HUMAN IMMUNOLOGY

Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility

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Innate natural killer (NK) cells are diverse at the single-cell level because of variegated expressions of activating and inhibitory receptors, yet the developmental roots and functional consequences of this diversity remain unknown. Because NK cells are critical for antiviral and antitumor responses, a better understanding of their diversity could lead to an improved ability to harness them therapeutically. We found that NK diversity is lower at birth than in adults. During an antiviral response to either HIV-1 or West Nile virus, NK diversity increases, resulting in terminal differentiation and cytokine production at the cost of cell division and degranulation. In African women matched for HIV-1 exposure risk, high NK diversity is associated with increased risk of HIV-1 acquisition. Existing diversity may therefore decrease the flexibility of the antiviral response. Collectively, the data reveal that human NK diversity is a previously undefined metric of immune history and function that may be clinically useful in forecasting the outcomes of infection and malignancy.

INTRODUCTION

Diversity is a fundamental characteristic of the lymphocyte lineage. It is typically considered in the context of adaptive B and T lymphocytes. These cells rearrange antigen recognition receptors during development, generating the diversity necessary to recognize a vast array of potential antigens. Adaptive lymphocyte diversity is shaped early in life—the repertoire of both the T cell receptor (TCR) and immunoglobulin begin to form early in fetal development (*1*). The TCR repertoire is completely formed at birth (*2*) and the immunoglobulin repertoire by 2 months of age (*3*, *4*). Natural killer (NK) cells, the third lymphocyte lineage, also have an extraordinarily diverse repertoire (*5*, *6*). However, unlike the somatic recombination of antigen-specific T and B cell receptors, NK cell diversity is generated by the combinatorial assortment of germline-encoded activating and inhibitory receptors expressed at the cell surface. These receptors include the following: killer immunoglobulin-like receptors (KIR), which recognize human leukocyte antigen A (HLA-A), HLA-B, HLA-C, and other ligands; leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1), which binds a conserved region in all HLA class I proteins; natural cytotoxicity receptors (NCRs), which recognize a variety of pathogen, tumor, and self-derived ligands; C-type lectin–like receptors, which recognize HLA-E; and signaling lymphocyte activation molecule (SLAM) family receptors, which recognize a variety of ligands in immune regulation. NK cells' unique rapid recognition and response system is shaped by the engagement of these receptors by ligands on virus-infected, malignant, or stressed cells (*7*). However, the roots and functional implications of this diverse repertoire remain poorly understood.

Because NK cells rely on combinatorial signaling from surface receptors, their phenotype and function are uniquely and closely linked. The canonical function of NK cells is cytolysis. Consistent with the critical nature of NK cell killing, impaired cytolysis is the primary diagnostic criterion in patients with functional NK cell deficiencies, whose uniting clinical feature is herpes virus susceptibility (*8*). NK cells also play both antiviral and regulatory roles via the release of soluble factors. Interferon-γ (IFN-γ) and tumor necrosis factor (TNF) secreted by NK cells can induce maturation and activation of T cells, macrophages, and dendritic cells (*9*). Thus, NK cells are both phenotypically and functionally diverse, yet unlike adaptive immune diversity, it is unclear what role this diversity plays in the immune response.

Viral infection provides a setting to interrogate the role of this diverse repertoire of NK cells in an immune response. NK cells are implicated in the control of many viral infections, including HIV type 1 (HIV-1), West Nile virus (WNV), Epstein-Barr virus (EBV), and human cytomegalovirus (CMV) (*10*–*15*). In particular, many studies have suggested that NK cells play a role in HIV-1 control through specific host–viral protein interactions. For instance, HIV-1–infected individuals with certain KIR-HLA genotypes differ in their NK cell responses to HIV-1–infected cells and progression to AIDS (*16*). Additionally, HIV-1–encoded proteins allow infected $CD4^+$ T cells to escape NK cell recognition by down-modulating their surface ligands for activating NK receptors (*17*–*19*). NK cells have also been implicated in the initial acquisition of HIV-1, but their role remains unclear. NK cell activation has been both positively (*20*) and negatively (*21*, *22*) associated with the risk of HIV-1 acquisition. These apparently conflicting data may be influenced by the inability of previous studies to examine the full spectrum of NK cell subpopulations. Here, we use mass cytometry to deeply interrogate the features that shape NK cell diversity and its implications for an antiviral response. Using an antibody panel comprising 41 NK cell–focused parameters, we interrogate the role of NK cells in the HIV-1 response in both in vitro experiments and a unique in vivo cohort. We validate our findings using a second pathogen, WNV, demonstrating the generalizability of our results. By integrating this large-scale epidemiological investigation, pioneering technology, and functional immunological assays, we uncover the functional and clinical significance of diversity within the human NK cell repertoire.

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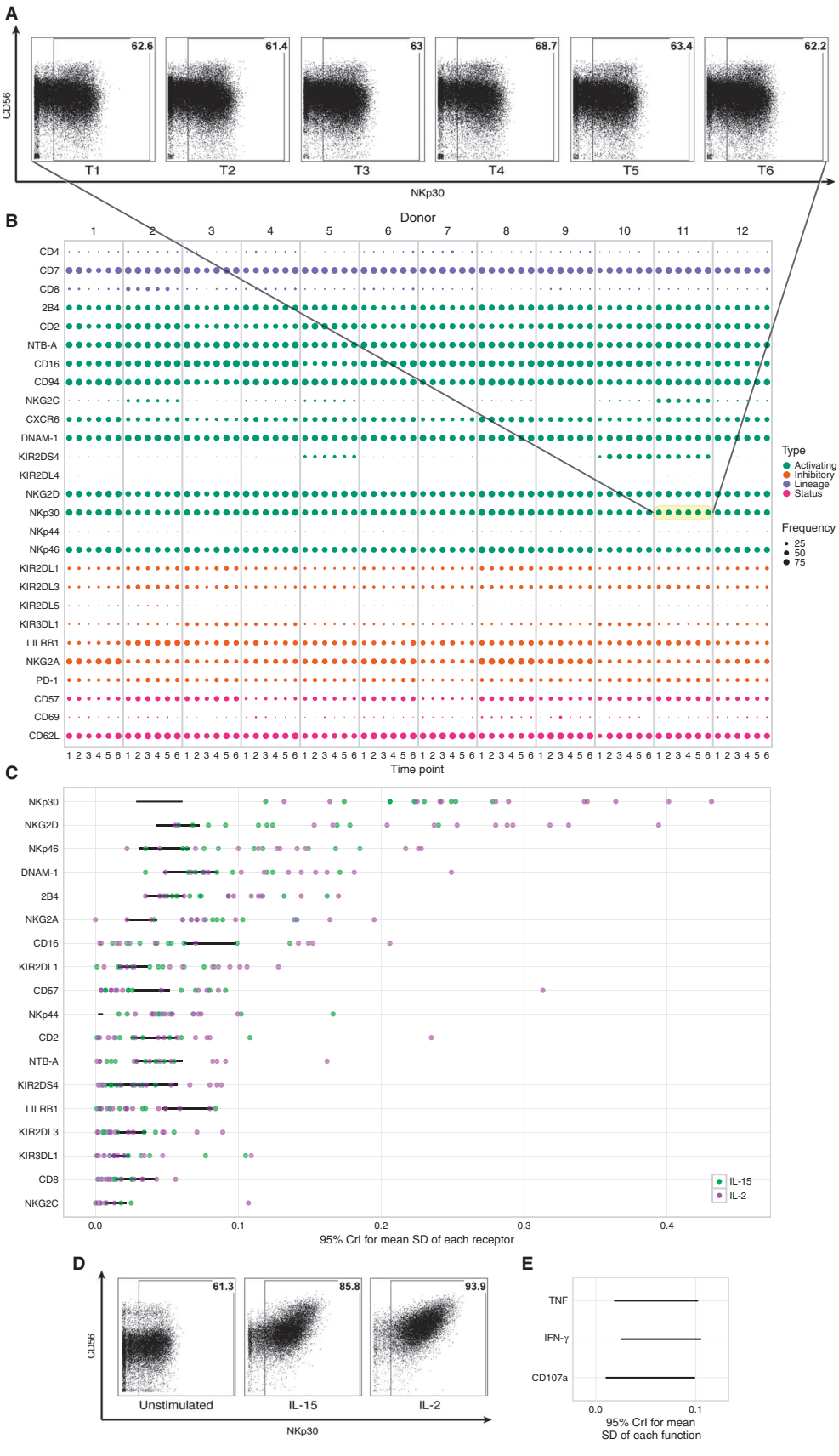

