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Human Cytomegalovirus-Induced NKG2C^{hi} CD57^{hi} Natural Killer Cells Are Effectors Dependent on Humoral Antiviral Immunity

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Recent studies indicate that expansion of NKG2C-positive natural killer (NK) cells is associated with human cytomegalovirus (HCMV); however, their activity in response to HCMV-infected cells remains unclear. We show that NKG2C^{hi} CD57^{hi} NK cells gated on CD3^{neg} CD56^{dim} cells can be phenotypically identified as HCMV-induced NK cells that can be activated by HCMV-infected cells. Using HCMV-infected autologous macrophages as targets, we were able to show that these NKG2C^{hi} CD57^{hi} NK cells are highly responsive to HCMV-infected macrophages only in the presence of HCMV-specific antibodies, whereas they are functionally poor effectors of natural cytotoxicity. We further demonstrate that NKG2C^{hi} CD57^{hi} NK cells are intrinsically responsive to signaling through CD16 cross-linking. Our findings show that the activity of pathogen-induced innate immune cells can be enhanced by adaptive humoral immunity. Understanding the activity of NKG2C^{hi} CD57^{hi} NK cells against HCMV-infected cells will be of relevance for the further development of adoptive immunotherapy.

Human cytomegalovirus (HCMV) causes severe disease in immunocompromised patients. While the antiviral roles of T cells have been extensively studied and monitored in patients, human studies proving the specific relevance of NK cells against HCMV infection are still very limited. Nevertheless, NK cells are supposed to be important for protection against CMV infections in humans (1). A case report indicated that NK cell deficiency was associated with active HCMV infection (2). Another case report showed that NK cells could control HCMV infection in the absence of T cell help in a T^{neg} B^{neg} NK^{pos} SCID patient (3). In transplant recipients, NK cell activity was shown to increase during both primary and recurrent HCMV infection, indicating that NK cells may contribute to recovery (4, 5). *In vitro* studies have shown that HCMV expresses multiple gene products and a microRNA to modulate the NK cell response, and the mechanisms by which these gene products act have been reviewed (6).

Although NK cells are prototypic innate immune cells, studies on mice show that NK cells also share characteristics of adaptive immune cells (7–9). During murine CMV infection, Ly49H^{+} NK cells proliferated preferentially, a characteristic of the adaptive immune response. These cells were shown to protect newborn mice from disease (9). In humans, studies showed that HCMV infection selectively expanded NKG2C-positive NK cells in healthy individuals (10, 11). Even in coinfections of HCMV with HIV (12, 13), hantavirus (14), and hepatitis B and hepatitis C viruses (15), the expansion of NKG2C-positive NK cells was exclusively dependent on the HCMV infection. Similar results were also obtained in studies using cells from patients with chronic lymphocytic leukemia (16) and after transplantation (11, 17, 18).

In solid-organ transplant (SOT) recipients with active HCMV infection, the percentage of CD57^{+} NKG2C^{hi} NK cells increased shortly after the detection of HCMV viremia (11). Clinical studies performed after hematopoietic stem cell transplantation (HCT) and umbilical cord blood (UCB) transplantation confirmed an expansion of NKG2C^{+} NK cells during the acute phase of HCMV reactivation (17, 18). In humans, CD56^{dim} and CD57 are expressed preferentially by subsets of NK cells with a mature phenotype which may define a subpopulation of highly differentiated NK cells (19, 20). CD57-positive NK cells exhibit a higher cytotoxic capacity, higher sensitivity to stimulation via CD16, and decreased responsiveness to cytokines (20). Thus, we hypothesized that NKG2C^{hi} CD57^{hi} NK cells may possess unique functional properties in HCMV infection.

Myeloid cells are an important site of HCMV latency and reactivation (21). Macrophages can act as antigen-presenting cells upon HCMV infection and can secret cytokines that lead to T and NK cell activation (22, 23). Furthermore, they can be obtained from peripheral blood mononuclear cells (PBMCs) to perform experiments *in vitro*. The HCMV strain TB40/E exhibits a broad cell tropism, including endothelial- and monocyte-derived cells (24, 25). In addition, TB40/E is available as a bacterial artificial chromosome (BAC) and is suitable for genetic manipulation (26).

NK cells are considered the main effectors of antibody-dependent cell-mediated responses, but evidence for a role of this process in immune defense against HCMV is sparse (27). Our new assay, using autologous macrophages and the HCMV strain TB40/E, enables us to investigate the role of the NKG2C^{hi} CD57^{hi} NK cell response to HCMV-infected macrophages and to measure the additional effect of HCMV antibodies.

MATERIALS AND METHODS

Ethics statement. All buffy coats were purchased from the Transfusion Center of the Ulm University Hospital (Institut für Klinische Transfusionsmedizin und Immungenetik Ulm GmbH, Ulm, Germany) and were

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randomly obtained from anonymized healthy blood donors. All blood donors gave written informed consent to approve and authorize the use of their blood for medical, pharmaceutical, and research purposes.

Cells. Human erythroleukemia cell line K562 (DMSZ), Burkitt lymphoma cell line Raji, and Fc receptor (FcR)-positive mouse mastocytoma cell line P815 (all from American Type Culture Collection) and monocytes, macrophages, PBMCs, and purified NK cells were cultured in RPMI 1640 medium (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS). Primary human foreskin fibroblasts (HFFs) were cultured in minimum essential medium (MEM) (GIBCO/Invitrogen) supplemented with 10% FBS. Macrophage colony-stimulating factor (M-CSF) (R&D Systems)-stimulated monocyte-derived macrophages were obtained from human buffy coats as previously described (22). PBMCs were frozen in 90% FBS containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. In order to preserve the cytotoxic activity of NK cells, the cryoprotectant was added and removed slowly, as previously described (28). NK cells were enriched by negative selection from thawed PBMCs using a human NK cell enrichment kit (Miltenyi).

Preparation of viral stocks and macrophage infections. HCMV strain TB40/E was propagated in HFFs. For preparation of virus stocks, infectious supernatants from HFF cultures were harvested at 5 to 7 days postinfection. Cellular debris was removed by centrifugation at 2,800 × *g* for 10 min, and virus particles were precipitated from the supernatants by ultracentrifugation (70,000 × *g* for 70 min at 10°C). Then, the pellet was resuspended in RPMI–10% FBS medium. Viral stocks were frozen at −80°C and thawed before use. The infectious titer of HCMV preparations was determined as the 50% tissue culture infective dose ($TCID_{50}$) using HFFs on 96-well plates. Macrophages were infected using a multiplicity of infection (MOI) of 5 PFU/macrophage for 24 h before further experiments.

