23–25). YFV belongs to the genus Flavivirus, which also includes Dengue virus, tick-borne encephalitis virus, and West Nile virus (27). YFV infects hepatocytes, monocytes, dendritic cells, and macrophages, and vaccination with YFV 17D induces distinct innate gene signatures that are predictive of subsequent T and B cell responses (22). The YFV 17D model allowed us to study the earliest NK cell responses to an acute viral infection, including the roles of NK cell differentiation and education.

Materials and Methods

Study population and blood samples

A total of 21 healthy volunteers, aged 18–50 y and not previously vaccinated against yellow fever, received a single dose of the 17D-204 yellow fever vaccine Stamaril (Sanofi Pasteur MSD). All donors gave informed written consent, and experiments were conducted according to the Declaration of Helsinki. The regional ethical review board in Stockholm, Sweden, approved the study (2008/1881-31/4). Blood was collected in EDTA-treated vacuum tubes (BD Biosciences) before vaccination (day 0), and at days 1, 3, 6, 10, 15, and 90 after vaccination. Plasma was collected for determination of viral load and plasma type I/III IFNs. PBMC were isolated by Lymphoprep gradient centrifugation (Axis-Shield) and cryopreserved in FCS with 10% DMSO.

KIR and KIR ligand genotyping

Genomic DNA was isolated using a DNeasy Blood & Tissue Kit (QIAGEN) from 100 μl whole blood. KIR genotyping and KIR ligand determination were performed using PCR-SSP technology with a KIR typing kit and a KIR HLA ligand kit (Olerup SSP), according to the manufacturer’s instructions. The KIR and HLA genotypes of the donors are listed in Supplemental Table I.

Flow cytometry

For phenotypic analyses, PBMCs were stained with two separate panels, depending on the KIR genotype of the donor. All donor PBMCs were cell surface stained with anti-CD3 ECD (clone UCHT1), anti-NKG2A allophycocyanin (clone Z199) (both from Beckman Coulter), anti-CD4 PE-Cy5 (clone RPA-T4; Affymetrix/eBioscience), anti-CD14 (clone M6P9) and anti-CD19 (clone HIB19) Horizon V500, anti-CD56 Qdot 705 (clone NCAM16.1), anti-CD69 allophycocyanin-Cy7 (clone FN50) (BD Biosciences), anti-CD57 Pacific Blue (clone HC57D7; BioLegend), anti-KIR2DL1/2/3/5 PE-Cy7 (clone 1EB6), anti-KIR2DL2/23/52 PE-Cy5.5 (clone GL183) (Beckman Coulter), and LIVE/DEAD Aqua. Subsequently, PBMCs were fixed and permeabilized using cytotox/Cytoperm (BD Biosciences) and stained with anti-CD56 (clone HCD57; BioLegend), anti-CD4 PE-Cy5 (clone UCHT1), anti-CD56 Brilliant Violet 650 (clone BD56; BioLegend) in permeabilization buffer (Affymetrix/eBioscience). For functional analyses, PBMCs were stained with anti-CD107a FITC (clone H4A3; BD Biosciences), anti-CD3 PE-Cy5 (clone UCHT1), anti-CD56 ECD (clone N901), anti-NKG2A–allophycocyanin–Cy7 (clone Z199), anti-KIR2DL2/3/52 PE-Cy5.5 (clone GL183), anti-KIR2DL1/1S1 (clone EB6) (all from Beckman Coulter), anti-KIR2DL1 Alexa Fluor 700 (clone 131411), anti-KIR2DL3 biotin (clone 180701), anti-KIR3DL1 Brilliant Violet 421 (clone DX9; BioLegend), anti-CD14 (clone M6P9) and anti-CD19 (clone HIB19) Horizon V500, and purified anti-CD57 (clone TB01; Affymetrix/eBioscience), followed by staining with streptavidin Qdot 605 (Invitrogen), anti-mouse IgM–eFluor 650NC (clone II/41; Affymetrix/eBioscience), and LIVE/DEAD Aqua. Subsequently, PBMCs were fixed and permeabilized using cytotox/Cytoperm (BD Biosciences) and stained with FlowJo version 9.5.2 (TreeStar).