Fig. 1. Human NK cell repertoire and function are stable for 6 months in an individual, yet can be rapidly mobilized by exogenous factors. (**A**) NKp30 expression over 6 months in donor HIP11. Time points T1 to T5 occurred weekly within a 6-week period; T6 occurred 6 months later. (**B**) Stability of the human NK repertoire based on single receptor expression patterns at T1 to T6. Columns represent six blood draws for a single donor; circle size represents frequency of receptor expression. $n = 12$. (**C**) Mean SDs of each receptor's expression over the 6-month period given by Bayesian inference hierarchical model (see Methods for details). Black bars, 95% CrI for mean SD of each receptor for T1 to T6; Healthy Immune Profiling (HIP) donors. Colored dots, mean SD for each donor after in vitro IL-15 ($n = 10$) or IL-2 ($n = 12$) stimulation for 72 hours; Stanford Blood Bank (SBB) donors. (**D**) Up-regulation of NKp30 after 72-hour treatment with IL-15 or IL-2 in donor SBB10. (**E**) Stability of NK cell function over 6 months. The diagram shows the 95% CrI for mean SD of each function (CD107a, IFN-γ, and TNF) after 721.221 stimulation at T1 to T6. $n = 3$.

RESULTS

The human NK repertoire is stable for 6 months in vivo, yet can be rapidly modified

NK cells are considered innate short-lived effectors with a turnover time of about 2 weeks (*23*, *24*). Although recent studies have highlighted the extreme diversity of the NK cell repertoire (*5*, *25*), a systematic assessment of the stability of their receptor repertoire over time, an essential baseline for understanding their role in viral acquisition, has never been established. We therefore built a longitudinal profile of healthy human NK repertoire stability. We used a 41-parameter NK-focused mass cytometry panel (table S1) to profile blood samples from 12 healthy subjects drawn weekly for five of six sequential weeks and again 6 months later (table S2). We used a serial gating strategy to define NK cells (fig. S1) and to evaluate the expression of each marker (fig. S2). As expected, especially for genetically variable receptors such as KIR2DS4, interindividual variability in receptor expression levels is apparent. However, the intraindividual expression of each receptor on NK cells over time is

extremely stable (Fig. 1, A and B and table S4). Using a hierarchical model built by Bayesian inference, we estimate an extremely low SD of each receptor over time on the basis of its posterior distribution [95% credible intervals (CrI) for mean SD <0.1; Fig. 1C, bars]. We identified similar levels of stability when the NK population was further subdivided into $CD56^{bright}$ and $CD56^{dim}$ populations (fig. S3). Therefore, even if individual NK cells are short-lived, homeostatic mechanisms maintain stable population-level expression of individual receptors in vivo.

To establish the boundaries of this stability, we elicited a short-term (72-hour) repertoire perturbation using cytokine treatment of human NK cells in vitro. Treatment with interleukin-15 (IL-15) or IL-2 results in marked modifications of the NK repertoire (Fig. 1C, circles and table S5). Differences between cytokine-treated and untreated cells are up to eightfold greater than SDs in the serially sampled stable peripheral blood NK repertoire. The most pronounced changes in response to cytokine treatment are increased levels of activating receptors, primarily NKp30 and NKG2D (Fig. 1, C and D). These results confirm cytokine-driven receptor induction (*26*, *27*) and further demonstrate that the diversity of the NK repertoire can be altered by cytokine stimulation in vitro.

To determine whether in vivo NK repertoire stability is also associated with stability in NK functional capacity, we cultured NK cells with major histocompatibility complex class I (MHC-I)–deficient 721.221 cells, which are targeted and lysed by NK cells. Production of IFN-γ, TNF, and CD107a in response to 721.221 cells is also stable over time (95% CrI for mean SD <0.11; Fig. 1E, fig. S4, and table S6).

This analysis illustrates that the average expression of each receptor and function is maintained within a population of NK cells but does not address whether the same receptor combinations are consistently maintained on the same cells. We therefore used an ecology-based approach to quantify diversity (*5*). We used Boolean gating to define positive populations for each marker (fig. S2), determined the frequency of each of the >11,000 single-cell phenotypic marker combinations, and calculated the inverse Simpson index to measure the diversity of the resulting combinatorial cellular phenotypes (Fig. 2). This analysis revealed that total NK diversity differs among individuals. Yet, as for each single receptor, intraindividual variability in diversity over six time points of the longitudinal profile is extremely low (Fig. 2; mean variance, 64.5; 95% CrI, 50 to 82). Further analysis of the top 10 most frequently detected subpopulations revealed exquisite stability even at this level of granularity (fig. S5). These data indicate that NK receptor levels are stable not only at the population level but also as single-cell combinatorial phenotypes.

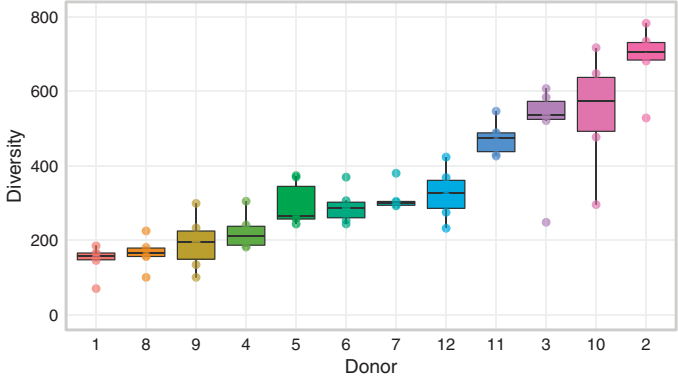

Fig. 2. NK cell diversity is stable and donor-specific. T1 to T6 box plot [box = median + interquartile range (IQR); whiskers = 1.5 × IQR] distributions of NK diversity (inverse Simpson Index) in HIP donors (n = 12).

NK cell diversity accumulates over the course of a lifetime

We next sought to understand the source of NK diversity within an individual. We observed that the range of NK diversity observed within our cohort correlates positively with each donor's mean expression of the NK terminal differentiation marker CD57 ($P = 7.8 \times 10^{-3}$, Fig. 3A) and negatively with mean expression of the NK immaturity marker NKG2A ($P = 7.5 \times 10^{-5}$, Fig. 3B). Because CD57 expression is induced on NK cells after cell or cytokine stimulation (*28*) and increases with age, directly opposing NKG2A expression (*29*), we hypothesized that NK diversity accumulates as a result of repeated stimulation over the course of a lifetime.