Immunofluorescence. To determine the infection rates, macrophages were fixed at 24 h postinfection with 80% acetone and incubated with HCMV immediate early antigen (IEA) antibodies (Argene-Biosoft), followed by staining with Alexa Fluor 555 (AF555)-conjugated goat anti-mouse immunoglobulins (Molecular Probes/Invitrogen). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). The number of IEA and DAPI signals was determined in three frames per well with an automated counting feature of the Zeiss AxioVision microscope. The infection rate was calculated as the ratio of IEA-positive nuclei to total DAPI-positive nuclei.

To determine HCMV antibody binding, infected macrophages were fixed 72 h postinfection with precooled methanol for 10 min at 4°C, incubated for 20 min at 4°C with FcR blocking reagent (Miltenyi), and incubated for 120 min at 4°C with the dilutions of anti-HCMV Ig or human serum/plasma controls indicated in Fig. 3. During the last 30 min of incubation, rabbit serum (1:10; Sigma) was added to block unspecific binding of secondary antibodies. After washing, cells were incubated for 1 h at 4°C with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako). Infected and noninfected cells were stained as described above. Microphotographs were generated with a Zeiss Observer.

Serum/plasma preparation and neutralization assay. Pooled immunoglobulins for intravenous use Gamunex (Talecris Biotherapeutics) was purchased commercially and used at a 1:200 dilution. Aliquots of autologous plasma were collected from buffy coats for subsequent cell isolation, and HCMV-IgG-negative and -positive individual serum/plasma samples were collected from whole blood by centrifugation at 4,000 rpm for 20 min. All sera/plasma were heated to 56°C for 30 min and stored at −20°C. HCMV IgG serology was determined with an enzyme-linked fluorescence assay (VIDAS CMV IgG; bioMérieux). For neutralization studies, medium containing TB40/E (MOI of 5) was incubated with the Ig preparations (using seronegative plasma/serum [1:10] as the control) indicated below for 30 min at 37°C, and then the indicated mixtures were added to the macrophages.

Flow cytometry. The following monoclonal antibodies (MAbs) were used: peridinin chlorophyll protein (PerCP)–Cy5.5–anti-CD3 antibody (UCHT1), allophycocyanin (APC)- or phycoerythrin (PE)–Cy7–anti-CD56 antibody (B159), PE- or FITC–anti-CD107a antibody (H4A3), PE–anti-NKp46 antibody (9E2/NKp46), PE–anti-NKG2D antibody (1D11), FITC–anti-leukocyte Ig-like receptor 1 (LIR-1) antibody (GHI/75), PE–anti-CD57 antibody (NK-1), PE–anti-CD158b antibody (CH-L), FITC–anti-CD69 antibody (FN50), FITC–anti-CD94 antibody (HP-3D9), Pacific Blue–anti-CD16 antibody (3G8) (all from BD), Alexa Fluor 488 (AF488)- or PE–anti-NKG2C antibody (134591; R&D Systems), PE–anti-NKG2A antibody (131411; R&D Systems), PE–anti-interleukin-12Rβ2 (IL-12Rβ2) antibody (305719; R&D Systems), APC–anti-CD57 antibody (HCD57; BioLegend), FITC–anti-2B4 antibody (C1.7; BioLegend), APC-Vio770- or PE–anti-gamma interferon (IFN-γ) antibody (45-15, Miltenyi), PE–Vio770–anti-CD56 antibody (AF12-7H3, Miltenyi), Aqua Live/Dead fixable dead cell dye (Invitrogen), and anti-DNAX accessory molecule 1 (DNAM-1) antibody (clone 4; gift from Stipan Jonjić, Department of Histology and Embryology, Medical Faculty, University of Rijeka, Croatia). The chimeric human/mouse anti-CD20 MAb rituximab (Roche; obtained from the Pharmacy Department at the Ulm University Hospital) was used with an optimum concentration of 5 μg/ml. The antibodies used for redirect degranulation assay were purified mouse anti-human NKG2C antibody (134591; R&D Systems), purified mouse anti-human CD16 antibody (3G8; BioLegend), and an isotypic control (MOPC-21; BioLegend). The optimal concentrations for the MAb coating (NKG2C, 1 μg/ml, and CD16, 100 μg/ml) were based on maximum staining by flow cytometry (29). Eight-color staining was modified from a published NK cell panel (30). Cells were fixed and analyzed using a FACSCalibur or FACSCanto II (BD Biosciences).

NK cell degranulation and IFN-γ production assay. After coculture of NK and target cells, monensin (GolgiStop, 2 μM; BD), brefeldin A (5 mg/ml; Sigma), and anti-CD107a MAb (20 μl/ml) were added for 5 h. Then, CD107a surface expression and IFN-γ production were analyzed by flow cytometry. The K562 cell line was used as a positive control for degranulation. To investigate the HCMV antibody-dependent NK cell-mediated response, HCMV antibodies were added together with anti-CD107a MAb. Raji cells were used as a positive control for the rituximab-dependent NK cell-mediated response.

Redirect degranulation assay. For redirect degranulation assays, P815 cells were coated with purified anti-NKG2C antibody, anti-CD16 antibody, and the corresponding isotypic control antibodies at 37°C for 60 min. Effectors and antibody-coated P815 cells were cocultured for 4 h at an effector/target ratio of 10:1 in 96-well round-bottom plates in the presence of anti-CD107a MAb.

Statistical analysis. The Mann-Whitney U test was performed for the data shown in Figure 1A. The nonparametric Wilcoxon signed rank sum test was used to compare the subset with NKG2C-negative NK cells paired from the same donor. Results were considered significant at a two-sided *P* value of 0.05.

