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Unconventional Repertoire Profile Is Imprinted during Acute Chikungunya Infection for Natural Killer Cells Polarization toward Cytotoxicity

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Abstract

Chikungunya virus (CHIKV) is a worldwide emerging pathogen. In humans it causes a syndrome characterized by high fever, polyarthralgia, and in some cases lethal encephalitis. Growing evidence indicates that the innate immune response plays a role in controlling CHIKV infection. We show here that CHIKV induces major but transient modifications in NK-cell phenotype and function soon after the onset of acute infection. We report a transient clonal expansion of NK cells that coexpress CD94/NKG2C and inhibitory receptors for HLA-C1 alleles and are correlated with the viral load. Functional tests reveal cytolytic capacity driven by NK cells in the absence of exogenous signals and severely impaired IFN-γ production. Collectively these data provide insight into the role of this unique subset of NK cells in controlling CHIKV infection by subset-specific expansion in response to acute infection, followed by a contraction phase after viral clearance.

Introduction

Emerging and re-emerging viral infectious diseases can cause devastating illnesses in humans and are accordingly one of the principal challenges in global health care today. Among these viruses, arthropod-borne arboviruses are especially important because many of them cause fatal diseases in humans and animals [1,2]. Chikungunya virus (CHIKV) is an Aedes mosquito-borne alphavirus from the Togaviridae family. Typically, a silent incubation period of 2–4 days usually follows infection by CHIKV, and symptoms arise afterwards. CHIKV-associated disease is an acute illness characterized by fever, skin rash, and severe incapacitating arthralgia [2–4]. Most clinical symptoms of CHIKV infection generally resolve within a few weeks, except for joint stiffness and pain, the hallmark of chronic CHIKV infection, which can persist for months or years. Intriguingly, CHIKV, which has historically been transmitted by Aedes aegypti mosquitoes, has repeatedly been associated in recent years with a new vector, Aedes albopictus, which has spread into tropical areas and dispersed worldwide [5]. CHIKV has been documented in nearly 40 countries and is currently present in Asia, the United States, Central Africa and Europe; the US National Institute of Allergy and Infectious Disease (NIAID) listed it as a category C priority pathogen in 2008 [2,6,7]. These recent outbreaks have resulted in more detailed descriptions of its clinical manifestations, including complications such as maternal-fetal transmission and fatal hemorrhagic and neurologic manifestations [8,9].

Although much is known about this disease, the study of immune response to CHIKV infection is in its infancy. Reports in humans and in a macaque model show that virus elimination occurs very rapidly, before the host can mount IgG and T cell responses and thus suggest an effective innate immune response. Type-I interferon (IFN) has been detected at high levels in the serum of infected individuals, its concentration correlated with viral load [10–14]. Natural killer (NK) cells are also an important mediator of the innate immune defense during early infectious events. NK cells mediate their antiviral effects through at least three different mechanisms: (1) the release of immunoregulatory cytokines, particularly IFN-γ, which enhance the innate immune response and help to shape the subsequent adaptive immune response, (2) the production of cytolytic granules for lysis of infected cells, and (3) the induction of target-cell death through cell surface receptors [15,16].

The variety of signaling pathways to stimulate NK cells equips these cells with multiple detection systems for sensing and responding to infection and thus makes it more difficult for viruses to escape detection and defense. NK-cell function is controlled by the integration of signals from various activating and inhibitory cell surface receptors [17–19]. Its direct cytolytic activity is associated with downregulation of major histocompatibility
Chikungunya virus (CHIKV) infection, which is responsible for devastating human illness, is rapidly becoming a global concern. The spread of this disease throughout tropical areas, where it now affects nearly 40 countries, underlines the need to improve our understanding of this infection. In 2008, CHIKV was listed as a US National Institute of Allergy and Infectious Disease (NIAID) category C priority pathogen. Natural killer (NK) cells are cytotoxic effector cells that play a vital role in the innate immune system by limiting acute infection, as previously described for several other diseases. This report describes the first phenotypic and functional analysis of NK cells soon after infection by this virus. The key element of this study was the detailed analysis of the expansion of NK cells. Coexpression of NKG2C activating receptors and HLA-C1 ligands is associated with viral load, impaired IFN-γ production, and significant cytolytic functions. We found that NK cells were able to sense CHIKV from the beginning of infection and contributed to the clearance of the infected cells through the expansion of a unique NK-cell subset.