To test this hypothesis, we profiled NK cells from 20 umbilical cord blood samples from healthy singleton pregnancies. These samples represent an undifferentiated NK population for comparison with the immune-experienced adult donors (table S7). NK cells from newborns express significantly less CD57 ($P = 8.9 \times 10^{-9}$, Fig. 3C) and significantly more NKG2A ($P = 3.7 \times 10^{-5}$, Fig. 3D) than adult NK cells, in accordance with previous work (*29*). They are also significantly less diverse ($P = 1.4 \times 10^{-4}$, Fig. 3E) than adult NK cells. These data expand on work in individual viruses (*10*, *12*) to suggest that experience matures and diversifies the human NK cell repertoire.

In our adult cohort, NK diversity is not significantly correlated with age (fig. S6), suggesting that diversity likely results not simply from aging but from the cumulative effects of an individual's uniquely divergent NK cell response history. To confirm this idea, we assessed the uniqueness of each adult individual's baseline repertoire. Notably, phenotypes of immature NK cells expressing NKG2A are highly shared among donors. The proportion of phenotypes expressing NKG2A is significantly positively associated with the number of donors sharing the phenotype ($P = 0.031$, Fig. 3F and table S8). By contrast, phenotypes of differentiated NK cells expressing CD57 are much more likely to be unique to an individual. The proportion of phenotypes expressing CD57 is significantly negatively associated with the number of donors sharing the phenotype ($P = 5.6 \times 10^{-5}$, Fig. 3F). We detected no specific effects of CMV serostatus on NK diversity, CD57 expression, or NKG2A expression (fig. S7). Considerable diversity in response to exogenous stimuli therefore yields a repertoire that grows progressively individualized.

NK cell diversity correlates with acquisition of HIV-1 in Kenyan women

Because NK repertoire diversity is consistently maintained in healthy humans and is associated with differentiation, we hypothesized that its role in the immune response is important. We therefore asked if it correlates with the strength of the antiviral response. We were unable to detect an association between NK diversity and the ability to suppress HIV-1 infection in an in vitro assay (fig. S8) and therefore used an in vivo cohort to ascertain the disease relevance of NK diversity. We labeled preinfection samples preserved from a case-control study of Kenyan women who did or did not acquire HIV-1 over a 9- to 12-month follow-up period (Mama Salama Study, table S3). Samples were selected from a cohort of 1304 women with a total of 25 incident HIV-1 infections, of which 13 had a preinfection peripheral blood mononuclear

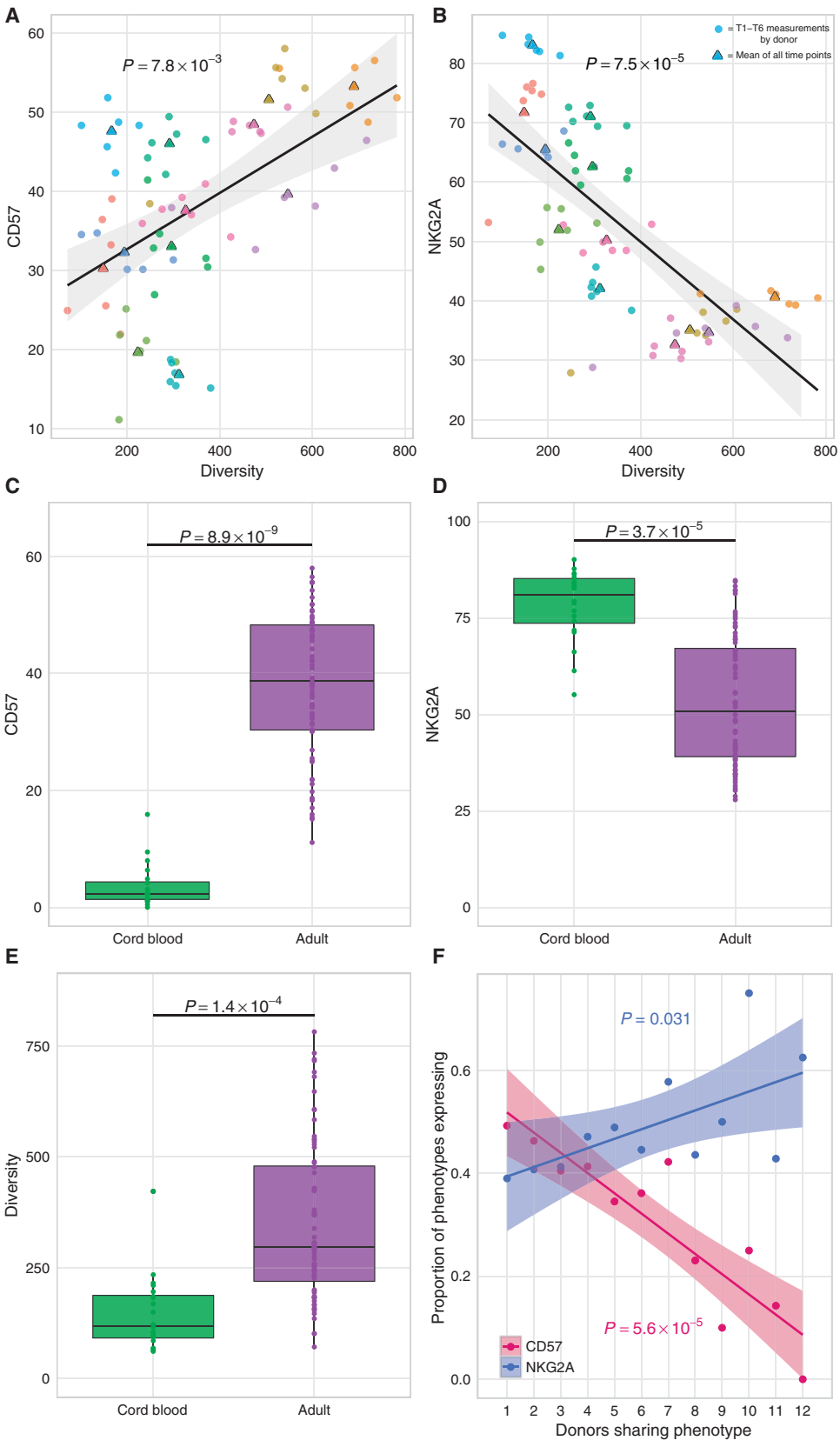

Fig. 3. Diverse NK repertoires contain differentiated NK cells unique to an individual. (**A** and **B**) CD57 and (A) NKG2A (B) frequency on NK cells versus NK cell repertoire diversity for HIP donors. Circles, individual time points. Triangles, mean of all time points in each individual. Linear regression, black; 95% CI, gray. Generalized estimating equation, AR-1 correlation structure: CD57, $P = 7.8 \times 10^{-3}$; NKG2A, $P = 7.5 \times 10^{-5}$. $n = 12$. (**C** to **E**) CD57 frequency (C), NKG2A frequency (D), and NK diversity (inverse Simpson index) (E) on umbilical cord bloods ($n = 20$) versus HIP donors ($n = 12$). Mann-Whitney U tests: CD57, $P = 8.9 \times 10^{-9}$; NKG2A, $P = 3.7 \times 10^{-5}$; diversity, $P = 1.4 \times 10^{-4}$. (**F**) Convergence analysis of NK phenotypes. Total proportion of phenotypes expressing CD57 (magenta) or NKG2A (blue) shared at baseline. Linear regression: CD57, $P = 5.6 \times 10^{-5}$; NKG2A, $P = 0.031$. $n = 12$, SBB donors.

cell (PBMC) sample available. Controls were matched to cases by a risk score calculated on the basis of epidemiologic and clinical parameters associated with increased HIV-1 acquisition risk in this region. These included the woman's age (continuous), knowledge of partner's receipt of an HIV-1 test (dichotomous), marriage status (dichotomous), and history of trading sex for money or goods (dichotomous) (*30*). In addition, controls and cases did not significantly differ in the frequency with which they tested positive for a sexually transmitted infection [2 (15%) of cases, 5 (23%) of controls; $P = 0.71$, χ^2] or the frequency with which they reported having unprotected sex in the past month [5 (38%) of cases, 12 (52%) of controls; $P = 0.49$, χ^2] (table S3). Pre-infection samples were selected at the time closest to viral encounter, with a mean estimated time to infection of 91 days (IQR, 52.5 to 187 days) (table S3).

