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NKG2C^{pos} NK Cells Regulate the Expansion of Cytomegalovirus-Specific CD8 T Cells

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Infection with the human CMV associates with phenotypic alterations in lymphocyte subsets. A highly reproducible finding in CMV-seropositive individuals is an expansion of NKG2C^{pos} NK cells. In this study, we analyzed if the altered NK cell compartment in CMV-seropositive human donors may affect CMV-specific CD8 T cells. Resting CMV-specific CD8 T cells were terminally differentiated and expressed high levels of the NKG2C ligand HLA-E. Activation of CMV-specific CD8 T cells with the cognate Ag further increased HLA-E expression. In line with a negative regulatory effect of NKG2C^{pos} NK cells on HLA-E^{high} CD8 T cells, depletion of NKG2C^{pos} NK cells enhanced Ag-specific expansion of CMV-specific CD8 T cells in vitro. In turn, the activation of NK cells in coculture with CMV-specific CD8 T cells promoted a selective loss of HLA-E^{high} CD8 T cells. To test if NKG2C^{pos} NK cells can target HLA-E^{high} CD8 T cells, Jurkat T cells with and without stabilized HLA-E on the surface were used. NKG2C^{pos} NK cells stimulated with HLA-E^{high} Jurkat cells released higher levels of Granzyme B compared with NKG2C^{neg} NK cells and NKG2C^{pos} NK cells stimulated with HLA-E^{low} Jurkat cells. Moreover, intracellular levels of caspase 3/7 were increased in HLA-E^{high} Jurkat cells compared with HLA-E^{low} Jurkat cells, consistent with higher rates of apoptosis in HLA-E^{high} T cells in the presence of NKG2C^{pos} NK cells. Our data show that NKG2C^{pos} NK cells interact with HLA-E^{high} CD8 T cells, which may negatively regulate the expansion of CMV-specific CD8 T cells upon activation. ***The Journal of Immunology*****, 2020, 204: 2910–2917.**

Cytomegalovirus is a β herpesvirus causing persistent infection despite eliciting a massive immune response, which has been shown to influence the majority of immune cell frequencies, functional responses, and serum protein concentrations (1). CMV has a worldwide prevalence ranging between 50 and 100% of the population, depending on socioeconomic factors. Although in most cases, CMV leads to a lifelong latent infection without symptoms (2), it can cause severe disease in the immunocompromised when reactivated from the latent state. Lytic replication of CMV is therefore a frequent and major complication following allogeneic hematopoietic stem cell transplantation and solid organ transplantation, causing a variety of organ-specific diseases, including pneumonia, encephalitis, and gastrointestinal disease (3–5). Therefore, understanding the mechanisms leading to immune control of CMV infection and the consequences of the altered immune cell compartment remains of interest.

The two most striking immunological features associated with CMV infection are massive inflation of terminally differentiated CMV-specific CD8 T cells, with up to 30% of total CD8 T cells being CMV specific (reviewed in Refs. 6 and 7) and expansion of NK cells carrying the activating NK cell receptor NKG2C (reviewed in Ref. 8). NKG2C^{pos} NK cells are potent producers of IFN-γ (9, 10) and highly efficient mediators of Ab-dependent cellular cytotoxicity (11, 12) and have long been associated with CMV control, especially in immunocompromised individuals (13–15). Interestingly, CMV-seropositive individuals with homozygous deletion of the NKG2C gene show an increase of terminally differentiated memory cells re-expressing CD45RA (TEMRA) CD8 T cells at a younger age (16), indicating a possible interaction between NKG2C^{pos} NK cells and TEMRA CD8 T cells in the context of CMV infection. It has been known for several years that NK cells are involved in modulating T cell responses. In fact, one of the first studies showing this was conducted in the murine CMV model and reported increased T cell proliferation and a higher numbers of T cells expressing IFN-γ when NK cells were depleted (17). In line with this, depletion of NK cells in mice postinfection with lymphocytic choriomeningitis virus (LCMV) was associated with superior immune control, which was mechanistically associated with increased T cell immunity in the absence of negative regulation by NK cells (18–20). Follow-up studies showed that type I IFNs can protect CD8 T cells from negative NK cell regulation by upregulating Qa1-b, the ligand for the inhibitory NK