NK cells were identified as CD56+ cells among the single, live, CD3− CD4− CD14− CD19− lymphocytes and were subsequently divided into CD56dim and CD56bright NK cells. The CD56dim NK cells were further divided into NKG2A+CD57−, NKG2A+CD57+, and NKG2A+CD57+ NK cells. Within each of these CD56+ NK cell subsets, within-donor cells expressed KIR2DL2/3, and KIR3DL1. Using a Boolean gate of KIR2DL1, KIR2DL3 and KIR3DL1, all combinations of KIR-expressing NK cells were identified, including those expressing no KIRs (KIR−) or expressing one, two, and three of the KIRs on the same cell. To identify NK cells expressing a single self- or nonself-HLA class I–specific KIR, we matched the KIR expression with the HLA class I of the donor. For YFV specific KIR, we matched the KIR expression with the HLA class I of the donor. For YFV specific KIR, we matched the KIR expression with the HLA class I of the donor. For YFV specific KIR, we matched the KIR expression with the HLA class I of the donor. For YFV specific KIR, we matched the KIR expression with the HLA class I of the donor.
formed with a High-Capacity cDNA Reverse-Transcription Kit including RNase inhibitor and a TaqMan Core Reagents Kit (Applied Biosystems), following the manufacturer’s protocol. Primers and the FAM-TAMRA-labeled probe (Applied Biosystems) for real-time PCR were specific for a sequence within the highly conserved NS5 gene of YFV (21). Final concentrations for the quantitative PCR were 6 nM MgCl₂, 800 nM each primer, 250 μM each of the dNTPs with the exception of dUTP (500 μM), 125 nM probe, and 0.625 U AmpliTaq Gold DNA polymerase in 25 μl reaction volume. Amplifications were performed on a 7500 Fast Thermocycler (Applied Biosystems) under the following thermal cycling conditions: 10 min at 95°C, 45 cycles of 15 s at 95°C, followed by 1 min at 60°C. Control RNA isolated from the YFV 17D vaccine (Stamaril) was used for quantification.

Statistical analyses
Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software). Repeated-measures ANOVA with the Bonferroni post hoc test was used to compare more than two groups, and the Student paired t test (for normally distributed data) or Wilcoxon matched-pairs signed-rank test (for nonnormally distributed data) was used to compare matched pairs of data. Spearman rank correlation was used to test the association among type I/III IFN levels, viral load, and NK cell activation.

Results
YFV induces robust NK cell responses in vivo
A total of 21 individuals was vaccinated with YFV 17D, and blood samples were collected before vaccination (day 0) and at days 1, 3, 6, 10, 15, and 90 after vaccination. To determine the kinetics of the NK cell responses to the vaccine, we first analyzed the expression of Ki67 and CD69 in NK cells ex vivo over time (Fig. 1A). The expression of Ki67 and CD69 increased in CD56dim and CD56bright NK cells in response to vaccination (Fig. 1A–C, 1E). Despite the increase in Ki67 expression, only minor changes were observed in the frequency of CD56bright and CD56dim NK cells, and we did not detect any significant changes in the absolute numbers of NK cells (data not shown). Ki67+ NK cells expressed lower levels of the antiapoptotic protein Bcl-2, indicating that they might be more susceptible to apoptosis (Fig. 1D). Ki67+ and CD69+ displayed different kinetics (Fig. 1B, 1C, 1E): the expression of Ki67 peaked at day 10 (Fig. 1B, 1C), whereas that of CD69 peaked at day 6 (Fig. 1E). Ki67 and CD69 were expressed in a virtually mutually exclusive pattern; few of the Ki67+ NK cells expressed CD69, and conversely, few of the CD69+ NK cells expressed Ki67 (Fig. 1A). The expression of CD69 at day 6 was strongly associated with the expression of Ki67 at day 10 (Fig. 1F), suggesting that NK cell activation at day 6 predicts NK cell proliferation at day 10.