Author Summary

Chikungunya virus (CHIKV) infection, which is responsible for devastating human illness, is rapidly becoming a global concern. The spread of this disease throughout tropical areas, where it now affects nearly 40 countries, underlines the need to improve our understanding of this infection. In 2008, CHIKV was listed as a US National Institute of Allergy and Infectious Disease (NIAID) category C priority pathogen. Natural killer (NK) cells are cytotoxic effector cells that play a vital role in the innate immune system by limiting acute infection, as previously described for several other diseases. This report describes the first phenotypic and functional analysis of NK cells soon after infection by this virus. The key element of this study was the detailed analysis of the expansion of NK cells. Coexpression of NKG2C activating receptors and HLA-C1 ligands is associated with viral load, impaired IFN-γ production, and significant cytolytic functions. We found that NK cells were able to sense CHIKV from the beginning of infection and contributed to the clearance of the infected cells through the expansion of a unique NK-cell subset.

Results

Changes in the proportion and activation status of different lymphocytic subsets from CHIKV-infected patients

Flow cytometry was used to assess the frequency of CD3+ T and CD3-CD56+ NK subsets in CHIKV-infected patients as well as in healthy Caucasian and Gabonese individuals. These lymphocyte subsets were found at similar frequencies in both healthy control groups, regardless of their origin. In contrast, and consistently with previous studies [14,23], the percentage of CD3+ T cells was significantly lower in CHIKV-infected patients (p<0.0001) than in controls (Figure 1A). Infection-associated profound T lymphopenia tended to spare CD3-CD56+ NK cells, and the proportions of these cells increased significantly after CHIKV infection, to 13.0±3.9% compared with 8.4±4.3% in healthy Gabonese controls (p=0.0006) (Figure 1A). The increase in NK-cell frequency was directly correlated with viral load (r=0.7337; p=0.0005) (Figure 1B). However, the absolute count of NK cells from the Caucasian CHIKV-infected patients (233±48 per mm3) was similar to that among the healthy Caucasian controls (275±66 per mm3). In contrast, the CD3+ T cell count was sharply lower in CHIKV-infected patients than in controls (Supplementary Table S1). These data are in line with a previous published study [23]. Of note, the modulation of frequencies of both CD3+ T and CD3-CD56+ NK cells was transient and rapidly returned to baseline levels after a two month-period (Figure 1C).

NK cells can be divided into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} populations, based on the cell-surface density of the CD56 molecules; these subgroups present distinct phenotypic and functional properties [28]. Intriguingly, the percentage of CD56\textsuperscript{bright} NK cells was very significantly lower in CHIKV-infected patients than in healthy controls (p<0.0001) (Figure 1D), a finding that supports the hypothesis of profound upheavals of the NK cell subset after CHIKV infection. The percentage of activated NK cells was also assessed by flow cytometric determination of the early activation marker CD69. As shown in Figure 1E, the NK cells from CHIKV-infected patients were activated; 77.2±9.9% of the NK cells expressed CD69 vs 15.4±6.2% in healthy donors (p<0.0001). Similar results were observed with HLA-DR, another activation marker (Figure 1E). Furthermore, within two months, the rate of NK cell activation reverted back to that of healthy donors (Figure 1F), in association with viral clearance (Supplementary Table S1).