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Abbreviations used in this article: CM, central memory; EM, effector memory; HBV, hepatitis B; IAV, influenza A virus; LCMV, lymphocytic choriomeningitis virus; TEMRA, terminally differentiated memory cell re-expressing CD45RA.

cell receptor NKG2A (21, 22). In the human system, a negative regulatory effect of NK cells on CD8 T cells has also been observed in the context of hepatitis B (HBV) infection (23). In this study, in the context of CMV infection, we aimed to analyze whether NKG2C^{pos} NK cells modulate CMV-specific CD8 T cell responses.

Materials and Methods

Study subjects

PBMCs from healthy donors were isolated from buffy coats obtained at the center for blood donation from the University Hospital Düsseldorf with approval of the local Ethics Committee. Seventy-eight percent of the study subjects were male. The median age was 42 y, ranging from 21 to 64 y.

All participants were tested negative for Abs against HBV, hepatitis C, hepatitis E, HIV, and *Treponema pallidum* on ADVIA Centaur XP (Siemens). All participants also tested negative for RNA/DNA of HBV, hepatitis C, hepatitis E, HIV, and CMV using Cobas6800 (Roche). Anti-CMV IgG was quantified using the LIAISON XL (DiaSorin).

Analysis of NK cells and T cells

All samples were stained with Fixable Viability Dye eFluor506 (catalog no. 65-0866-18; eBioscience). The following Abs were used to analyze NK cells: CD3 eFluor450 (catalog no. 48-0037-42, clone: OKT3), CD14 eFluor450 (catalog no. 48-0149-42, clone: 61D3), CD16 PE/Cy5 (catalog no. 302010, clone: 3G8; BioLegend), CD16 allophycocyanin-eFluor780 (catalog no. 47-0168-42, clone: eBioCD16), CD19 eFluor450 (catalog no. 48-0198-42, clone: SJ25C1), CD56 PE-Cy7 (catalog no. 25-0567-42, clone: CMSSB), CD56 allophycocyanin (catalog no. 17-0566-42, clone: TULY56), CD57 eFluor450 (catalog no. 48-0577-41, clone: TB01), CD158 PE/Cy7 (catalog no. 339512, clone HP-MA4; BioLegend), CD158b allophycocyanin (catalog no. 312612, clone: DX27; BioLegend), CD161 Brilliant Violet 605 (catalog no. 339916, clone: HP-3G10; BioLegend), LILRB1 allophycocyanin/Fire 750 (catalog no. 333718, clone: GHI/75; BioLegend), NKG2C PE (catalog no. FAB128P, clone: 134591; R&D Systems), FcεR1γ FITC (catalog no. FCABS400F, clone: 3G6; MilliporeSigma), CD107a PE-Cy7 (catalog no. 451348, clone: H4A3; BD), CD107a Alexa Fluor 700 (catalog no. 561340, clone: H4A3; BD), TNF-α Brilliant Violet 711 (catalog no. 502940, clone: MAb11; BioLegend), Granzyme B PE/Dazzle 594 (catalog no. 372216, clone: QA16A02), and Perforin Brilliant Violet 711 (catalog no. 308130, clone: dG9) (all eBioscience). The following Abs were used to characterize T cells: CD3 PerCP-Cy5.5 (catalog no. 45-0037-42, clone: OKT3), CD3 eFluor450 (catalog no. 48-0037-42, clone: OKT3), CD4 allophycocyanin-eFluor780 (catalog no. 47-0049-42, clone: RPA-T4), CD8 eFluor450V (catalog no. 48-0088-42, clone: RPA-T8), CD8 allophycocyanin (catalog no. 17-0088-42, clone: RPA-T8), CD8 FITC (catalog no. 11-0088-42, clone: RPA-T8), CD27 PerCP-eFluor710 (catalog no. 46-0279, clone: O323), CD38 PerCP-eFluor710 (catalog no. 46-0388-42, clone: HB7), CD45RA allophycocyanin (catalog no.17-0458-42, clone: HI100) (all eBioscience), HLA-E PE-Cy7 (catalog no. 342608, clone: 3D12), CCR7 BV421 (catalog no. 353207, clone: G043H7) (both BioLegend), and KLRG1 PE (catalog no. 130-103-703; Miltenyi Biotec). For identification of CMV-specific (epitope: NLVPMVATV) and influenza A virus (IAV)–specific (epitope: GILGFVFTL) CD8 T cells, PE-conjugated HLA-A*02/peptide multimers were obtained from Immudex (Copenhagen, Denmark) and Proimmune (Oxford, U.K.). NK cells were analyzed via flow cytometry using a FACSCanto or LSRFortessa (BD Biosciences).