To analyze how NK cell activation related to viral replication and whether the vaccination induced an IFN response, we measured the plasma viral load and levels of type I/III IFN. Viral RNA was detected at day 6 (Fig. 1G) in a majority of the donors, concomitant with an increase in the levels of type I/III IFN in plasma (Fig. 1H). Thus, the increase in CD69 expression on NK cells coincided temporally with the increase in plasma viral load and type I/III IFN, suggesting that the IFN response may induce the activation of NK cells. Indeed, the levels of type I/III IFN in plasma at day 6 correlated positively with NK cell activation, measured as CD69 expression at day 6 (Fig. 1I, left panel) and Ki67 expression at day 10 (Fig. 1I, middle and right panels). In addition, the viral load at day 6 correlated positively with the frequency of Ki67+CD56dim+ NK cells at day 10 (Fig. 1J). To test whether YFV could activate NK cells in vitro, we stimulated PBMCs with YFV and subsequently measured CD69 expression on NK cells. Coincubation of PBMCs with YFV induced a marked upregulation of CD69 (Fig. 1K), whereas coincubation of YFV with purified NK cells alone did not (Fig. 1L). The results indicated that activation of NK cells by YFV required the presence of other cell types and was not induced by YFV acting directly on the NK cells. The upregulation of CD69 on NK cells after stimulation of PBMCs with YFV was abrogated by inhibiting signaling via the JAK pathway with Pyridone 6 (Fig. 1M), further supporting the notion that NK cell activation was dependent on type I/III IFN.

The in vivo activation of NK cells following YFV infection also translated to changes in NK cell function, because YFV induced increased NK cell degranulation and IFN-γ production after stimulation with IL-12 alone, IL-12 and IL-18 in combination, or K562 cells (Fig. 2). The increase in functional responsiveness was already apparent at day 3, peaked at day 6, and returned to baseline levels at day 15 after vaccination (Fig. 2). Together, these results indicated that NK cells were activated early post-infection with YFV, allowing us to study the regulation of NK cell responses in this model.

Less-differentiated NK cells respond more strongly to YFV in vivo
More-differentiated NK cells, as defined by the expression of CD57 (15) or lack of CD62L (14), respond less well to cytokine stimulation and are less prone to proliferate in vitro. In addition, CD62L+CD56dim NK cells were recently reported to express higher levels of Ki67 ex vivo, both before and 7 d after the administration of YFV (14). However, it remains unknown whether the kinetics of Ki67 and CD69 upregulation in more-differentiated NK cells differ from those in less-differentiated NK cells or whether the regulation of NK cell activation measured by the expression of other markers (e.g., CD69) differs from that of Ki67. Therefore, to determine the in vivo impact of NK cell differentiation on NK cell responses to YFV, we analyzed the expression of Ki67 and CD69 in conjunction with the expression of NKG2A and CD57 on CD56dim NK cells (Fig. 3A, 3B). We chose to divide NK cells into four subsets based on CD57 and NKG2A expression, because NK cell differentiation is characterized by an upregulation of CD57 and a loss of NKG2A (15). However, it should be noted that the four subsets analyzed do not necessarily reflect a strict linear differentiation of NK cells, but rather represent distinct subsets along a continuum of NK cell differentiation. In addition, NKG2A has a strong impact on NK cell education, and as such, dividing the CD57+ and CD57− NK cells into NKG2A− and NKG2A+ subsets helped to discriminate between the effects of differentiation and education. CD57− NK cells responded more strongly to vaccination compared with CD57+ NK cells, as measured by Ki67 expression at day 10, whereas there were no significant differences between NKG2A+ and NKG2A− NK cells in either the CD57+ or CD57− subsets (Fig. 3C, 3D). Moreover, the different subsets responded with similar kinetics (Fig. 3D), showing that the increased responsiveness of less differentiated NK cells was not dependent on the time of sampling. Interestingly, NK cell responses measured by upregulation of CD69 were strongest in NKG2A+CD57− NK cells, whereas there were no significant differences among NKG2A−CD57−, NKG2A−CD57+ and NKG2A+CD57+ NK cells (Fig. 3E), suggesting that the requirements for upregulation of CD69 and Ki67 are slightly different. Similar to Ki67, the kinetics of upregulation of CD69 did not differ among different subsets (Fig. 3F). NKG2A+CD57− NK cells also responded better than NKG2A−CD57− NK cells after stimulation of PBMCs with YFV 17D in vitro (Fig. 3G), thus recapitulating the increased responses by NKG2A+CD57− NK cells in vivo. However, it should be noted that CD57+ NK cells also responded to vaccination, as measured by Ki67 and CD69 expression (Fig. 3B–F). To analyze the contribution of the different NK cell subsets to the total pool of responding NK cells, we compared the frequency of NKG2A−CD57−, NKG2A−CD57+, NKG2A+CD57−, and NKG2A+CD57+ subsets between Ki67+ and Ki67− NK cells. CD57+ NK cells made up ∼40–50% of the responding Ki67+ NK cells.
FIGURE 1. NK cells are activated in vivo after vaccination against YFV. (A) FACS plots showing Ki67 and CD69 expression on CD56dim (upper panels) and CD56bright (lower panels) NK cells before and at different days after vaccination. NK cells were identified by gating on live, single, CD3− CD4− CD14− CD19− lymphocytes and further divided into CD56bright and CD56dim NK cells based on the expression level of CD56. Frequency of CD56bright (B) or CD56dim (C) NK cells expressing Ki67 before and after vaccination (n = 21). (D) Mean fluorescence intensity of Bcl-2 expression in Ki67+ and Ki67− CD56dim NK cells. (E) Frequency of CD56dim NK cells expressing CD69 before and after vaccination (n = 17). (F) Correlation between the expression of CD69 at day 6 and Ki67 at day 10 in CD56dim NK cells (n = 17). Plasma viral load (G) and levels of plasma type I/III IFNs (H) at different time points after vaccination (n = 20). (I) Correlation between the increase in CD69 (left panel) and Ki67 expression (middle and right panels) by NK cells and levels of plasma type I/III IFN (n = 20). (J) Correlation between Ki67 expression in NK cells and plasma viral load. CD69 expression on NK cells after coculture of PBMCs (K) and purified NK cells (L) with YFV 17D. (M) Expression of CD69 on NK cells after coculture of PBMCs with YFV 17D, with or without the JAK1/2/3-inhibitor Pyridone 6. Correlations were tested by Spearman analysis. *p < 0.05, ***p < 0.001, Wilcoxon matched-pairs signed-rank test.
cells compared with ~65% of the Ki67− NK cells (Fig. 3H). Thus, despite being less responsive at the single-cell level, CD57+ NK cells contributed to the overall NK cell response at the bulk level.