In this cohort, donors with high NK cell diversity were significantly more likely to acquire HIV-1 (Fig. 4A). Each 100-point increase in diversity, within a range of nearly 500, is associated with a 2.5-fold increased risk of HIV-1 acquisition [95% confidence interval (CI), 1.2 to 6.2]. Diversity of NK receptors expressed on $CD4^+$ or $CD8^+$ T cells is not associated with HIV-1 acquisition (Fig. 4B), nor is any single marker expressed on $CD4^+$ T, $CD8^+$ T, or NK cells (Fig. 4C). These data indicate that, in this population, high NK cell diversity is significantly associated

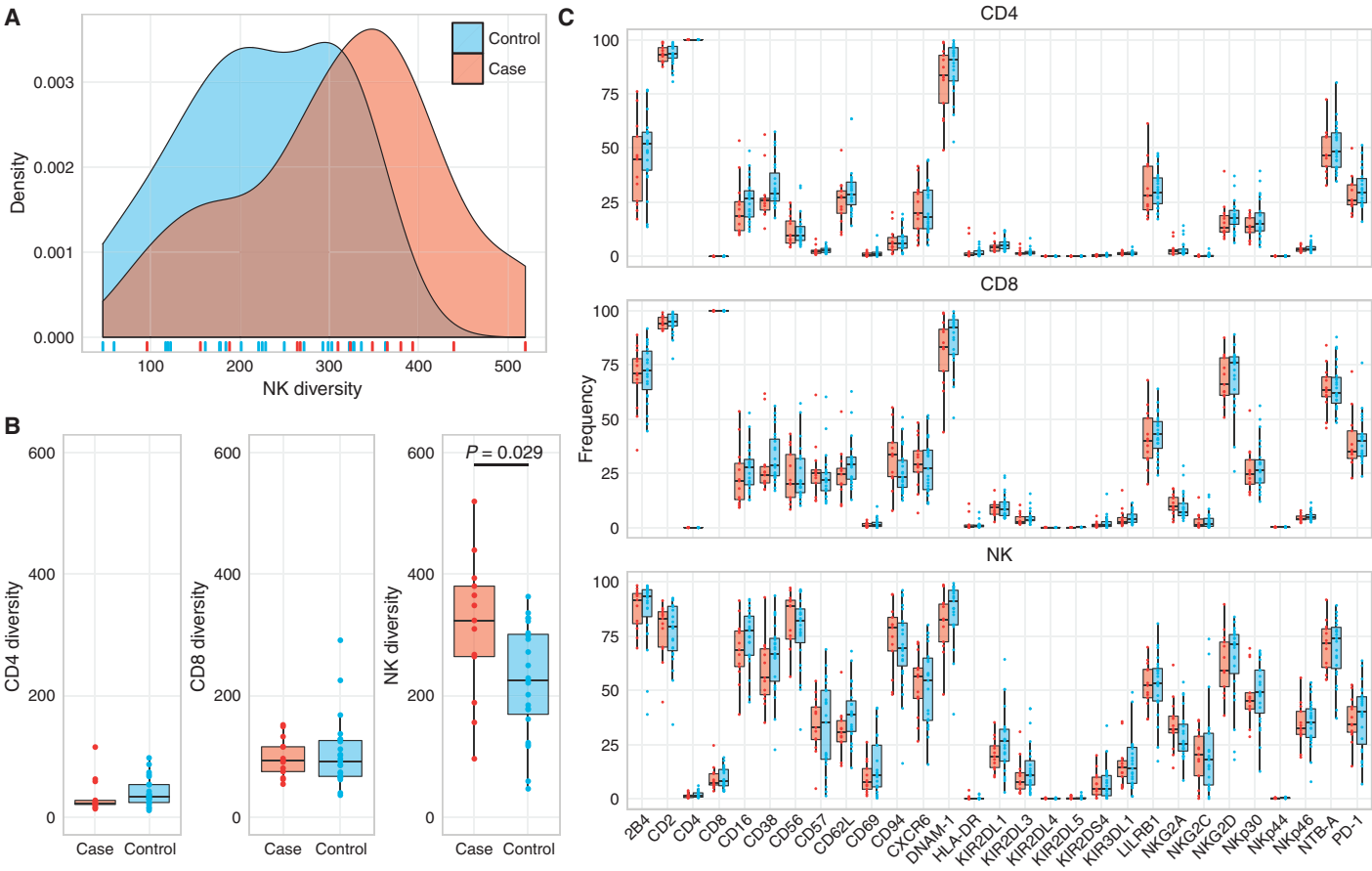

Fig. 4. NK cell repertoire diversity correlates with acquisition of HIV-1 in Kenyan women. (**A**) Distributions of NK cell repertoire diversity (inverse Simpson index) in cases (n = 13) and matched controls (n = 23) (logistic regression odds ratio per 100-point diversity increase, 2.5; 95% CI, 1.2 to 6.2). (**B**) Box plots of NK receptor diversity on $CD4^+$ T, $CD8^+$ T, and NK cells in cases and controls (logistic regression of diversity versus HIV-1 acquisition for $CD4^+$ and $CD8^+$ T cells is not significant; NK, P = 0.029). (**C**) Box plots show distributions of expression frequency of each receptor on $CD4^+$ T, $CD8^+$ T, and NK cells in cases versus controls. All case-control receptor comparisons are not significant by logistic regression.

with increased risk of HIV-1 acquisition. They further suggest that, unlike T and B cell diversity, high NK diversity may actually be disadvantageous in a clinical setting.

Diverse NK repertoires contain differentiated NK cells skewed toward cytokine production

We next sought to explain how NK diversity and maturity could influence disease outcomes. We therefore evaluated the function of differentiated NK cells in an antiviral response. We again chose HIV-1 as a model virus because NK cells have been strongly implicated in its pathogenesis, yet unlike other chronic infections such as CMV (*10*, *12*), HIV-1 does not appear to be associated with a specific NK cell subpopulation. Consistent with this idea, no unique phenotypic subset of NK cells preferentially responds to autologous HIV-1–infected $CD4^+$ T cells (fig. S9). However, in response to HIV-1–infected cells, $CD57^+$ NK cells produce significantly more IFN-γ ($P = 3.42 \times 10^{-3}$) and TNF (P = 0.021) than do $CD57^-$ cells (Fig. 5, A and B and table S9). $CD57^+$ cells also degranulate less (CD107a, P = 0.027; Fig. 5C) and show a trend toward dividing less [5′-iododeoxyuridine (IdU) (*31*), P = 0.077; Fig. 5D] than $CD57^-$ NK cells. Terminally differentiated repertoires thus favor cytokine production and not degranulation or division in response to HIV-1–infected cells. Similarly, within each donor, cytokine-producing NK cells are more diverse than non–cytokine-producing NK cells (fig. S10). These data suggest that differentiated NK cells occupy a specific niche within the NK population specialized for cell-cell communication rather than viral control or cellular expansion.