ELISpot

CMV-specific IFN-γ responses were determined using T-SPOT.*CMV* (Oxford Immunotec, Abingdon, Oxfordshire, U.K.). Two hundred fifty thousand PBMC per cell culture were seeded in each of four wells of eight-well strips, according to the manufacturer's instructions. The cells were stimulated for 16–20 h at 37˚C with CMV-specific peptides (IE-1 and pp65), parallel with negative and positive controls. The resultant spots, each representing a single IFN-γ–releasing cell, were quantified using an ELISpot plate reader (AID FluoroSpot; Autoimmun Diagnostika, Strassberg, Germany). Negative controls were subtracted from CMV-specific values, resulting in spot-forming cells.

Depletion of NK cells and NKG2C^{pos} NK cells

For depletion of total NK cells or NKG2C^{pos} NK cells, PBMCs were stained using an anti-CD56 allophycocyanin Ab (eBioscience) alone or in combination with an anti-NKG2C PE Ab (R&D Systems). CD56 allophycocyanin-positive cells or CD56 allophycocyanin, NKG2C PE double-positive cells were depleted using a FACSAria II (BD Biosciences).

Expansion of CMV-specific CD8 T cells and NK cell activation

PBMCs were cultured for 10 d in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES buffer containing 25 IU/ml IL-2 (Roche) and 1 μg/ml peptide (CMV: NLVPMVATV). Fresh medium and IL-2 was added after 7 d. For activation of NK cells, irradiated K562 cells (30 Gy) were added at an effector/target ratio of 10:1.

T cell activation in vitro

For activation of CD8 T cells, PBMCs were cultured with 2 μl of Dynabeads Human T-Activator CD3/CD28 (Life Technologies) per 10^6 PBMCs for 3 d.

Analysis of caspase 3/7 activity in Jurkat cells

PBMCs were prestimulated with low dosage IL-15 (1 ng/ml) overnight. Wild-type Jurkat cells were incubated overnight at 26˚C with an HLA-E–stabilizing peptide (VMAPRTLFL, 100 μM) (HLA-E^{high}). As a control, Jurkat cells were incubated without peptide (HLA-E^{low}). HLA-E expression was analyzed via flow cytometry (FACS Fortessa [BD Biosciences]). Jurkat cells were traced with either CellTrace Violet Cell Proliferation Kit (HLA-E^{low}) or CellTrace Far Red Cell Proliferation Kit (HLA-E^{high}) (both from Thermo Fisher Scientific). PBMCs were cocultivated with traced HLA-E^{low} and HLA-E^{high} Jurkat cells for 24 h at 26˚C at an effector/target ratio of 10:1:1. The caspase 3/7 activity in Jurkat cells were analyzed via CellEvent Caspase 3/7 Green Flow Cytometry Assay Kit from Thermo Fisher Scientific, according to manufacturers' instructions. As a control, active caspase 3/7 was measured after 24 h in two replicas of mixed HLA-E^{high}– and HLA-E^{low}–expressing Jurkat cells in the absence of PBMCs. The median expression of active caspase 3/7 was 11.6% for HLA-E^{high}– and 11.3% for HLA-E^{low}–expressing Jurkat cells. Subsequently, these background levels were subtracted.