Major phenotypic features of NK cells from CHIKV-infected patients

To explore whether CHIKV infection was associated with the pattern of natural killer receptor (NKR) expression, flow cytometric analysis was performed with anti-CD3 and anti-CD56 mAbs, in combination with a panel of reagents for NKRs. CHIKV-infected and healthy individuals did not differ significantly in their proportions of NK cells bearing NKG2D, Nkp46, CD94/NKG2A, Nkp30, and Nkp44 (Supplementary Figure S1). In contrast, the frequency of fluorescence intensity and mean fluorescence intensity (MFI) of Nkp30 and Nkp46, two specific natural cytotoxic receptors (NCR) constitutively expressed on virtually all NK cells, decreased significantly after CHIKV-infection (Figure 2A and Supplementary Figure S1). In contrast, Nkp44, a marker usually expressed only upon activation, was upregulated in most infected patients, as were other CD69 and HLA-DR activation markers (Figures 1E and 2A). More importantly, the balance of inhibitory NKG2A to activating NKG2C cells switched during acute CHIKV infection. Thus, all infected patients showed a significant loss of both the frequency (p<0.0001) and MFI...
Figure 1. Distribution and activation status of NK cells from CHIKV-infected patients. (A) FACS analysis of percentages of CD3+ T cells and CD3 CD56+ NK cells from 15 Caucasian (CAU) and 15 Gabonese (GAB) healthy donors, and 25 CHIKV-infected patients (CHIK). Samples were gated on the lymphocyte gate for flow cytometric analysis. Horizontal bars represent the median values. Representative samples of each group are presented in the lower panels. Numbers are the percentage of CD3-CD56+ NK cells in the CD45+ lymphocyte gate. The region used to determine the proportion of CD3 CD56+ NK cells is shown. (B) Correlation between viral load and frequency of NK cells from CHIKV-infected patients. (C) Longitudinal studies of the percentage of CD3 CD56+ NK cells from 3 Caucasian CHIK-infected patients. (D) Frequency of CD56bright cells among the CD3-CD56+ NK cells. (E) Expression of the activation markers CD69 or HLA-DR on NK cells. Samples were gated on the CD3-CD56+ NK-cell subset for flow cytometric analysis. Horizontal bars indicate the median. Representative samples of each group are presented in the lower panels. Numbers are the percentage of CD69+ cells in the CD3-CD56+ NK-cell gate. (F) Longitudinal study of the percentage of CD3 CD56+ NK cells expressing CD69, from 3 CHIKV-infected Caucasian patients. **: p<0.001; ***: p<0.0001.

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Figure 2. Patterns of specific NK receptors on CD3-CD56+ NK cells from CHIKV-infected patients. PBMC were collected from 15 Caucasian (CAU) and 15 Gabonese (GAB) healthy donors, and 25 CHIKV-infected patients (CHIK) to perform large-scale five-color flow cytometric analysis of CD3-CD56+ NK cells. (A) Frequency of the expression of NK-cell receptors including: NCR (NKp30, NKp44 and NKp46), NKG2A and NKG2C, ILT-2, as well as CD161, CD57, and CD8α. Horizontal bars indicate the median. *: p < 0.05; **: p < 0.001; ***: p < 0.0001. (B) Hierarchical clustering of the
Clonal Expansion of NK Cells in CHIKV Infection

Preferential modulation of receptors for HLA-C1 alleles on NK cells from CHIKV-infected patients

As previously shown, KIRs are stochastically expressed on the surface of NK cells and are critical for monitoring alterations in MHC expression during viral infection, as shown in the KIR gene cluster in the NCBI dbLRC database [http://www.ncbi.nlm.nih.gov/docs/pubs/abstracts/abs05/10.html]. While acute CHIKV infection is associated with a high frequency of NK cells (Figure 1A), as described [23], it is uncertain whether this expansion occurs specifically in KIR-expressing NK cells. We therefore assessed the overall change in KIR+ NK cells with a pool of antibodies combining CD158a, CD158b, CD158c, and NKB1, and observed a similar frequency in both CHIKV-infected and healthy individuals (data not shown). However, when KIR markers were split during single KIR analysis, CHIKV-infected and control individuals had significantly different levels of receptors for HLA-C alleles. These included KIR2DL1, specific for HLA-C2 alleles, and KIR2DL2/DL3, specific for HLA-C1 alleles. Profiles for both frequency and MFI of inhibitory ligands for HLA-C molecules were similar (Figure 4A and Supplementary Figure S3A). Thus, compared with healthy controls, NK cells from CHIKV+ patients expressed significantly less KIR2DL1 (p = 0.0027) and significantly more KIR2DL2/DL3 (p = 0.0008) (Figure 4A). Thus, up to 76% of the NK cells in CHIKV-infected patients were stained with an anti-KIR2DL2/DL3 mAb. Importantly, KIR2DL1 expression in NK cells from CHIKV-infected patients was significantly inversely correlated with their KIR2DL2/DL3 expression (p = 0.0318, r = -0.5201) (Supplementary Figure S3B). We therefore conducted several analyses to identify the underlying factors that might account for these differences observed in KIR2DL1 and KIR2DL2/DL3 expression during acute CHIKV infection. Intriguingly, we observed significant correlations between these receptors for HLA-C alleles and NKG2C: KIR2DL1 expression was inversely correlated with NKG2C expression (r = -0.3398, p = 0.0053), whereas KIR2DL2/DL3 expression was directly correlated with it (r = 0.7514, p < 0.0001) (Figure 4B). These findings were strengthened by significant correlations between the viral load and expressions of both KIR2DL1 (r = -0.2397, p = 0.0438) and KIR2DL2/DL3 (r = 0.7110, p = 0.0009) (Figure 4C).