RESULTS

Identification of the NKG2C^{hi} CD57^{hi} NK cell subset in HCMV-seropositive healthy donors. The identification of a CD57^{+} NKG2C^{hi} NK cell subset has been reported after gating on CD3^{neg} CD56^{dim} CD16^{+} cells (11). To avoid underestimation of the role of NKG2C^{hi} CD57^{hi} NK cells in functional studies due to CD16 downregulation (31) as a consequence of the gating strategy, we applied a four-color staining strategy. We gated NKG2C^{hi} CD57^{hi} NK cells within CD3^{neg} CD56^{dim} populations from freshly isolated PBMCs. As shown in Figure 1A, NKG2C^{hi} CD57^{hi} NK cells can be detected exclusively in many of the HCMV-seropositive healthy donors. In contrast, the occurrence of NKG2C^{hi} CD57^{hi} NKT cells and NKG2C^{hi} CD57^{hi} T cells is not dependent on the HCMV serostatus (Fig. 1A) and no correlation between the percentages of NKG2C^{hi} CD57^{hi} NK cells and T cells was found in the

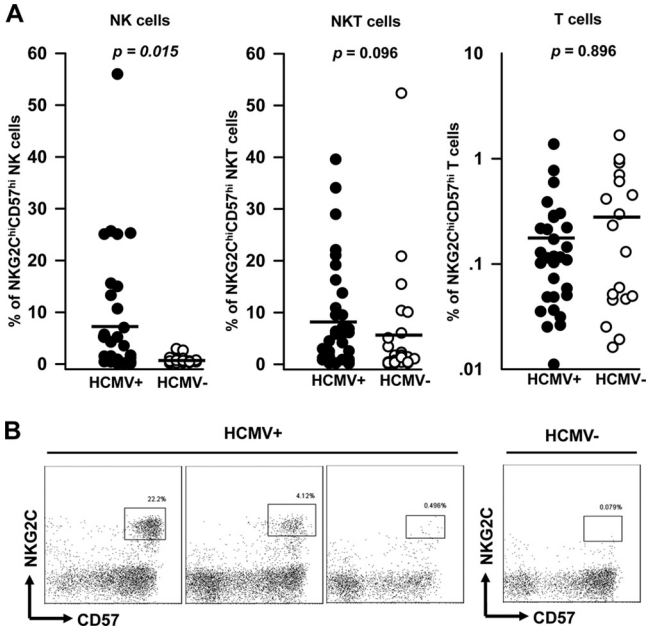

FIG 1 NKG2C^{hi} CD57^{hi} NK cells are associated with HCMV seropositivity. (A) Freshly isolated PBMCs from HCMV-seropositive (n = 35) and -seronegative (n = 24) healthy donors were stained with PerCP–Cy5.5–anti-CD3 (UCHT1), APC–anti-CD56 (B159), PE–anti-CD57 (NK-1), and AF488-NKG2C (134591) antibodies. After gating on lymphocytes, the percentages of NKG2C^{hi} CD57^{hi} NK cells gated on CD3$^-$ CD56^{dim} (left), NKG2C^{hi} CD57^{hi} NKT cells gated on CD3$^+$ CD56$^+$ (middle), and NKG2C^{hi} CD57^{hi} T cells gated on CD3$^+$ CD56$^-$ (right) are shown. Statistical significance is indicated at the top (Mann-Whitney U test). The horizontal lines represent the mean for each group. (B) Results of representative four-color staining to identify NKG2C^{hi} CD57^{hi} NK cells from 3 HCMV-seropositive donors and 1 seronegative donor are shown.

seropositive donors. As shown in Figure 1B, HCMV-seropositive donors possessed substantially different amounts of NKG2C^{hi} CD57^{hi} NK cells. Concerning our further functional analyses, we defined donors with at least 3% NKG2C^{hi} CD57^{hi} NK cells within the CD3^{neg} CD56^{dim} population as NKG2C^{hi} CD57^{hi} NK cell positive. By using this cutoff, 15 of 35 (42%) HCMV-seropositive donors were NKG2C^{hi} CD57^{hi} NK cell positive, but none of the 24 HCMV-seronegative donors were positive for this subset (Fig. 1A). In all NKG2C^{hi} CD57^{hi} NK cell-positive donors, NKG2C^{hi} NK cells expressed a high level of CD57 (clone NK-1). Therefore, we define the subset as CD57^{hi} rather than CD57$^+$.

NKG2C^{hi} CD57^{hi} NK cells express distinct inhibitory and activating receptors and show low responsiveness to HCMV-infected autologous macrophages. We compared the expression levels of inhibitory and activating receptors on NKG2C^{hi} CD57^{hi} and NKG2C-negative NK cells. As shown in Figure 2A, where the paired results from the same donor are connected by a line, NKG2C^{hi} CD57^{hi} NK cells (Fig. 2A, black circles) expressed significantly different amounts of the inhibitory CD158b, LIR-1, and NKG2A receptors than NKG2C-negative NK cells (Fig. 2A, white circles). CD158b is the most frequently expressed inhibitory killer cell Ig-like receptor (KIR) in NKG2C-positive NK cells (15, 17). The inhibitory leukocyte Ig-like receptor 1 (LIR-1) shows a high binding affinity for the HCMV glycoprotein UL18, which has been shown to act as a major histocompatibility complex (MHC) class I homolog (32). The inhibitory receptor NKG2A interacts specifically with the nonclassical MHC class I molecule HLA-E (33). For the first time, we could also show that, compared to NKG2C-negative NK cells, NKG2C^{hi} CD57^{hi} NK cells expressed lower levels of the activating receptor NKp46 (Fig. 2A, bottom), which has recently been described to be important for the NK cell response to HCMV-infected macrophages (23). In agreement with previous data (10, 11, 15), the expression levels of the activating receptors 2B4, DNAM-1, NKG2D, and CD16 were not significantly different (Fig. 2A, bottom).

In order to assess the functional capacity of these cells, we compared NKG2C^{hi} CD57^{hi} and NKG2C-negative NK populations with respect to their degranulation induced either by TB40/E-infected autologous macrophages (Fig. 2B) or MHC class I-negative K562 cells (Fig. 2C). We found that NKG2C^{hi} CD57^{hi} NK cells were less responsive than NKG2C-negative NK cells to both targets (Fig. 2B and C). NKG2C-positive NK cells also showed less degranulation than NKG2C-negative NK cells in response to HCMV-infected dendritic cells (34). However, a recent study suggested that NKG2C-positive NK cells may be involved in the resolution of CMV DNAemia episodes (35). The obvious question was why NKG2C^{hi} CD57^{hi} NK cells from HCMV-seropositive donors did not exhibit an enhanced antiviral effect.