Functional analysis of NK cells

PBMCs were stimulated in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, and 10 ng/ml brefeldin A (Sigma-Aldrich), with Jurkat cells at an effector/target ratio of 10:1 for 5 h. Granzyme B and Perforin release experiments were carried out in the absence of brefeldin A. Subsequently, NK cells were analyzed via flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.04 software (GraphPad Software, San Diego, CA). For the comparison of two groups, either a parametric or nonparametric *t* test was performed. Three groups were compared by Kruskal–Wallis test. The applied statistical tests are indicated in the figure legends.

Study approval

Written informed consent was obtained from all participants, and the study was approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University (study no. 2018-131-KFogU).

Results

NKG2C^{pos} NK cells are involved in regulating CMV-specific CD8 T cells

We first analyzed the expression of NKG2A and NKG2C on NK cells of CMV-seropositive and CMV-seronegative individuals. Whereas the frequencies of NKG2C^{neg}NKG2A^{neg}, NKG2C^{pos}NKG2A^{pos}, and NKG2C^{neg}NKG2A^{pos} NK cells were comparable in CMV-seropositive and -seronegative individuals, CMV-seropositive individuals had significantly higher frequencies of NKG2C^{pos}NKG2A^{neg} NK cells (Fig. 1A, $p = 0.0007$). An exemplary staining of NKG2A and NKG2C expression on NK cells of a CMV-seropositive individual and a CMV-seronegative individual is shown in Fig. 1B. Of note, not all CMV-seropositive individuals showed an expansion of NKG2C^{pos} NK cells. In line with previous reports, the expanded NKG2C^{pos} NK cells of CMV-seropositive donors showed an adaptive NK cell phenotype (CD57^{high}, LILRB1^{high}, CD161^{low}, and