**YFV infection increases IL-12 responsiveness primarily in less-differentiated NK cells**

We next investigated the effects of NK cell differentiation on functional NK cell responses after vaccination with YFV 17D. In all subsets, NK cell degranulation was significantly higher at day 6 after vaccination compared with baseline after stimulation with IL-12 alone, IL-12 and IL-18, or K562 cells (Fig. 4A, 4B). CD56bright and the least-differentiated (NKG2A+CD57−) CD56dim NK cells displayed the strongest responses to IL-12 alone, measured both as degranulation and IFN-γ production, whereas the responses were gradually weaker in the more-differentiated subsets (Fig. 4A, 4B). NKG2A+CD57− cells displayed the greatest increase in degranulation from day 0 to day 6 after stimulation with IL-12 (Fig. 4C), mirroring the strong in vivo responses by this subset (Fig. 3C, 3E). This NK cell subset also displayed a similar trend toward greater IFN-γ responses (Fig. 4C).

In contrast, there were only minor or no differences in the increase (day 6 − day 0) in degranulation among subsets after stimulation with IL-12 and IL-18 or K562 cells (Fig. 4C). Taken together, YFV 17D stimulates an increased NK cell responsiveness to both cytokines and target cells, with the most pronounced increase observed in the least-differentiated NK cells after stimulation with IL-12 alone.

**Educated and noneducated NK cells contribute to a similar extent to the NK cell response against YFV**

NK cells are both inhibited and educated via interactions between inhibitory receptors and their HLA class I ligands. In addition, increased NK cell differentiation is associated with expression of multiple KIRs on single NK cells, in a process parallel to education (15). Therefore, we next asked whether expression of inhibitory KIRs affected NK cell responsiveness to YFV and whether this was linked to NK cell differentiation or education. We first analyzed the expression of Ki67 in NKG2A+CD57−, NKG2A−CD57−, NKG2A−CD57+, and NKG2A−CD57− NK cells expressing no, one, two, or three KIRs (out of KIR2DL1, KIR2DL2/3, and KIR3DL1), irrespective of the HLA class I specificity of the KIRs (Fig. 5A, 5B). There were no significant differences in Ki67 expression among NK cells expressing no, one, two, or three KIRs (out of KIR2DL1, KIR2DL2/3, and KIR3DL1), irrespective of the HLA class I specificity of the KIRs (Fig. 5A, 5B). There were no significant differences in Ki67 expression among NK cells expressing no inhibitory KIRs (KIR2DL1− KIR2DL2/3− KIR3DL1−), a single self-HLA class I–specific KIR.
KIR (sKIR), or a single nonself-HLA class I–specific KIR (nsKIR) in each of the four subsets defined by NKG2A and CD57 (Fig. 5C). Furthermore, analyses of the frequency of KIR2, sKIR+, and nsKIR+ NK cells among Ki67+ and Ki67NK cells did not reveal any significant bias toward self- or nonself-KIR–expressing NK cells among the responding Ki67NK cells (Fig. 5D). Several reports demonstrated that a subset of NKG2C+ NK cells expressing sKIRs is expanded in certain viral infections, including CMV, HIV, hantavirus, and Chikungunya virus. However, we did not detect any alterations in NKG2C expression on NK cells after vaccination (data not shown). Together with the lack of a bias toward sKIR NK cells, our data indicate that infection with YFV...
17D does not induce any pronounced skewing of the NK cell population.