Short-term exposure to virus-infected cells augments NK diversity

If a diverse repertoire of functionally altered NK cells accumulates over the course of a lifetime that includes viral infections, we hypothesized that a population of NK cells responding to virus-infected cells in culture should diversify. We therefore evaluated the NK repertoire after exposure of NK cells to HIV-1–infected $CD4^+$ T cells in vitro.

We used correspondence analysis, designed to capture the complexity of incidence data, to reduce dimensionality and to complement our ecological diversity analyses (Fig. 6, A to F, and fig. S11). Figure 6A shows the projection of >11,000 Boolean phenotypes detected from all donors in the absence of HIV-1–infected $CD4^+$ T cells. In the presence of HIV-1–infected $CD4^+$ T cells, the cloud of NK phenotypes expands as they differentiate from each other (Fig. 6B). The sum of squared

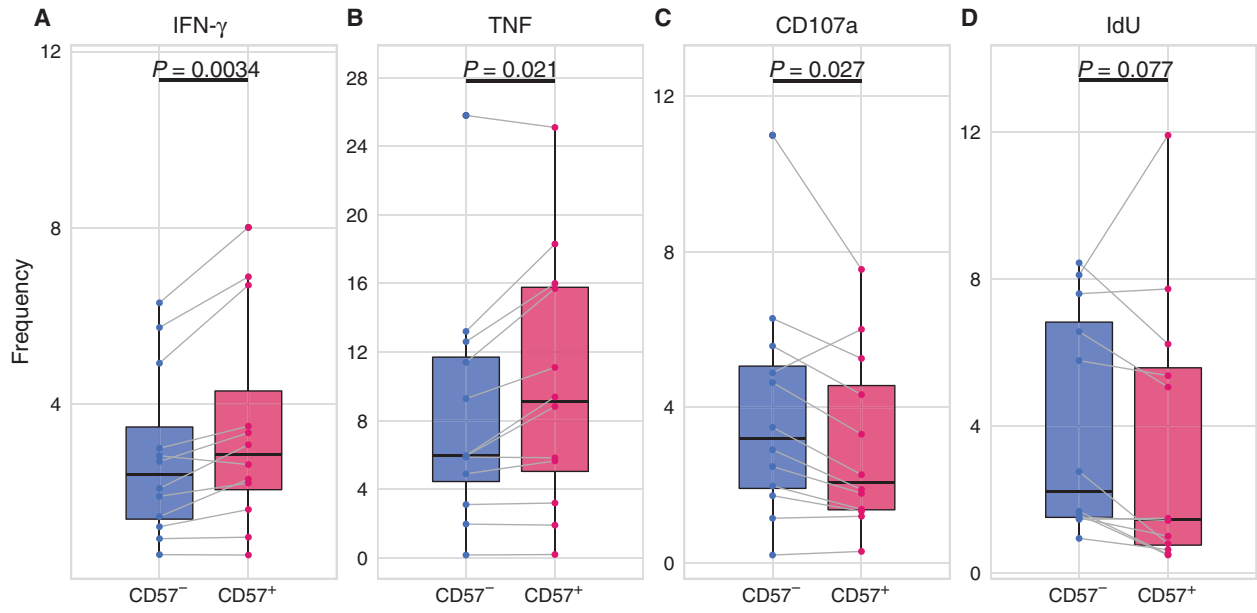

Fig. 5. Differentiated NK repertoires are skewed toward cytokine production in response to virus-infected cells. (**A** to **D**) Frequency of cells producing IFN-γ (A), TNF (B), CD107a (C), and IdU (D) in CD57$^-$ or CD57$^+$ NK cells after stimulation with IL-2 and HIV-1–infected target cells. Wilcoxon signed-rank tests: IFN-γ, $P = 3.4 \times 10^{-3}$; TNF, $P = 0.021$; CD107a, $P = 0.027$; IdU, $P = 0.077$. SBB donors, $n = 11$.

distances from the centroid, a measure of the total dispersion of the NK repertoire, is significantly higher in the presence of HIV-1–infected cells ($P = 0.038$, Fig. 6C), demonstrating short-term in vitro repertoire diversification.

To confirm that viral-induced NK diversity is not an HIV-1–specific phenomenon, and to demonstrate that the diversity generated in our Kenyan cases was not HIV-1–specific, we used a parallel assay to test NK diversification in response to WNV. WNV is a mosquito-borne flavivirus that is estimated to have infected more than 3 million Americans and can cause neuroinvasive disease (*32*). Despite its distinction from HIV-1 in classification, pathogenesis, and method of transmission, NK cells have also been implicated in WNV control in humans (*13*, *33*). NK repertoire dispersion is also observed during the response to WNV ($P = 1.0 \times 10^{-4}$; Fig. 6, D to F). Total NK cell diversity is also significantly increased in the presence of HIV-1–infected cells ($P = 0.034$, Fig. 6G and table S10) and WNV-infected cells ($P = 0.0033$, Fig. 6H and table S11). These data show that short-term exposure to virus-infected cells augments NK diversity.

Together with our functional studies, these data are consistent with a model in which previous response drives differentiation and divergence in the NK repertoire (Fig. 6I). At birth, NK cells have very low diversity but have high capacity for a range of responses. In response to viral stimulation over the course of a lifetime, the NK repertoire diversifies, becoming functionally specialized and increasingly unique to each individual. As a result, it lacks the adaptability required for responses to novel antigens.

DISCUSSION

Here, we demonstrate that human NK cell diversity has functional significance in the setting of viral infection. NK receptors, functions, and diversity are stable for 6 months in healthy adults, yet are rapidly modifiable in response to cytokine stimulation in vitro. We found that NK diversity is low at birth and higher in adults and that individuals become increasingly unique as their repertoires diversify. This is exemplified by in vitro diversification in response to productively infected HIV-1– and WNV-infected cells. Differentiated NK repertoires are associated with functional skewing toward cytokine production at the cost of degranulation and division. High NK diversity correlates with HIV-1 acquisition in a case-control cohort of Kenyan women in vivo. NK diversity therefore appears to be a functional immune metric that reflects an individual's unique history of pathogen encounter and correlates with future susceptibility to disease.

Diversity of antigen-specific receptors is a well-documented asset in adaptive immunity (*34*), where greater diversity increases the likelihood of a responsive subpopulation of lymphocytes or immunoglobulin. Adaptive immunity thus maintains a flexible diversity reserve both for initial pathogen encounters and to preserve reactivity in the case of immune evasion. However, the innate system has neither of these requirements. Instead, the capacity for rapid differentiation may be more advantageous to quickly adjust its receptor repertoire to a given threat. NK cell diversity may therefore be a side effect of accumulated short-term responses, the only goal of which is rapidity. Thus, the adaptive immune-based concept of diversity as an asset may not reflect the prioritization of speed over specificity that is characteristic of the innate response.