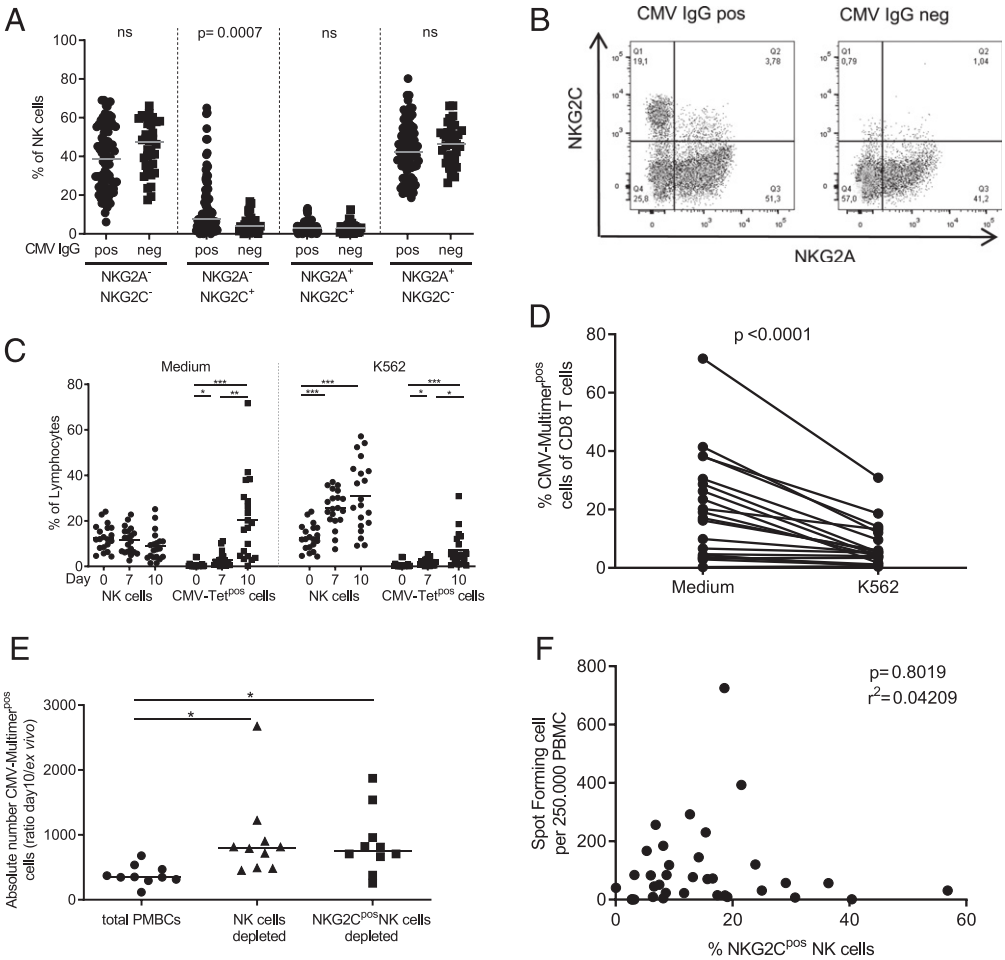

FIGURE 1. Analysis of CMV-specific T cells in CMV-seropositive individuals. (**A**) The frequency of NKG2A^{neg}NKG2C^{neg}, NKG2A^{neg}NKG2C^{pos}, NKG2A^{pos}NKG2C^{pos}, and NKG2A^{pos}NKG2C^{neg} NK cells was analyzed in 88 CMV-seropositive and 36 CMV-seronegative individuals. CMV-seropositive individuals had significantly higher frequencies of NKG2A^{neg}NKG2C^{pos} NK cells then CMV-seronegative individuals (*t* test). (**B**) Exemplary staining of NKG2A- and NKG2C-expressing NK cells of one CMV-seronegative and one CMV-seropositive individual. (**C**) CMV-specific CD8 T cells were expanded in the presence of whole PBMCs. NK cells either were activated by K562 or not activated (medium). The frequencies of NK cells and CMV-specific CD8 T cells of 20 individuals on day 0, 7, and 10 are shown (Kruskal–Wallis test, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$). (**D**) On day 10, there were significantly fewer CMV-specific CD8 T cells in the presence of activated NK cells (paired *t* test). (**E**) CMV-specific CD8 T cells of 10 CMV-seropositive individuals were expanded in the presence of NK cells (total PBMCs), in the absence of NK cells (NK cells depleted), or in the absence of NKG2C^{pos} NK cells (NKG2C^{pos} NK cells depleted). The absolute cell numbers of CMV-Multimer^{pos} CD8 T cells were determined ex vivo and after 10 d of expansion. The expansion ratio (absolute cell number day 10/absolute cell number day 0) is displayed (Kruskal–Wallis test, $*p \leq 0.05$). (**F**) PBMCs of 38 CMV-seropositive individuals were stimulated with overlapping peptides of the CMV protein pp65. The ELISpot assays showed no association between the number of spot-forming cells and the frequencies of NKG2C^{pos} NK cells.

FcεR1γ^{low}), accompanied by the expression of self-specific KIRs (24, 25) (data not shown). Next, we analyzed whether NK cells influence the expansion of CMV-specific CD8 T cell responses. We hypothesized that both NK cells and T cells receive stimulatory signals during lytic CMV infection and therefore performed coculture experiments of peptide-stimulated CMV-specific CD8 T cells in the presence of activated NK cells. Therefore, PBMCs of 30 HLA-A*02–positive CMV-seropositive individuals were cultured in the presence of the HLA-A*02–restricted CMV-pp65–derived NLVPMVATV peptide. Irradiated K562 cells at an effector/target ratio of 10:1 were added for the activation of NK cells. As expected, Ag-specific CD8 T cells were expanded in the presence or absence of K562 cells, whereas NK cells were only expanded in cultures containing irradiated K562 cells (Fig. 1B). After 10 d, CMV-specific CD8 T cells were identified with the corresponding HLA-A*02/peptide multimer. Twenty-eight of the HLA-A*02–positive CMV-seropositive individuals showed an expansion of CMV-specific CD8 T cells in vitro. It is of note that the activation of NK cells by K562 cells significantly inhibited the expansion of CMV-specific CD8 T cells ($p < 0.0001$, Fig. 1D). Because a CMV infection is strongly associated with an expansion of NKG2C^{pos} NK cells, we next analyzed if NKG2C^{pos} NK cells influence the expansion of CMV-specific CD8 T cells. Therefore, total NK cells (CD56^{pos}) or NKG2C^{pos} NK cells (CD56^{pos}NKG2C^{pos}) of 10 CMV-seropositive individuals were depleted prior to expansion of CMV-specific CD8 T cells. The absolute number of CMV-specific CD8 T cells in culture was determined on day 0 and 10 and is shown as an expansion ratio (absolute cell number day 10/absolute cell number day 0). Depletion of total NK cells resulted in a more robust expansion of CMV-specific CD8 T cells with a median expansion ratio of 813-fold compared with a median of 350-fold in the presence of NK cells (Fig. 1E, $p < 0.05$). Depletion of NKG2C^{pos} NK cells alone was sufficient to increase the frequencies of CMV-specific CD8 T cells to the same extent as the depletion of total NK cells (a median expansion ratio of 820-fold, Fig. 1E, $p < 0.05$). Next, we aimed to analyze whether there is a direct correlation between the