Effective cytotoxicity of NK cells from CHIKV-infected patients

To evaluate the functional capacities of NK cells from CHIKV-infected patients, non-activated and IL-2 activated PBMC were analyzed in a degranulation assay against K562, 721.221, and HLA-E-transfected 721.221 (AEH) target cells (Figure 5). In the absence of IL-2 activation, a minimal level of CD107a expression was detected at the surface of NK cells from healthy controls, regardless of the target cell line. In contrast, the degranulation capacity of NK cells from CHIKV-infected patients was strongly enhanced against HLA-class I negative K562 (p = 0.0005), and...
Figure 3. Correlation between viral load and NKR expression and kinetic expression of NKR for CHIKV infection. (A) Correlation between viral load and NKG2A or NKG2C, both in frequency and MFI, on CD3-CD56⁺ NK cells from CHIKV-infected patients. NS: not significant. (B) Three Caucasian CHIKV-infected patients were tested at 0, 2, and 4 months after symptom onset, to determine the frequency of various NKR, including NKG2A, NKG2C, NKG2D, ILT-2, NKp30, and NKp46, on CD3-CD56⁺ NK cells.

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Figure 4. Specific modulation of HLA-Cw ligands on CD3+CD56+ NK cells from CHIKV-infected patients. PBMC were collected from 15 Caucasian (CAU) and 15 Gabonese (GAB) healthy donors, and 25 CHIKV-infected patients (CHIK). (A) Frequency and MFI of KIR2DL1 and KIR2DL2/DL3 on CD3+CD56+ NK cells. Horizontal bars indicate the median. *: p < 0.05; **: p < 0.001. (B) Correlation between expression of NKG2C and HLA-Cw ligands KIR2DL1 and KIR2DL2/DL3 on NK cells from the CHIKV-infected individuals. (C) Correlation between the viral load and KIR2DL1 or KIR2DL2/DL3 on CD3+CD56+ NK cells, both in frequency and MFI.

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721.221 (p = 0.0006) target cells (Figures 5A and 5B). More importantly, in the presence of HLA-E+ target cells, NK cells from CHIKV+ patients displayed greater cytotoxic activity than did NK cells from healthy donors (Figure 5C). These results are in accordance with the high expression level of major activation markers, including CD69, HLA-DR and Nkp44, but also with NKG2C, the receptor of HLA-E. The intracellular production of perforin and granzyme-B did not differ between CHIKV-infected and healthy individuals (data not shown). Following IL-2 activation, CD107a expression on NK cells from CHIKV+ patients was similar to that in controls in the presence of K562 or 721.221 target cells. In contrast, in the presence of HLA-E+ target cells, degranulation capacity was enhanced in NK cells from CHIKV+ patients, compared with those from controls (p = 0.0414) (Figures 5C and 5D). To confirm the key role of NKG2C in the lytic capacity of NK cells from CHIKV+ patients, we repeated these experiments in the presence of neutralizing NKG2C mAb. The degranulation capacity of NK cells from five CHIKV+ patients decreased strongly in the presence of anti-NKG2C, to less than 12.4±4.6%, compared with 44.6±5.4% in the presence of an IgG isotypic control (Figure 5E).

**Discussion**

This paper describes an extensive phenotypic and functional study of NK cells from CHIKV-infected individuals and provides the first evidence that CHIKV infection may selectively shape the NKR repertoire of healthy individuals. NK cells in patients shortly after CHIKV infection had significantly lower levels of NKP30 and NKP46, a reduction undoubtedly linked to their high expression of activation markers (CD69 and HLA-DR), as previously described after HCMV and HIV-1 infections [30–32]. An investigation of a large panel of phenotypic markers of the NK cell subset strengthened the evidence of NKR repertoire modulation, showing that CHIKV-infected patients have phenotypes quite distinct from those of either Caucasian or Gabonese healthy donors. This modulation likely reflects the challenge exerted by CHIKV on the innate immune system and thus may become a useful indicator for exploring the complex host-pathogen relation during the acute phase of infection.