NK cell-mediated response to HCMV-infected autologous macrophages is antibody dependent. We hypothesize that the low degranulation capacity shown by NKG2C^{hi} CD57^{hi} NK cells *in vitro* was due to the lack of an important component normally present in the blood of donors and that HCMV antibodies might be required for an efficient NKG2C^{hi} CD57^{hi} NK cell response against HCMV-infected cells. Since there was no accepted standardized assay to measure HCMV-specific antibody-dependent NK cell-mediated responses, we established an autologous assay system using three essential components: HCMV antibodies binding to infected cells, target cells expressing HCMV antigens, and autologous NK cells. First, we demonstrated that plasma or serum from HCMV-positive donors can efficiently neutralize HCMV infection of macrophages. As shown in Figure 3A, comparable percentages of macrophages expressed immediate early HCMV antigens (Fig. 3A, red fluorescence) when infected with the virus alone or in the presence of plasma or serum obtained from HCMV-seronegative individuals. When macrophages were infected in the presence of plasma or serum obtained from HCMV-seropositive donors, the viral infectivity was completely abolished. We also tested a commercially available HCMV antibody preparation produced from a large pool of human plasma, because it contained a broad spectrum of HCMV antibodies (36). Second, although the fluorescence results on infected macrophages were relatively faint at a 1:10 dilution, some of the infected macrophages (Fig. 3B, red fluorescence) showed clear binding of human anti-HCMV IgG (Fig. 3B, green fluorescence). This indicates that the HCMV-infected macrophages did express HCMV antigens which could be recognized by HCMV antibodies. Third, we measured IFN-γ production and CD107a expression as indicators of the biological activity of the autologous NK cells. All three HCMV antibody preparations can efficiently enhance NK cell production of IFN-γ, as well as degranulation, in response to infected macrophages (Fig. 3C, last three graphs), while this effect is not induced by plasma or serum obtained from HCMV-seronegative donors (Fig. 3C, second and third graphs). HCMV antibodies could not elicit any response with uninfected macrophages (data not shown).

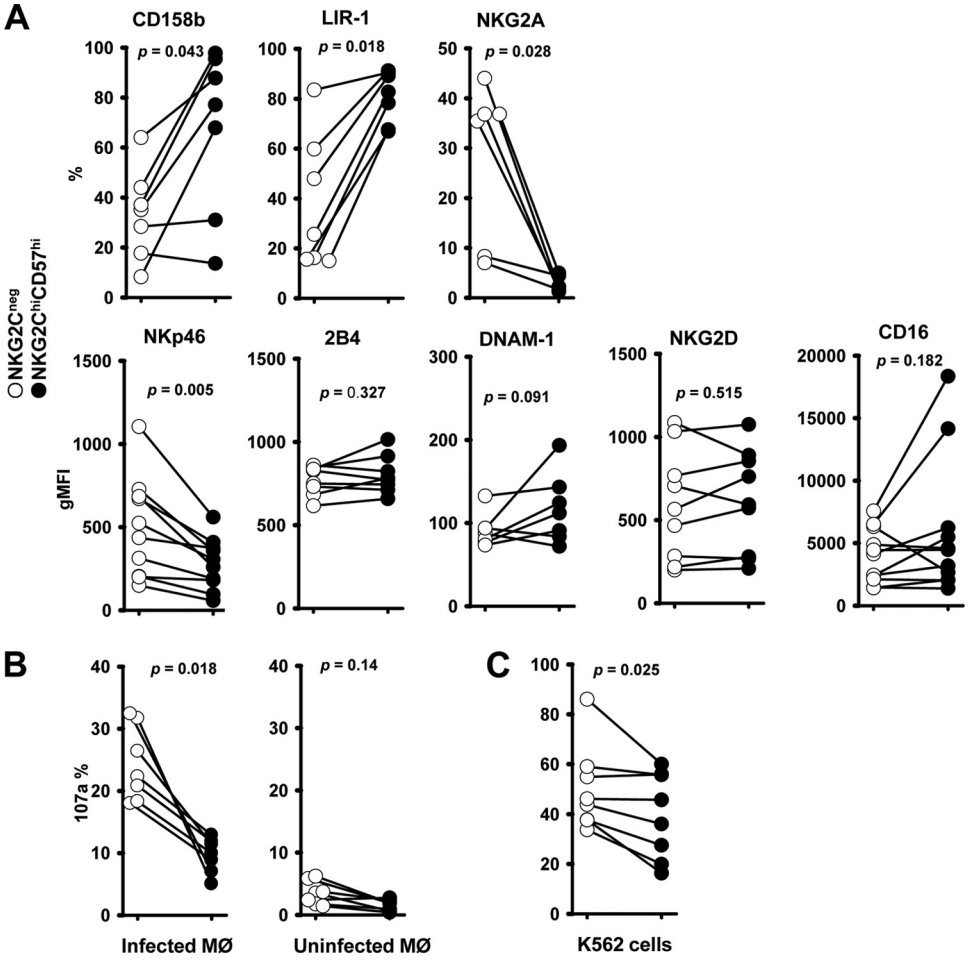

FIG 2 Expression of inhibitory and activating receptors on NKG2C^{hi} CD57^{hi} NK cells (A) and their degranulation to HCMV-infected autologous macrophages (B) and K562 cells (C). (A) Expression of CD158b, LIR-1, NKG2A, NKp46, 2B4, DNAM-1, NKG2D, and CD16 on NKG2C^{hi} CD57^{hi} NK cells and NKG2C-negative NK cells from NKG2C^{hi} CD57^{hi} NK cell-positive donors. The paired circles from one donor are connected by a line. Percentages (CD158b, LIR-1, and NKG2A) or geometric mean fluorescence intensities (gMFI) (NKp46, 2B4, DNAM-1, NKG2D, and CD16) are shown. (B) Thawed PBMCs (1×10^6) were cocultured with TB40/E-infected macrophages (1×10^5) or uninfected macrophages for 48 h. Then, surface expression of CD107a on NK cells was assessed 5 h after the addition of anti-CD107a. (C) Thawed PBMCs (1×10^6) were cultured for 48 h and afterwards cocultured with K562 cells (1×10^5) for 5 h in the presence of anti-CD107a, and then surface CD107a expression was assessed. (A, B, and C) The percentages of positive cells were determined on NKG2C^{hi} CD57^{hi} NK cells and NKG2C-negative NK cells. The nonparametric Wilcoxon signed rank sum test was used to compare NKG2C^{hi} CD57^{hi} NK cells with NKG2C-negative NK cells matched from the same donor.