CMV is the only virus known to drive the expansion of a specific NK population (CD57$^+$NKG2C$^+$) in humans (*10*, *12*). However, rather than being a restricted, clonal population, the pool of "memory-like" NK cells elicited by this virus is considerably heterogeneous with regard to the expression of other markers (*5*). Taken with our data that viral challenge augments NK diversity in vitro, this finding suggests that expansion of NK subpopulations, and potentially NK memory, may be associated with increased diversity. This contrasts with the decreased diversity associated with oligoclonal expansions resulting from

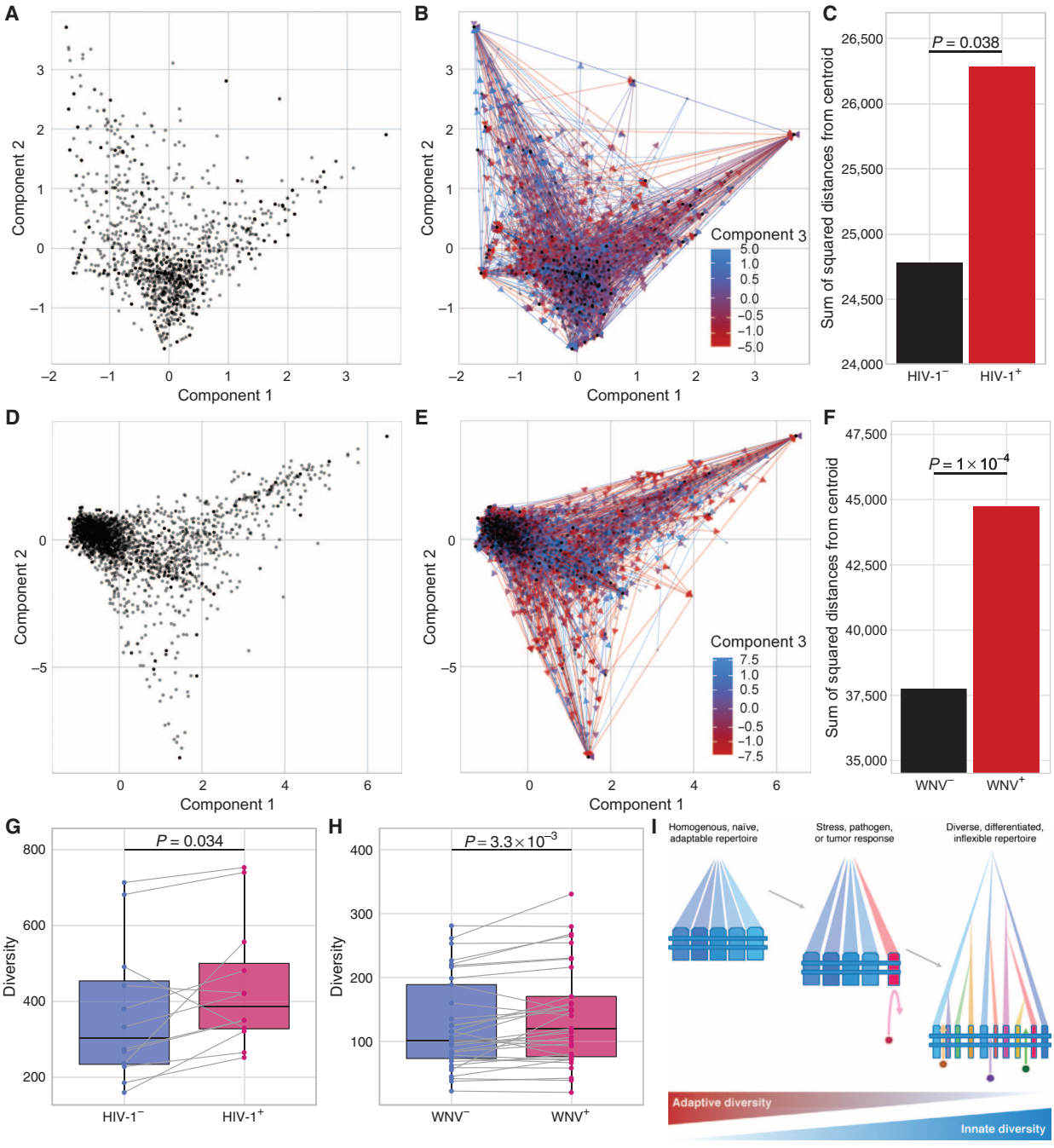

Fig. 6. Short-term exposure to virus-infected cells augments NK diversity. (**A** to **F**) Correspondence analysis of Boolean phenotypes for HIV-1 (A to C, $n = 12$) or WNV (D to F, $n = 33$). (A and D) Components 1 and 2 of analysis of all phenotypes after culture with IL-2 alone (HIV-1) or unstimulated (WNV). For HIV-1, 11,523 total phenotypes were detected, and for WNV, 8397 phenotypes. (B and E) Components 1 and 2 and total change in component 3 (colored arrows) after culture with IL-2 + HIV-1–infected $CD4^+$ T cells (B) or after PBMC infection with WNV (E). (C and F) Sum of squared distances to centroid of point cloud in NK cells cultured with IL-2 alone ($HIV\text{-}1^-$) or IL-2 + HIV-1–infected $CD4^+$ T cells ($HIV\text{-}1^+$) (C) or PBMCs uninfected with WNV (WNV^-) or with WNV infection (WNV^+) (F). Permutation test (10,000×) on sum of squared distances from centroid: HIV-1, $P = 0.038$; WNV, $P = 1.0 \times 10^{-4}$. (**G** and **H**) Diversity of NK cells cultured with IL-2 alone ($HIV\text{-}1^-$) or IL-2 + HIV-1–infected $CD4^+$ T cells ($HIV\text{-}1^+$) (G, $n = 12$) or uninfected with WNV (WNV^-) or with WNV infection (WNV^+) (H, $n = 33$); Wilcoxon signed-rank tests: HIV-1, $P = 0.034$; WNV, $P = 0.0033$. (**I**) Model for NK cell diversity and differentiation. A naïve NK repertoire is an effective fence for infection prevention. As the NK repertoire encounters novel pathogens over the course of a lifetime, it diversifies with each response mounted. Its increasingly branched, diffuse nature increases the chance that a newly encountered virus will penetrate the barrier.

adaptive memory. Experiments in murine models, including humanized mice, will be important for addressing this hypothesis.

Mass cytometry has enabled in-depth examination of NK repertoire diversity in a manner akin to tetramer and sequencing techniques for the interrogation of T and B cell repertoires. T and B cell diversity peaks early in life and decreases with age, as oligoclonal memory populations of both T and B cells pervade, diminishing diversity for both TCR (*35*, *36*) and immunoglobulin (*37*, *38*). This honing of the repertoire allows for rapid recall responses to previously encountered antigens at the cost of the ability to respond to new threats. By contrast, NK cell diversity appears to accumulate as a result of environmental exposures. This evolutionary "bet-hedging" strategy provides a logical advantage in maintaining an effective immune response over the course of a lifetime. This would have been especially relevant during most of our evolutionary history, when human life span was more limited. A loss of responsiveness to novel antigens would have had fewer years to be disadvantageous than in modern times.

Our model is supported by recent murine work showing that NK cells specific for murine CMV or alloantigens no longer respond to heterologous stimuli (*39*). Similarly, we show that women with higher NK diversity have an increased likelihood of successful infection with HIV-1, implying that they may be less able to mount an effective antiviral response. NK cells are present in the female genital tract and are important for antiviral defense (*40*, *41*). Impaired NK degranulation and cytotoxicity in this early mucosal response could increase the likelihood of productive infection. A diverse NK repertoire may additionally be associated with a state of immune differentiation associated with decreased reactivity beyond the NK compartment.

The stability of the NK repertoire within an individual on the order of months demonstrates its potential for utility as a clinical marker. We postulate that the increase in diversity after viral exposure in humans is very gradual in adulthood, explaining why we did not observe increases in adult NK diversity over 6 months. Following individuals during childhood and over many years will improve this resolution.