In this study, we also demonstrated that CHIKV infection appears to impair the ability of NK cells to produce IFN-γ, as previously reported during such viral infections as HIV-1 and viral hepatitis [30,33,34], particularly at the peak of viremia. This impairment may be linked to the modulation in these patients of the subset of cells that produce IFN-γ most effectively — the CD56bright NK-cell subset. Experimental evidence indicates that NK-cell development proceeds from a CD56bright to CD56dim phenotype [35–37]. Several other changes in NKR expression were previously described after HCMV and HIV-1 infections [30–32]. A hierarchical clustering analysis of a large panel of phenotypic markers of the NK cell subset strengthened the evidence of NKR repertoire modulation, showing that CHIKV-infected patients have phenotypes quite distinct from those of either Caucasian or Gabonese healthy donors. This modulation likely reflects the challenge exerted by CHIKV on the innate immune system and thus may become a useful indicator for exploring the complex host-pathogen relation during the acute phase of infection.
Clonal Expansion of NK Cells in CHIKV Infection

A

W/o IL-2

IL-2 treatment

% CD107a+  

CAU GAB CHIK

**

% CD107a+  

CAU GAB CHIK

K562 cells

B

% CD107a+  

CAU GAB CHIK

**

% CD107a+  

CAU GAB CHIK

721.221 cells

C

% CD107a+  

CAU GAB CHIK

**

% CD107a+  

CAU GAB CHIK

AEH cells

D

CAU 10.8%  

GAB 15.7%  

CHIK 38.0%

E

% CD107a+  

CAU GAB CHIK

IgG-treated cells

Anti-NKG2C-treated cells
Consistent with these data, CHIKV infection completely shifted the NK-cell repertoire to create a specific subset of highly mature CD56dim NK cells mostly unresponsive to cytokine stimulation, but with strong cytolytic capacity for killing diseased host cells, according to their cell-activation phenotype. These data are in line with the recent study of Bjorkström et al [39] showing prompt NK cell activation and expansion in humans infected with hantavirus.

Our data show that NKG2C+ NK cells expand after CHIKV infection. However we cannot exclude an induction of NKG2C upon preexisting NK cells, or the elimination of NK cells that do not express NKG2C. This is closely associated with major modifications in the activation, differentiation, and cytolytic capacities of NK cells from CHIKV-infected viremic individuals. NKG2 receptor expression switched from inhibitory to activating after acute CHIKV infection, as previously described in other human infections, including HIV-1, CMV, HBV, and HCV [40–42]. The loss of NKG2A+ NK cells accompanied the expansion of NKG2C+ NK cells, which exist at very low frequencies in uninfected individuals. The frequency of NKG2C+ cells observed among CHIKV-infected individuals varied widely, but it is especially notable that it was significantly correlated with viral load. Interestingly, the rapid increased of NKG2C in response to the acute CHIKV infection was followed by a contraction phase, after viral clearance.

We cannot, however, reach a definitive conclusion about either the expansion of a specific NK cell subset or a difference in the lymphopenia rate in patients after CHIKV-infection, although both have been observed in a restricted population of murine NK cells expressing the activating receptor Ly49H+ after MCMV infection [43–45]. In this model, NK cells were shown to possess features previously attributed to cells of the adaptive immune system, including the expansion of a specific subset followed by contraction and formation of cells with memory-like characteristics [29,45,46]. NKG2C expression during acute hantavirus and HIV-

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**Figure 5. Robust degranulation efficacy of NK cells from CHIKV-infected patients against HLA-E+ target cells.** PBMC were collected from 10 Caucasian (CAU) and 10 Gabonese (GAB) healthy donors, and 10 CHIKV-infected patients (CHIK) to determine the cytolytic capacities of NK cells before and after a two-day treatment with IL-2. Degranulation activity was determined by CD107a expression against K562 (A), 721.221 (B), or HLA-E-transfected 721.221 (AEH-cells) (C) target cells. Results are shown for an effector/target (E/T) cell ratio of 1/1. Horizontal bars indicate the median. **: p<0.001. (D) Representative samples of each group were gated on the IL-2 activated CD3-CD56+ NK-cell subset in the presence of AEH HLA-E-expressing target cells. Numbers denote the percentage of CD107a+ cells among the CD3-CD56+ NK cells. (E) Neutralization of the degranulation in the presence of anti-NKG2C mAb. PBMC from 5 healthy Caucasian (CAU) and 5 healthy Gabonese (GAB) donors, and 5 CHIKV-infected patients (CHIK) were tested in the presence of neutralizing anti-NKG2C mAb (closed bars) or IgG isotypic control (Open bars) against HLA-E-transfected 721.221. Results are expressed in mean ±SD. doi:10.1371/journal.ppat.1002268.g005