NKG2C^{hi} CD57^{hi} NK cells are highly responsive to infected autologous macrophages through HCMV antibody-dependent stimulation. We next examined whether the NKG2C^{hi} CD57^{hi} NK cell subset response is also modulated by HCMV antibodies. We found that NKG2C^{hi} CD57^{hi} NK cells exhibit enhanced degranulation and IFN-γ production in response to HCMV-infected autologous macrophages in the presence of HCMV antibodies (Fig. 4A, right). This enhanced effect was observed in all donors tested. To examine whether this response was also dependent on the spectrum and amount of HCMV antibodies, we compared the effect of the autologous plasma with that of commercial pooled human Ig in our assay. No differences in the response of NKG2C^{hi} CD57^{hi} NK cells to HCMV-infected macrophages could be found (data not shown). The concentration of HCMV antibodies needed in our functional assay was even lower than the physiological concentration in humans. It is known that antibody-dependent NK cell-mediated responses occur in parallel with the CD16 downregulation of NK cells (31). This loss of CD16 is thought to prevent a continuous stimulation of NK cells and activation-induced cell death (37, 38). In the HCMV antibody-mediated NK cell response, CD16 was clearly downregulated (Fig. 4A). Similar downregulation was also observed in the rituximab-mediated NK cell response to Raji cells, which we included as a control. Our data also indicate that CD16-downregulated NKG2C^{hi} CD57^{hi} NK cells show enhanced degranulation and IFN-γ production (Fig. 4B, top and middle). Furthermore, the CD107a expression correlates with the IFN-γ production of NKG2C^{hi} CD57^{hi} NK cells (Fig. 4B, bottom). We also studied purified NK cells and found that NKG2C^{hi} CD57^{hi} NK cells were again highly responsive to infected macrophages in the presence of HCMV antibodies (data not shown). To study NKG2C^{hi} CD57^{hi} NK cell responses to superantigen stimulation, we applied staphylococcal enterotoxin B (SEB), which can induce lymphokine release (39). The SEB-stimulated responses were only observed in