In addition to its short duration, our study also has several other limitations. Our assessment of functional stability included only NK responses to MHC-I–deficient target cells. Stimulations to assess the stability of additional types of NK responses will add an important level of detail to this understanding. In addition, although the Mama Salama Study is one of the very few HIV-1 acquisition studies of adequately large scale to test our model of immune differentiation, additional acquisition cohort studies with samples stored before infection with other viruses will be required to further test this model. Additional cohorts will also facilitate the disentanglement of exposure versus risk, a challenge inherent in case-control studies. Tracking NK diversity during acute and chronic infections, especially in a study of HIV-1 elite controllers, will further illuminate its dynamics over the course of an antiviral response.

Harnessing parametric dimensionality, rather than relying on large sample sizes, makes this approach especially advantageous in a clinical setting. Many analytical techniques use serial partitioning to detect changes in increasingly rare cell populations. Here, we show that the totality of the NK repertoire, rather than a particular cell subpopulation, correlates with HIV-1 susceptibility. Recent work in many fields, including genomics, has demonstrated the ubiquity of biological heterogeneity and rarity of disease outcome prediction from a single marker. Our work provides a framework for the adoption of this paradigm and should serve as a valuable model as the number of parameters captured by single-cell technologies continues to expand.

In summary, we used high-dimensional single-cell data to discover the functional significance of NK cell diversity in the setting of viral infection. Unlike T and B cell diversity, NK cell diversity increases with experience and does not appear to be beneficial to de novo antiviral responses. These results indicate that assessing the previous experience of the innate immune system could have functional and clinical utility in forecasting the vigor of its response. The upcoming challenge will be to further define the implications of NK diversity in a variety of disease contexts, as well as to rationally design therapeutic strategies that can modulate NK diversity in the settings of infection and malignancy.

MATERIALS AND METHODS

Study design

The objective of this study was to define the stability and functional significance of NK cell diversity in the setting of viral infection. To this end, we used several different approaches. First, we enrolled 12 healthy adults at Stanford University into the HIP cohort to assess the phenotypic and functional stability of the NK cell repertoire. Second, cord blood samples were obtained from singleton pregnancies at Stanford Hospital to understand the impact of development on the NK cell repertoire. Third, samples from a prospective cohort study of HIV-1 acquisition in pregnant and postpartum Kenyan women (Mama Salama Study) (*30*) were used to assess immune parameters associated with HIV-1 acquisition. The Mama Salama Study enrolled 1304 women and was powered to accrue 60 to 80 women acquiring HIV-1, though only 25 acquired HIV-1 during the study. Of those women, 13 had samples available from before infection, and all of these women were included in our study. A case-control design was used to select two cases for each control on the basis of a risk score for HIV-1 acquisition, as detailed below. Fourth, blood samples were purchased from the SBB to assess the anti–HIV-1 NK cell response in in vitro functional experiments. Fifth, healthy volunteers were enrolled at Yale University to study responses to WNV. For all studies except the Mama Salama Study, sample sizes were determined both by feasibility and for representation of diverse KIR and HLA genotypes. All studies were observational; randomization and blinding were not used. All subjects gave fully informed and written consent. All studies were performed in accordance with the Declaration of Helsinki.

Subjects and sample processing

The repertoire stability study was performed at Stanford University and approved by the Institutional Review Board of Stanford University. Twelve healthy volunteers (6 male and 6 female, ages 22 to 45 years) were recruited specifically for this study (HIP cohort, table S2), designed to analyze the stability of the human NK repertoire. Five blood draws over a 6-week period were collected, each spaced at least 1 week apart. A final sample was collected 6 months later. None of the individuals in the study reported acquisition of any chronic infections over the course of the study. HIP5 reported an upper respiratory infection at time point T4. PBMCs were separated using Ficoll density gradient centrifugation and were cryopreserved. All samples from each donor at all time points were thawed, stained, and acquired on a mass cytometer simultaneously. CMV serostatus was

determined by enzyme-linked immunosorbent assay (Gold Standard Diagnostics).

The cord blood study was designed to understand the effect of differentiation on the NK repertoire. Cord blood samples were obtained from singleton pregnancies at Stanford Hospital under a protocol approved by the Stanford Institutional Review Board. Twenty healthy mothers were enrolled and cord blood was collected at birth using sterile cord blood collection units (Pall Medical). Cord blood mononuclear cells were separated using Ficoll density gradient centrifugation and were cryopreserved.

For HIV-1 in vivo studies, cryopreserved PBMC samples were obtained from the Mama Salama Study, a prospective cohort study of HIV-1 acquisition in pregnant and postpartum women (table S3). Women in this study were enrolled during pregnancy and tested for HIV-1 by nucleic acid amplification tests at enrollment, 28 and 36 weeks gestation, and 6, 14, 24, and 36 weeks postpartum; 25 women were identified as acquiring HIV-1. Thirteen cases were included in this analysis on the basis of acquiring incident HIV-1 infection and the availability of a PBMC sample at a visit before detection of infection. Controls were selected from women who did not acquire HIV-1 infection during the study and had a PBMC sample from the same pregnancy or postpartum study visit as the case. Cases were matched to two controls with the closest risk score for HIV-1 acquisition and study visit. Risk scores were generated based on age, knowledge of partner being tested for HIV-1, marital status, and history of trading sex. Cases and controls did not significantly differ in the frequency of sexually transmitted infections (syphilis, gonorrhea, chlamydia, or trichomoniasis). All PBMC samples from matched cases and controls were thawed, stained, and acquired on a mass cytometer simultaneously. Three controls with inadequate NK cell counts (<1200) were excluded before analysis. The Mama Salama Study was approved by the Institutional Review Boards of the University of Washington and Kenyatta National Hospital in Kenya.

For the HIV-1 in vitro studies, designed to interrogate the functional significance of the NK repertoire in the setting of HIV-1 suppression, leukoreduction system chambers were purchased from the SBB (SBB cohort, table S2). Subjects were fully de-identified. All experiments were performed on fresh PBMCs separated using Ficoll density gradient centrifugation.

For the WNV in vitro studies, designed to interrogate the functional significance of the NK repertoire in the setting of WNV suppression, healthy volunteers were enrolled after written informed consent approved by the Yale School of Medicine. Thirty-three subjects were included (19 female and 14 male; mean age, 44.8 years; SD, 23.7 years).

Staining and mass cytometry acquisition

Detailed staining protocols have been described (*42*, *43*). Briefly, cells were transferred to 96-well deep-well plates (Sigma), resuspended in 25 μM cisplatin (Enzo Life Sciences) for 1 min (*44*), and quenched with 100% serum. Cells were stained for 30 min on ice, fixed (BD FACS Lyse), permeabilized (BD FACS Perm II), and stained with intracellular antibodies for 45 min on ice. Staining panels are described in table S1. All antibodies were conjugated using MaxPar X8 labeling kits (DVS Sciences). Cells were suspended overnight in iridium interchelator (DVS Sciences) in 2% paraformaldehyde in phosphate-buffered saline (PBS) and washed 1× in PBS and 2× in H_2O immediately before acquisition. Stained cells were analyzed on a mass cytometer (Fluidigm).

Virus production and titration

Replication-competent viruses were made by transfection as described (*45*). Briefly, HIV-1 Q23-FL plasmids (subtype A, isolated about 1 year after infection) were transfected into 293 T cells using FuGENE (Promega). The medium was replaced after 24 hours. After 48 hours, supernatants were harvested, filtered (0.22 μm, Millipore), and stored at −80°C. The virus was titered on TZM-bl cells (*45*).