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**Figure 6. Significant down-modulation of intracellular IFN-γ expression in NK cells from CHIKV-infected patients.** (A) PBMC were collected from 10 Caucasian (CAU) and 10 Gabonese (GAB) healthy donors, and 10 CHIKV-infected patients (CHIK) to determine intracellular production of IFN-γ after overnight treatment with IL-12 and IL-18. Horizontal bars indicate the median. ***: p<0.0001. (B) Representative samples of each group are shown. Samples were gated on the CD3-CD56+ NK-cell gate for flow cytometric analysis. Numbers denote the percentage of intracellular IFN-γ production among CD3-CD56+ NK cells. (C) Correlation between NKG2C expression by CD3-CD56+ NK cells and IFN-γ production in the sera of CHIKV-infected patients determined with Luminex Technology (Bio-Rad), as recently published [14]. doi:10.1371/journal.ppat.1002268.g006
1 infection has been associated with HCMV, but not EBV or HSV seropositivity [31,39,47]. Here we found that NKG2C expression was associated with HCMV serostatus in the three Caucasian CHIKV-infected patients. Evaluation of the HCMV status of the Gabonese patients was not relevant, as seroprevalence approaches 100% in this country [40].

More intriguingly, the NK cell repertoire appeared to be unusual with strong and specific correlations between NKG2C expression and specific self-inhibitory KIRs. In contrast to the overall NK cell repertoire, which contains a random distribution of KIRs, the NKG2C⁺ NK cells in CHIKV-infected patients are associated with HLA-Cw allele receptors. The correlation with KIR2DL1, recognizing C2 HLA-C subtypes, is inverse, while that with KIR2DL2/ DL3, recognizing C1 subtypes, is direct. This suggests that NK cells coexpressing NKG2C and receptors for HLA-C1 alleles expand during an acute viral infection. More importantly, HLA-C genotyping revealed that 11/12 CHIKV-infected patients examined in detail had HLA-C1/C1 or HLA-C1/C2 alleles. This finding suggests that expansion of highly cytotoxic NKG2C⁺ NK cells is associated with imbalanced expression of unique self-specific receptors and may dampen autoreactivity and limit immunopathology in CHIKV-infected patients.

Analysis of a large number of CHIKV-infected individuals, combining genetic analysis of KIR and HLA, kinetic study of phenotypic and functional features of NK cells, and the long-term evolution of clinical indicators, might make it possible to draw statistically powerful conclusions. The presence of a single inhibitory HLA-C binding KIR showed that the KIR overexpressed during CHIKV-infection mediates NK-cell licensing in the highly functional NKG2C⁺ NK cell subset, protecting against autoreactivity. This finding is supported by our recent observation of a persistent indolent proliferation of CD3⁺CD56⁺ large granular lymphocytes expressing NKG2C⁺ [49]. These data are also consistent with a case report of acute HCMV infection in a 3-month-old girl whose genes encoded a deficient alpha-chain of the IL-7 receptor known to be associated with a SCID-phenotype; phenotype characterization showed an extreme transient amplification of NKG2C-bearing NK cells positive for KIR2DL2/DL3, which accounted for more than 80% of the leukocytes [50]. More recently, Björkström et al [39] also showed a clonal selection of educated CD56⁺NKG2C⁺KIR⁺ NK cells in 5 hantavirus-infected patients. Taken together, these important data suggest a certain level of clonality in the response of NK cells against acute infection, including CHIKV infection. However, the underlying mechanism mediating this effect remains to be defined.

Collectively, our data suggest that the clonal expansion of a unique subset of NK cells coexpressing NKG2C and receptors for HLA-C1 alleles and correlated with the viral load, suggests that NK cells are able to sense CHIKV from the beginning of infection and may thus contribute to viral clearance. Understanding how these complex innate responses affect the outcome of CHIKV infection will help in the development of vaccines or other therapeutic strategies that could use innate immunity to enhance viral control with minimal pathogenesis.

Materials and Methods

Human ethics statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki and with French statutory and regulatory law. Patients received information about research performed on biological samples and provided written informed consent to participate. The hospital’s institutional review board (Comité de protection des personnes Île-de-France VI) approved the study (Hôpital Pitié-Salpêtrière, 47 boulevard de l’Hôpital, 75013 Paris).