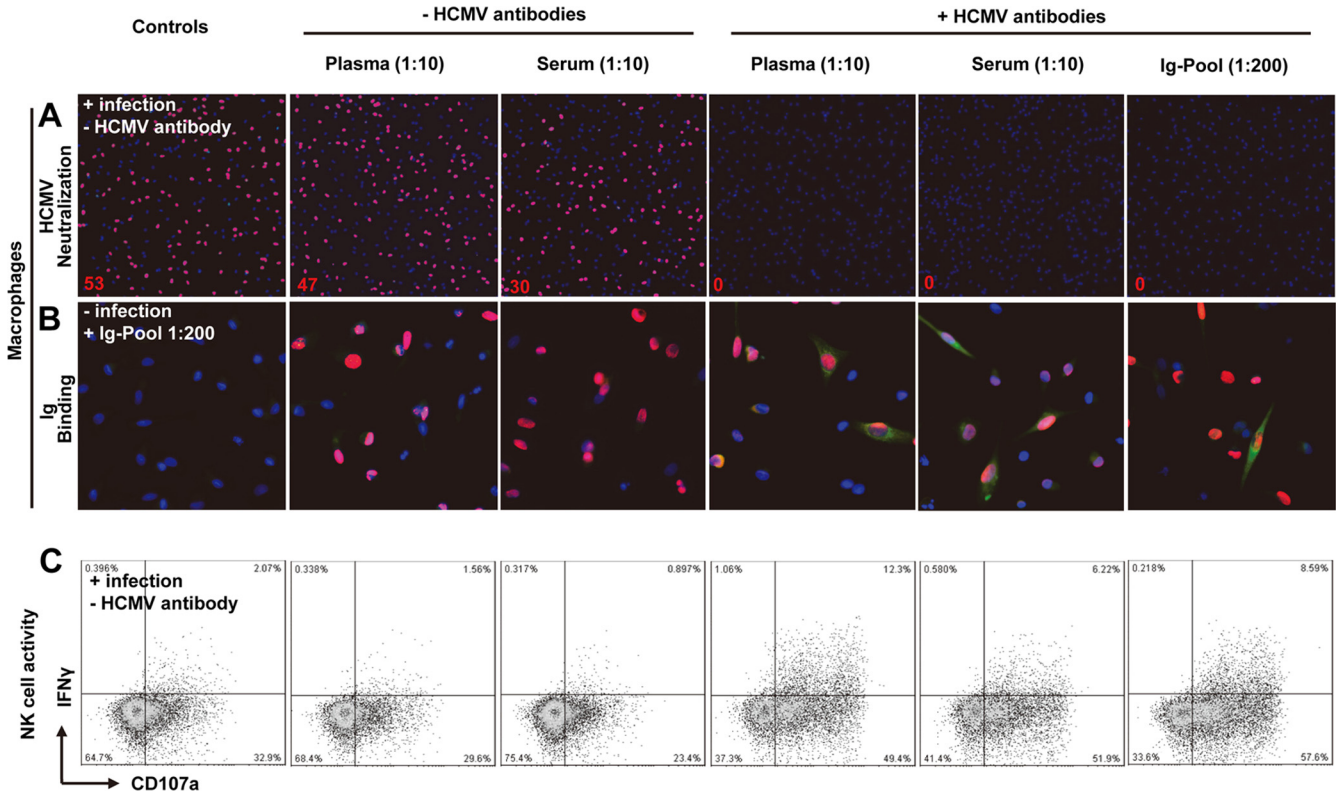

FIG 3 Establishment of HCMV antibody-dependent NK cell-mediated responses to infected autologous macrophages. (A) Two hundred-microliter cell-free suspensions of TB40/E with or without HCMV antibodies were used in neutralization experiments on macrophages. Virus suspensions and indicated Ig preparations (autologous HCMV-antibody-positive plasma/serum with 1:10 dilution or Ig pool with 1:200 dilution) were incubated for 30 min and then inoculated onto the cells. The infection rates were assessed after 24 h of incubation. The presence of HCMV IEA (red fluorescence) indicates infected macrophages, and cell nuclei are stained in blue (DAPI). The infection rates are indicated in red numbers. Original magnification, ×40. (B) Macrophages were used for staining 3 days postinfection. Methanol-fixed macrophages were incubated with the indicated Ig preparations, followed by FITC–anti-human IgG staining. The presence of HCMV IEA (red fluorescence) indicates infected macrophages, human IgG binding on macrophages is shown by green fluorescence, and nuclei are stained in blue (DAPI). Original magnification, ×100. (C) Thawed PBMCs (1×10^6) were cocultured with TB40/E-infected macrophages (1×10^5) for 48 h. Then, surface expression of CD107a and IFN-γ production of NK cells was assessed after incubation with the indicated HCMV-specific antibodies (seronegative plasma and serum used as controls) and anti-CD107a for an additional 5 h. The results of one representative experiment from an HCMV-seropositive donor without NKG2C^{hi} CD57^{hi} NK cells are shown.

NKG2C-negative NK cells, and SEB stimulation did not downregulate CD16 expression (data not shown). This is consistent with the published data showing that NKG2C-positive NK cells respond differently from NKG2C-negative NK cells after cytokine stimulation (15).

NKG2C receptor contributes to NKG2C^{hi} CD57^{hi} NK cell activation, and co-cross-linking of NKG2C enhances CD16-mediated response. To elucidate whether a greater responsiveness to signaling through the CD16 was an intrinsic property of the NK cell subset and whether the NKG2C receptor augmented CD16-mediated stimulation, we monitored the induction of CD107a expression on NK cells after coculture with CD16 and/or NKG2C antibody-coated P815 cells. It has been shown before that antibody-coated cells induce the NK cell response better than CD16 antibody-coated plastic plates (40). The NKG2C receptor contributed to CD57^{+} NKG2C^{hi} NK cell activation when stimulated on NKG2C antibody-coated plates (11). In Figure 5, we show the representative results for one donor (Fig. 5A) and the cumulated results from all experiments (Fig. 5B). In our assay, NKG2C cross-linking also induced degranulation of NKG2C^{hi} CD57^{hi} NK cells, while it did not activate NKG2C-negative NK cells (Fig. 5). NKG2C^{hi} CD57^{hi} NK cells were more responsive to CD16-coated P815 cells (Fig. 5), and we could show that both NK cell subpopulations express the same amount of CD16 (Fig. 2A). This indicates that NKG2C^{hi} CD57^{hi} NK cells are intrinsically more responsive to signaling through CD16. Furthermore, we show that co-cross-linking of NKG2C resulted in enhanced degranulation of NKG2C^{hi} CD57^{hi} NK cells in response to CD16-coated cells. Such an enhanced effect was not observed in NKG2C-negative NK cells. These data suggest that NKG2C can initiate NKG2C^{hi} CD57^{hi} NK cell activation and may also contribute to antibody-dependent stimulation.

DISCUSSION

Increasing evidence indicates that NK cells play an important role in the control of HCMV and that HCMV infection shapes the NK cell receptor repertoire (41). Our data add important new information to this field, in particular by demonstrating that NKG2C^{hi} CD57^{hi} NK cells exhibit high responsiveness to HCMV-infected autologous macrophages only in the presence of antiviral antibodies.

We demonstrate that, despite a high interdonor variation con-

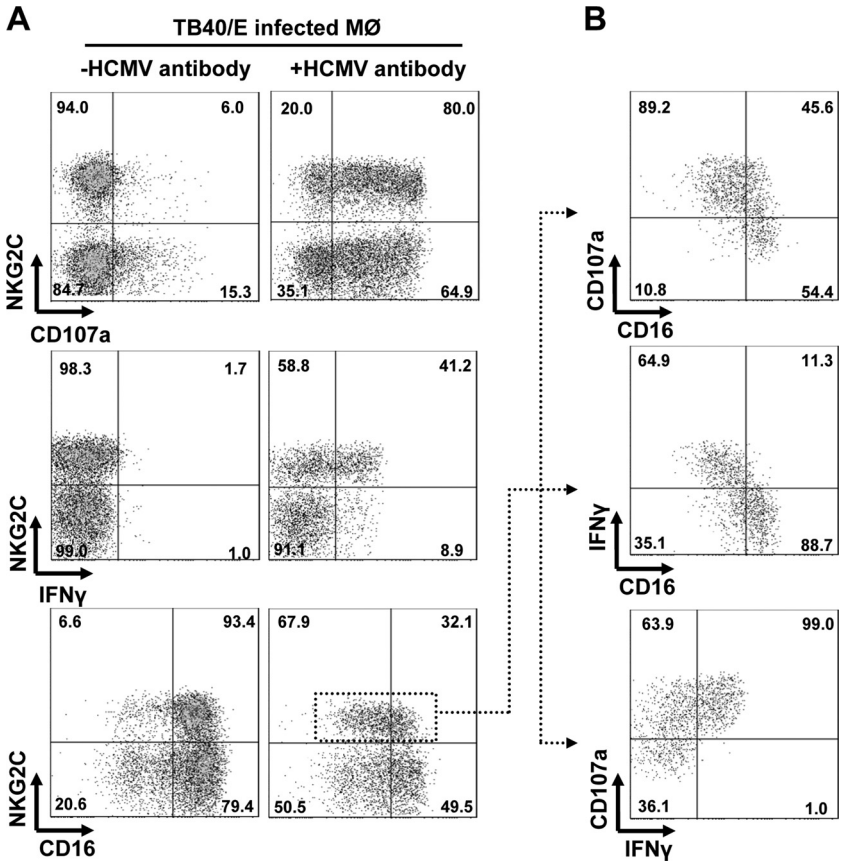

FIG 4 NKG2C^{hi} CD57^{hi} NK cells are highly responsive to HCMV antibody-dependent stimulation. (A) PBMCs (1×10^6) were cocultured with TB40/E-infected macrophages (1×10^5) for 48 h. Then, surface expression of CD107a, CD16, and IFN-γ production of NK cells was assessed after 5 h in the absence (left) or presence (right) of autologous plasma (1:10 dilution) and anti-CD107a. The indicated percentages of positive cells were determined as percentage of NKG2C^{hi} CD57^{hi} NK cells and NKG2C-negative NK cells. (B) CD16 and CD107a expression and IFN-γ production of the NKG2C^{hi} CD57^{hi} NK cell subset. First, NKG2C^{hi} CD57^{hi} NK cells were gated. Then, the percentages of positive cells were determined as percentage of CD16-positive and CD16-negative NK cells (top and middle) or as percentage of IFN-γ-positive and IFN-γ-negative NK cells (bottom). (A, B) The results of one representative experiment of four from NKG2C^{hi} CD57^{hi} NK cell-positive donors are shown.