Cell purification, stimulation, and infection

Autologous NK cells and $CD4^+$ T cells were purified by magnetic-activated cell sorting via negative selection (Miltenyi). NK cells were cultured with IL-15 (2 ng/ml, PeproTech) or IL-2 (300 U/ml, Novartis) or left unstimulated for 2.5 days. $CD4^+$ T cells were activated with plate-bound anti-CD3 (OKT3, eBioscience), soluble anti-CD28/CD49d (BD Biosciences), and phytohemagglutinin (Sigma) for 2 days. All cells were cultured in RPMI + 10% fetal calf serum (FCS) + 2 mM L-glutamine + antibiotics [penicillin (100 U/ml), streptomycin (100 mg/ml); Life Technologies BRL/Life Technologies] at 37°C with 5% CO_2. For HIV-1 experiments, $CD4^+$ T cells were infected with Q23-FL via ViroMag R/L magnetofection (Oz Biosciences) [typical multiplicity of infection (MOI), 30 to 70; range of infection as measured by intracellular p24 was 0 to 12.7% of $CD4^+$ T cells] in 48-well plates. Magnetic bead–bound virus was allowed to infect $CD4^+$ T cells overnight on a magnetic plate. For WNV experiments, PBMCs were infected with virulent WNV (CT-2741) at MOI 1 for 24 hours as described (*46*). WNV studies were conducted in a biosafety level 3 facility licensed by the state of Connecticut and Yale University.

HIV-1 viral suppression assay

For mass cytometry experiments, unstimulated, IL-15–stimulated, or IL-2–stimulated NK cells were cocultured for 4 hours with or without autologous infected or uninfected $CD4^+$ T cells in the presence of brefeldin A (eBioscience), monensin (eBioscience), and anti-CD107a–APC-H7 (BD Biosciences) at a 1:1 ratio in 48-well plates. IdU (Sigma) was added at 5 μM for the final 15 min of co-incubation (*31*), followed by 20 mM EDTA (Hoefer). For fluorescence cytometry experiments, purified NK cells were cocultured for 48 hours with autologous infected $CD4^+$ T cells at a 3:1 ratio in 96-well plates. Cells were stained with CD3-V450 (BD Biosciences), CD8-FITC (BD Biosciences), and KC57-RD1 (Beckman Coulter) antibodies and were analyzed on a MACSQuant analyzer (Miltenyi). Viral suppression was calculated as [(%p24 in $CD4^+$ T cells only − %p24 in $CD4^+$ T with NK cells)/(%p24 in $CD4^+$ T cells only) × 100] on background-subtracted data.

Data processing and diversity calculations

FCS files were normalized within each cohort using Normalizer v0.1 MCR (MATLAB Compiler Runtime) (*47*). Gating was performed in FlowJo v9 (TreeStar). Diversity was computed on the basis of the Boolean expression of 16 receptors: NTB-A, DNAM-1, CD57, CD16, KIR2DS4, LILRB1, NKp46, NKG2D, NKG2C, 2B4, NKp30, KIR3DL1, KIR2DL1, KIR2DL3, NKG2A, and CD56. It was calculated using the Inverse Simpson Index as described (*5*). For WNV calculations, NTB-A was omitted because it was not included in the panel.

Statistics

For continuous data, if the data were non-normal by the Shapiro-Wilk test, a nonparametric Mann-Whitney *U* test was used. For paired data, Wilcoxon signed-rank test was used. If the data were normal, a *t* test

was used. For normal data, if variances were equal by F test, Student's t test was used. Otherwise, Welch's t test was used. For the case-control study, logistic regression was used to account for 2:1 control/case matching. For nominal data, Pearson's χ^2 test with simulated P value was used. Two-sided tests were used in all cases. All statistical analyses were conducted in the open-source statistical package R. Correspondence analysis was performed using the ade4 package.

Hierarchical modeling using Bayesian inference

A simple Bayesian hierarchical model was used to model receptor stability. Let x_{ijk} represent the difference between the level of receptor i in individual j at time k, and let σ_{ij} represent the average level of receptor i in individual j. Let μ_i be the mean of a β distribution with parameters a_i and b_i [$\mu_i = a_i/(a_i + b_i)$], and let $c_i = a_i + b_i$. The model is

$$x_{ijk} \sim N(0,\ \sigma_{ij})$$
$$\sigma_{ij} \sim \mathrm{Beta}(a_i,\ b_i)$$
$$\mu_i \sim \mathrm{Unif}(0, 1)$$
$$c_i \sim \mathrm{Exp}(0.1)$$

Markov chain Monte Carlo simulation as implemented in the JAGS (Just Another Gibbs Sampler) software package was used to sample from the posterior distribution of this model. This yielded 95% CrI for the parameter μ_i, the average SD for each receptor. This parameter represents an average stability of the receptor over all individuals in the population.

SUPPLEMENTARY MATERIALS

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Fig. S1. Serial negative gating strategy used to define NK, $CD4^+$ T, and $CD8^+$ T cells from PBMCs.
Fig. S2. Representative gates to evaluate marker expression on NK cells.
Fig. S3. Stability analysis of $CD56^{bright}$ versus $CD56^{dim}$ NK cells.
Fig. S4. Human NK cell repertoire and function are stable for 6 months in an individual.
Fig. S5. Subpopulation analysis for the 10 most frequently detected NK subpopulations.
Fig. S6. Donor age does not correlate with NK cell diversity.
Fig. S7. CMV serostatus does not correlate with NK cell diversity or the frequency of NKG2A or CD57.
Fig. S8. NK diversity does not correlate with viral suppression in vitro.
Fig. S9. No correlative features distinguish NK cells responding to HIV-1–infected $CD4^+$ T cells.
Fig. S10. Cytokine-producing NK cells are more diverse than non–cytokine-producing NK cells.
Fig. S11. Proportion of variance explained in correspondence analysis.
Table S1. Mass cytometry antibody panels used in each experiment.
Table S2. HIP and SBB cohort information.
Table S3. Mama Salama Study cohort information.
Table S4. Frequencies of NK markers used in repertoire stability analysis.
Table S5. Frequencies of NK markers after 72-hour culture with IL-15 or IL-2, or without stimulation, for the SBB cohort.
Table S6. Frequencies of functional markers used in stability analysis.
Table S7. CD57 and NKG2A frequencies and NK diversity scores for HIP and cord blood cohorts.
Table S8. Proportion of phenotypes expressing CD57 and NKG2A.
Table S9. Functional frequencies of $CD57^+$ and $CD57^-$ NK cells.
Table S10. Diversity of NK cells in the presence or absence of HIV-1–infected $CD4^+$ T cells.
Table S11. Diversity of NK cells in the presence or absence of WNV-infected cells.
Reference (*48*)

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Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility

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Editor's Summary

The downside of diversity

The adaptive immune system exemplifies the benefits of diversity, allowing for individual responses to specific pathogens. Natural killer (NK) cells are diverse at the single-cell level, but the contribution of this diversity to NK cell–mediated immunity has been unclear. Strauss-Albee *et al*. found that contrary to adaptive immune cells, human NK cell diversity is lower at birth than in adults. Moreover, diversification as a result of antiviral response decreases the flexibility of future antiviral responses. Indeed, high NK cell diversity was associated with increased risk of HIV-1 acquisition in African women. These data suggest that preexisting NK cell diversity should be considered in the context of viral infections.

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