Patients and healthy controls

Peripheral blood samples from 25 CHIKV-infected patients (mean age 32±15 years, 60% women) were obtained during the CHIKV outbreak that occurred in Gabon, Central Africa, between March and August 2007. The outbreak began first in Libreville, the capital, and then spread to several small towns located on the road towards Cameroon in northern Gabon, generating approximately 20,000 cases. These samples were collected from patients whose symptoms included fever, arthralgia, and asthma and who visited specific medical centers in Libreville during the first five days after the onset of symptoms. Diagnosis of CHIKV infection was confirmed for each of these patients, as described [7,14]. These CHIKV-infected patients were negative for dengue fever, yellow fever, West Nile fever, Rift Valley fever, and malaria. Gabonese (n = 15) and Caucasian (n = 15) sex- and age-matched healthy volunteers from Franceville (Gabon) and our hospital blood bank (EFS, Pitié-Salpêtrière Hospital, Paris, France) were used as controls. Kinetic studies were performed on 3 Caucasian CHIKV-infected patients from the Department of Infectious Diseases of the Pitié-Salpêtrière Hospital (Paris, France) in 2006.

Viral RNA extraction and quantification

Viral RNA was extracted from 90 µl of plasma from CHIKV-infected patients with an EZ1 advanced XL system (Qiagen, Courtabœuf, France) and the EZ1 virus kit V2.0 (Qiagen). With the high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA, USA) and according to the manufacturer’s recommendations, 25 µl of extracted RNA was immediately reverse transcribed into cDNA. Real-time PCR was carried out in a 7500 real-time PCR system (Applied Biosystems), with the Universal PCR master mix kit (Applied Biosystems) according to the manufacturer’s recommendations with the specific primers F-CHIK and R-CHIK and the probe P-CHIK (10 mM) designed by Pastorino et al. [51]. A quantified synthetic CHIKV RNA transcript, kindly provided by the University of the Méditerranée (Pr X. De Lamballerie, Marseilles, France), was used as a standard. All amplifications were performed in duplicate.

Flow cytometric analysis

NK cells were analyzed after staining with an appropriate antibody cocktail: anti-CD45 (J33), anti-CD3-ECI (UCHT1), anti-CD56-PE (N901), anti-CD8a-APC (B9.11), anti-CD159a/ NKG2A-APC (Z199), anti-CD336/NKp46-PE (Z231), anti-CD35/NKp46-PE (BAB281), anti-NKG2D-PE (ON72), anti-CD85/ILT2-PE (HP-F1), anti-CD69-APC (FN50), and anti-HLA-DR-PE (Imm357), KIR2DL1/KIR2DS1-PE (EB6B), anti-KIR 2DL2/KIR2DL3/KIR2DS2-PE (GL183), KIR3DL1/KIR3DS1- PE (Z27) from Beckman Coulter; anti-CD94-FTTC (HP-3D9), anti-CD57-FTTC (S-HCL-1), and anti-CD161-FTTC (DX12) from Becton Dickinson; anti-NKG2C-PE (134591), KIR2DS4-PE (179915), and anti-KIR2DL4-APC (181703) from R&D systems; anti-CD337/NKp30-PE (AF29-4D12), anti-KIR2DL1/KIR2DL3-APC (DX27), and anti-KIR 3DL1-APC (DX9) from Miltenyi Biotech; and anti-KIR2DL5- Alexa Fluor 647 (UP-R1) from e-biosciences. For intracellular staining, PBMC were fixed and permeabilized with a cytofix/cytoperm kit (Becton Dickinson) and stained with perforin-PE (d9), or granzyme-B-FITC (GB1), as described [36]. Isotype-matched immunoglobulins served as negative controls. Lymphocytes were identified by characteristic forward and side scatter
parameters and CD45 expression. Populations of interest were
gated on patterns of CD56/CD3 staining within the CD45+ lymphocyte population. Results are expressed as the percentage of positive cells within the gated population. At least 20,000 CD45+ cells were analyzed on a Navios cytometer (Beckman Coulter).

Expression of each NKR was measured as a percentage of the total CD3-CD56+ NK cells. Hierarchical clustering was applied, and the results were displayed with the use of the Genesis program (software available at www.genome.tugraz.at), as previously described [52–54].