cerning the relative frequencies, NKG2C^{hi} CD57^{hi} NK cells exclusively appear in HCMV-seropositive donors. The reason for the variability in the occurrence of this NK cell subset in HCMV-seropositive donors is not yet clear but might be related to the status of HCMV latent infection, e.g., the time of primary infection or whether and when the individual donor experienced an HCMV reactivation. This could be assumed in analogy to the fact that, in a study including SOT recipients with reactivated active HCMV infection, a significant number of CD57$^+$ NKG2C^{hi} NK cells appeared within 1 to 2 weeks after the detection of HCMV viremia in all patients (11). Another possibility might be that the NKG2C^{hi} CD57^{hi} NK cell expansion is associated with a particular setup of killer cell immunoglobulin-like receptors (KIR). A cohort study showed that self-specific KIR NK cell expansion was most pronounced in the NKG2C-positive NK cells. Most of the expanded subsets expressed at least one HLA-C-binding inhibitory KIR (41). Furthermore, the NKG2C gene copy number may also contribute to the percentage of viable NKG2C^{hi} CD57^{hi} NK cells. Recently, a study in children reported that a homozygous NKG2C$^{+/+}$ genotype was associated with increased absolute numbers of NKG2C-positive NK cells (42).

It is known that NK cell activity is regulated by activating and inhibitory receptors. It has been shown in antibody-blocking experiments that the activating receptors NKp46, 2B4, and DNAM-1 contribute to the NK cell response to HCMV-infected macrophages (23). The receptor NKp46 is an important mediator of NK cell cytotoxicity. It has been reported to interact with hemagglutinins derived from influenza and parainfluenza viruses (43). 2B4 (also called CD244) expressed on mature human NK cells is clearly activating or coactivating (44). It is a member of the SLAM family of membrane receptors. The ligand for 2B4 is CD48, a cell surface glycoprotein expressed broadly on hematopoietic cells. DNAX accessory molecule 1 (DNAM-1) receptor (also called CD226) is a member of the Ig superfamily. CD112 (also known as polio virus receptor [PVR]) and CD155 (also called nectin-2) have been identified as ligands for DNAM-1. It regulates both NK cell migration and cellular activation (45). We could show that NKG2C^{hi} CD57^{hi} NK cells expressed lower levels of the activating receptor NKp46 but equal levels of 2B4 and DNAM-1 compared to the levels in NKG2C-negative cells. A lower level of expression of NKp46 may contribute to the reduced responsiveness of NKG2C^{hi} CD57^{hi} NK cells against infected macrophages in the absence of HCMV antibodies. We also show that NKG2C^{hi}

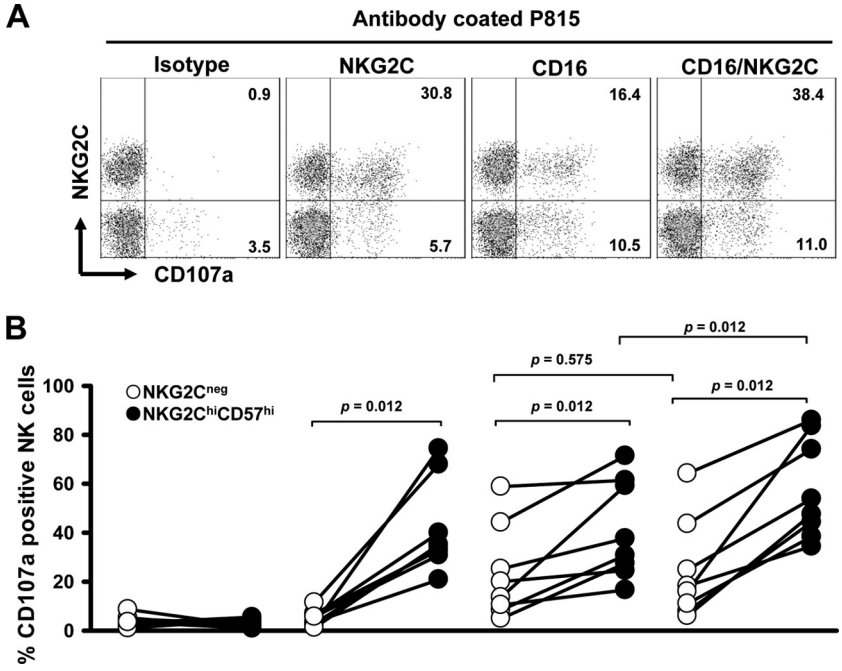

FIG 5 NKG2C induces degranulation of NKG2C^{hi} CD57^{hi} NK cells and augments degranulation through CD16 stimulation. (A) Representative staining of redirected degranulation against antibody-coated P815 cells. Thawed PBMCs (1×10^6) were cocultured with P815 cells (1×10^5) which had been precoated with the isotype control MAb (IgG1), anti-NKG2C MAb, or/and anti-CD16 MAb for 4 h. The percentages of CD107a-positive cells were determined on NKG2C^{hi} CD57^{hi} NK cells and NKG2C-negative NK cells. (B) Percentages of CD107a-positive cells on NKG2C^{hi} CD57^{hi} NK cells and NKG2C-negative NK cells from different donors are shown. The paired circles from one donor are connected by a line.

CD57^{hi} NK cells express more inhibitory CD158b. CD158b (predominately KIR2D/L3) is the most frequently expressed KIR in NKG2C-positive NK cells (15, 17). It is also possible that CD158b might downregulate the response of the NKG2C^{hi} CD57^{hi} NK subset. Previous data support the idea that self-inhibitory KIRs could damp the NKG2C-mediated activation (15). The exact contributions of NKP46 and CD158b need further investigation.