Cytokine stimulation in vitro was shown to alleviate the hypo-responsiveness of noneducated NK cells to stimulation with K562 cells (15). Thus, although there was no significant effect on the expression of Ki67 in NK cells ex vivo, it remained possible that YFV infection could differentially affect the in vitro function of educated and noneducated NK cells. To test this, we analyzed degranulation and IFN-γ production in subsets of NK cells lacking expression of KIR (KIR2DL1, KIR2DL3, KIR3DL1), expressing a self-KIR, or a nonself-KIR. After stimulation with IL-12 or K562 cells, all subsets degranulated at significantly higher levels at day 6 compared with day 0 (Fig. 6A, 6B, upper panels), indicating that both noneducated and educated NK cells increased...
their functional responsiveness during the response to YFV. Educated NK cells responded better than noneducated NK cells after stimulation with K562 cells at days 0 and 6 (Fig. 6A). However, there were no significant differences in the increase in responsiveness (day 6 − day 0) among the NK cell subsets (Fig. 6C). Although the least-differentiated NK cells (NKG2A−CD57−) responded better to stimulation with IL-12 (Fig. 6B), there were only minor or no differences in the response to IL-12 stimulation dependent on the expression of KIRs (Fig. 6D). Taken together, our data indicate that educated and noneducated NK cells contribute to a similar level to the response to YFV in vivo and that YFV does not abrogate the functional hyporesponsiveness of noneducated NK cells.

**Discussion**

It is well established that certain virus infections can result in the expansion of distinct subsets of human NK cells (3, 5, 6, 8). However, it remains largely unknown how NK cells respond and are regulated at the earliest time points after an acute viral infection in humans. In this article, we show that human NK cells mounted a robust response to acute experimental YFV 17D infection. NK cells responded in distinct temporal phases after vaccination, with an early upregulation of CD69 expression and a later wave of upregulation of Ki67, suggestive of a proliferative response. Despite the increase in Ki67 expression, we did not detect any significant increase in NK
cell numbers or frequency in peripheral blood, indicating that the activated NK cells either undergo apoptosis or home to tissues following vaccination. Our data also revealed that the in vivo NK cell response correlated positively with the plasma type I/III IFN levels, and it was strongest in the least-differentiated NKG2A+CD57− NK cells. In contrast, the expression of inhibitory HLA class I–binding KIRs had negligible effects on the NK cell response, indicating that educated and noneducated NK cells contribute to a similar level to the NK cell response to YFV.

NK cells are strongly activated by cytokines, such as IFN-α, IL-12, IL-15, and IL-18. YFV was shown to alter the expression of a large number of genes involved in the innate immune response, with a peak in gene expression alteration at day 7 after vaccination (19). Indeed, we detected an increase in type I/III IFNs at day 6 after vaccination in most donors, which coincided with the peak in viral load, as well as with the peak in functional NK cell responses. The finding that the NK cell response, as measured by CD69 expression at day 6 and Ki67 expression at day 10, correlated with the plasma levels of type I/III IFNs indicated that the NK cell responses postinfection with YFV 17D were induced by IFN. This notion was further supported by the fact that purified NK cells did not respond to YFV 17D, whereas NK cells in PBMCs stimulated with YFV 17D upregulated CD69 in a JAK-dependent manner.