Functional analysis
Degranulation was assessed by the detection of LAMP1/CD107a, on PBMC cultured in the absence or in the presence of 1000 IU/ml of Proleukin-2 (Chiron) for 48 h, against K562, 721.211 or HLA-E-transfected 721.221 (AEH) target cell lines. Briefly, PBMC were resuspended in the presence of anti-CD107a mAb (HA33, Becton Dickinson) with target cells at an effector:target (E:T) cell ratio of 1:1. After 1 h of incubation, monensin (Sigma Aldrich) was added at 2 mM for an additional 4 h of incubation [36,38].

To stimulate IFN-γ production, PBMC were incubated overnight in the presence of IL-12 (10 ng/ml) and IL-18 (100 ng/ml) (R&D Systems). Cells were then fixed and permeabilized with a cytofix/cytoperm kit (Becton Dickinson) and stained with anti-IFN-γ mAb (B27; Becton Dickinson), as described [36].

HLA-Cw typing
Genomic DNA was extracted from PBMC of CHIKV-infected patients with the QIAmp DNA mini kit (Qiagen). HLA-C alleles were sequenced with the SBT kit (Aria Genetics). Sequences were read with a 3100 Genetic analyzer (Applied Biosystems) and computer-assisted Conexio genomics software.

Statistical analysis
All statistical analyses were performed with Prism-5 software (GraphPad Software). Intergroup comparisons were assessed with the non-parametric Kruskal-Wallis test, with Dunn’s post test to define the significance between results from 3 independent groups of subjects. Significance was defined by P less than 0.05 with a 2-tailed test. *p<0.05, **p<0.01, ***p<0.001. Nonparametric correlations were assessed by determination of the Spearman’s rank correlation coefficient.

Supporting Information
Figure S1 Cell-surface expression of NK cell markers on NK cells from Caucasian (CAU) and Gabonese (GAB) healthy donors, and CHIKV-infected patients (CHIK).

(A) PBMC from one representative sample of each group of samples were stained with specific antibodies against NKG2A, NKG2C, ILT-2, Nkp30 and Nkp46, and then gated on the CD3/CD56+ NK-cell gate for flow cytometric analysis. Numbers denote the percentage of positive cells in the CD3/CD56+ NK-cell gate. (B) Correlation of the expression of NK-cell receptors including NKG2A, NKG2C, ILT-2, Nkp30 and Nkp46. Horizontal bars indicate the median. *: p<0.05; **: p<0.001; ***: p<0.0001.

Figure S2 Expression of KIR in CHIKV-infected patients. (A) Expression of KIR2DL1 and KIR2DL2/DL3 on NK cells from one representative sample of each group, including Caucasian (CAU) and Gabonese (GAB) healthy donors, and CHIKV-infected patient (CHIK). Numbers denote the percentage of positive cells in the CD3/CD56+ NK-cell gate. (B) Correlation between KIR2DL1 and KIR2DL2/DL3 expression on CD3/CD56+ NK cells from CHIKV-infected patients. (C) Frequency and MFI values of KIR2DL4, KIR2DL5, and KIR3DL1 on NK cells from Caucasian (CAU), and Gabonese (GAB) healthy donors, and CHIKV-infected patients (CHIK).

Figure S3 Expression of KIR in CHIKV-infected patients. (A) Expression of KIR2DL1 and KIR2DL2/DL3 on NK cells from one representative sample of each group, including Caucasian (CAU) and Gabonese (GAB) healthy donors, and CHIKV-infected patient (CHIK). Numbers denote the percentage of positive cells in the CD3/CD56+ NK-cell gate. (B) Correlation between KIR2DL1 and KIR2DL2/DL3 expression on CD3/CD56+ NK cells from CHIKV-infected patients. (C) Frequency and MFI values of KIR2DL4, KIR2DL5, and KIR3DL1 on NK cells from Caucasian (CAU), and Gabonese (GAB) healthy donors, and CHIKV-infected patients (CHIK).

Table S1 Viral load, absolute count and frequency of CD3+ T and CD3+CD56+ NK cells from Caucasian CHIKV-infected patients.

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Author Contributions
Conceived and designed the experiments: EML, VV. Performed the experiments: CP PB NW VB VV. Analyzed the data: CP PB NW PB PD EML, VV. Contributed reagents/materials/analysis tools: EML. Wrote the paper: NW EML VV.

References