The response of NKG2C-positive NK cells could be different in healthy subjects than in patients suffering from active HCMV infection. We and others could show that, in the absence of antibodies, NKG2C-positive NK cells are less responsive to HCMV-infected cells and K562 cells (15, 34). Another study showed that NKG2C-positive NK cells produced increasing amounts of IFN-γ after exposure to K562 cells when the NK cells were obtained from transplant recipients after HCMV reactivation (17). However, the underlying mechanism for this upregulation remains unknown. To study whether acute infection contributes to the responsiveness of NKG2C-positive NK cells, the assay presented here will be helpful.

We demonstrate in functional studies that infected autologous macrophages can be utilized to quantitatively determine HCMV antibody-dependent NK cell-mediated responses. In our assay, NKG2C^{hi} CD57^{hi} NK cells are highly responsive to HCMV antibody-dependent stimulation. It had been shown that CD57-positive NK cells do respond better than CD57-negative NK cells to CD16 stimulation (20); however, in our assay, we did not find NKG2C^{neg} CD57^{pos} NK cells to be highly responsive in response to HCMV antibody stimulation. This discrepancy might be explained by different experimental setups or by the fact that the previous study neither took into account the HCMV serostatus of their donors nor included the analysis of NKG2C-positive NK cells. We also found that CD16 was clearly downregulated on activated NK cells after HCMV antibody stimulation. This demonstrates that including CD16 to identify the NKG2C^{hi} CD57^{hi} NK cell subset would indeed lead to an underestimation of the role of these cells in antibody-dependent assays. Moreover, NKG2C^{hi} CD57^{hi} NK cells are intrinsically more responsive to signaling through CD16. Still, the exact mechanism for the enhanced responses to CD16 stimulation is unknown. During the preparation of the manuscript, a study was published in February that is mainly in line with our findings (46). Zhang et al. describe a molecular signature for HCMV-induced NK cells. Their study shows that FcRγ-deficient NK cells, which appear in HCMV-positive donors and also mostly express the activating receptor NKG2C, show an enhanced response to both herpes simplex virus 1 (HSV-1)- and HCMV-infected allogeneic fibroblasts in the presence of virus-specific antibodies. The cross-reactivity with HSV-infected fibroblasts through HSV-specific antibody stimulation may be explained by our finding that the HCMV-induced NK cells are intrinsically more responsive to CD16 stimulation. The published FcRγ deficiency may provide the clue for the finding that HCMV-induced NK cells respond more robustly through CD16 stimulation. We could show that NKG2C^{hi} CD57^{hi} NK cells are also more responsive to NKp46 cross-linking (data not shown). Both CD16 and NKp46 share the same signaling adaptors, FcRγ and CD3ζ. FcRγ deficiency may enhance the signaling when CD16 and NKp46 have to exclusively use CD3ζ, which contains three immunoreceptor

tyrosine-based activation motifs (ITAM) (47). A recent study showed that 38% of 151 HCMV-positive donors exhibited expanded HCMV-induced NK cells, among whom 8 donors had expanded NKG2C-negative NK cells (41). These NKG2C-negative cells expressed activating KIRs but shared the same functional profiles as NKG2C-positive NK cells. It would be interesting to investigate whether these HCMV-induced NKG2C-negative NK cells are FcRγ deficient. These outliers may help to refine the phenotype of HCMV-induced NK cells. Altogether, these phenotypic results have to be related to a functional characterization of the cells. Using autologous HCMV-infected macrophages, we demonstrate that HCMV-induced NK cells are functionally poor effectors of natural cytotoxicity but become strong effectors in the presence of HCMV-specific antibodies. In our experience, the coculture of NK cells with infected fibroblasts for 2 days provides less-reproducible results than using infected autologous macrophages. NKG2C, self-inhibitory KIRs, and FcRγ are most likely to relate to their expansion and function (10, 11, 41, 46). The most intriguing question is how these receptors and adaptors are modulated by HCMV. Understanding this mechanism may reveal fundamental properties of human NK cells.

The role of HCMV antigens for NK cell expansion and function has not been identified. HCMV encodes the MHC class I homolog glycoprotein UL18, which can bind directly to NKG2C (48). The glycoprotein UL40 can upregulate the cell surface expression of HLA-E (49), which in turn is recognized by NKG2C (33). Furthermore, HCMV US genes have been characterized for their capacity to damp the surface expression of MHC class I in infected cells (6). We have preliminary results using HCMV US 2, 3, 6, and 11 and UL18 and UL40 minus mutants in our experimental settings and have not found a contribution of these genes so far.

Although our *in vitro* assay mimics the physiological role of NK cells during HCMV infection, it has several limitations. First, due to limited resources, we were not able to include other types of autologous cells that are also important for HCMV infection (e.g., endothelial cells). Second, we randomly obtained buffy coats from anonymized healthy blood donors, and therefore, we could not perform kinetic studies on the function of this NK cell subset.

NKG2C-positive NK cell populations are stable in healthy adult donors (41, 46), and they expand and contract dynamically in active CMV infection (11, 17). NK cells undergo apoptosis after long-term IL-12 exposure *in vitro* (50), and CD57-positive NK cells express less IL-12Rβ2 chain mRNA than CD57-negative NK cells (20), which suggests that NKG2C^{hi} CD57^{hi} NK cells might also be less responsive to IL-12. We also found that NKG2C^{hi} CD57^{hi} NK cells survived preferentially after long-term IL-12 exposure with HCMV-infected macrophages (data not shown). This resistance to apoptosis may be an important feature of NKG2C-positive NK cells during active HCMV infection.

Our study demonstrates that it is extremely critical to take into account the donor HCMV serostatus in further human NK cell research, especially for studies related to NKG2C, CD57, and ADCC. It also suggests that large cohorts should be analyzed in future studies to clarify the occurrence of the NK cell subsets in different populations and their potential clinical relevance. Therefore, it would be necessary to analyze (i) the genotypic and phenotypic profiles of NKG2C, CD16, KIR receptors, and FcRγ adaptor, (ii) the longitudinal functional profiles of NKG2C^{hi} CD57^{hi} NK cells, and (iii) the correlation of the *in vitro* functional studies with the outcomes of patients with HCMV infections.

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