Although it seems likely that the NK cell response was primarily induced by type I/III IFNs, it remains possible that other cytokines may also contribute. However, we were unable to detect any significant increases in IL-12, IL-15, or IL-18 in plasma after vaccination (data not shown), in line with a previously published study (22). The in vitro cytokine responsiveness of NK cells is known to be strongly influenced by the degree of NK cell differentiation (15): less-differentiated NK cells respond more robustly to stimulation with cytokines. Our finding that the less-differentiated NK cells responded more strongly to vaccination in vivo indicates that NK cell differentiation is an important factor in determining the NK cell response to YFV in vivo. This finding is further supported by a recent report suggesting that preferentially less-differentiated NK cells expand in humanized mice infected with EBV (29). However, it should be noted that, although more-differentiated NK cells responded less well to YFV infection in the current study, they contributed to a large extent to the overall response, because they made up a large part of the total NK cell population.

It is generally believed that downregulation of HLA class I upon virus infection is one way that NK cells can detect virus-infected cells. In line with this notion, CMV infection in humans drives an expansion of educated KIR-expressing NK cells (3, 16), whereas, surprisingly, murine CMV infection is controlled by noneducated NK cells (30). In this study, we addressed whether

FIGURE 6. The increase in functional responsiveness in vitro after vaccination against YFV is largely independent of KIR-mediated education. Paired analyses of the frequency of CD107a+ (upper panels) or IFN-γ+ (lower panels) among NKG2A+CD57−, NKG2A+CD57−, NKG2A+CD57−, and NKG2A+CD57− CD56dim NK cells lacking the expression of KIR (KIR−), expressing a single sKIR, or expressing a single nsKIR after stimulation with K562 cells (A) or IL-12 (B). Change in frequencies of degranulating NK cells between days 0 and 6 after vaccination after stimulation with K562 cells (C) or IL-12 (D). Background degranulation and IFN-γ production in the medium control were subtracted for each donor. *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon matched-pairs signed-rank test (A and B). ANOVA with Bonferroni post hoc test (C) (n = 19).
expression of inhibitory KIRs played a role in the NK cell response to YFV. Infection with some flaviviruses results in rapid upregulation of MHC class I expression (31–33) and, as such, could be expected to evade NK cell recognition via ligation of inhibitory KIRs or CD94/NKG2A on educated NK cells. However, educated and noneducated NK cells contributed to a similar extent to the NK cell response after vaccination, demonstrating that both subsets of NK cells are involved in the response to YFV early postinfection.

The activation of NK cells in response to YFV vaccination implies that these cells could contribute to the immune response against YFV in vivo. NK cells can contribute to viral control directly through killing of virus-infected cells or indirectly through the production of cytokines. Mouse NK cells also were reported to act as rheostats of antiviral T cell responses, both through direct interactions with T cells and indirectly via interactions with dendritic cells (34). In this study, we did not detect any significant correlation between the early NK cell responses and the subsequent CD4+ or CD8+ T cell responses, as measured by Ki67 (data not shown), indicating that the NK cell response is not a major determinant of T cell responses in vivo. However, it remains possible that the response by NK cells could skew the quality of subsequent T cell responses (e.g., by increasing Th1 polarization through IFN-γ priming of dendritic cells). Moreover, it remains unknown whether NK cells can directly target YFV-infected monocytes, dendritic cells, or hepatocytes. Thus, future studies are warranted to investigate the direct and indirect effects of NK cells on the control of YFV infection.

Mouse NK cells can exhibit memory-like properties, including enhanced recall responses to viruses (35, 36) and hapten (36). Interestingly, a brief in vitro priming of human NK cells with IL-12 and IL-18, followed by resting conditions, was recently reported to result in enhanced IFN-γ responses to cytokines and target cells up to 3 wk after the priming, indicating a long-lasting imprint on the NK cell function by the priming (37). The increased functional responses to stimulation with IL-12 observed after YFV infection indicated that NK cells were indeed primed in vivo (Fig. 2). However, the increase in function coincided with the peak in type I/III IFN and viral load, and it decreased rapidly back to the levels observed at baseline, suggesting that vaccination against YFV may not result in sustained memory-like imprints in NK cell function. In conclusion, our data provide a framework for understanding the regulation of human NK cell responses to YFV. The NK cell response to experimental YFV infection in vivo is IFN dependent and regulated primarily by NK cell differentiation, independently of the education or inhibition via inhibitory KIRs.

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Disclosures

The authors have no financial conflicts of interest.

References

### Supplementary table I: KIR and KIR-ligand genotype of donors in the study.

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