frequency of CMV-specific T cells and NKG2C^{pos} NK cells ex vivo. Therefore, PBMCs of 38 CMV-seropositive individuals with frequencies of NKG2C^{pos} NK cells ranging from 0 to 56.8% were incubated with overlapping peptides of the CMV-derived proteins pp65 and IE-1. There was no correlation between the frequencies of NKG2C-expressing NK cells and the number of spot-forming cells for either protein (IE-1, data not shown; pp65: Fig. 1F, $p = 0.8$). Taken together, our data indicate that NKG2C^{pos} NK cells influence activated, expanding CMV-specific CD8 T cells but not resting CMV-specific T cells.

HLA-E is upregulated on activated and terminally differentiated CD8 T cells

To further characterize the interaction between NKG2C^{pos} NK cells and CD8 T cells, we next analyzed the expression of the NKG2C ligand HLA-E on resting CMV- and IAV-specific CD8 T cells. Fourteen of twenty-eight CMV-seropositive individuals showed detectable IAV-specific CD8 T cells after staining with an HLA class I multimer complexed with the HLA-A*02–restricted IAV epitope GILGFVFTL (Fig. 2A). Interestingly, CMV-specific CD8 T cells expressed significantly more HLA-E on the cell surface than IAV-specific CD8 T cells (Fig. 2A). Based on the differentiation state, CD8 T cells can be subdivided into naive, central memory (CM), effector memory (EM), and TEMRA. It has been described that the majority of CMV-specific CD8 T cells in the peripheral blood are phenotypically TEMRA CD8 T cells (CCR7$^-$, CD45RA$^+$, CD27$^-$, KLRG-1^{++}) (reviewed in Refs. 6 and 26). In line with an expansion of CD8 TEMRA cells in CMV-seropositive individuals, significantly more CD8 T cells with the TEMRA phenotype were detected in CMV-seropositive individuals as compared with CMV-seronegative individuals (Fig. 2B). To elucidate whether HLA-E expression is associated with the T cell differentiation stage, we identified naive T cells (CCR7$^+$, CD45RA$^+$, CD27^{+++}, and KLRG-1$^-$), CM (CCR7$^+$, CD45RA$^-$, CD27^{++}, and KLRG-1$^-$), EM (CCR7$^-$, CD45RA$^-$, CD27$^{+/-}$, and KLRG-1$^{+/-}$), and TEMRA (CCR7$^-$, CD45RA$^+$, CD27$^-$, and KLRG-1^{++}) based on the expression of CCR7, CD45RA, CD27, and KLRG-1. In addition, HLA-E expression on the cell surface on both CMV-seropositive and CMV-seronegative individuals was quantified and revealed that HLA-E expression increased stepwise with differentiation (Fig. 2C). It was noticeable that HLA-E expression levels within each differentiation stage did not differ between CMV-seropositive and CMV-seronegative individuals (data not shown). Because it was previously described that CMV-seropositive individuals with a homozygous NKG2C deletion have increased frequencies of TEMRA CD8 T cells (16), we next compared the frequencies of naive, CM, EM, and TEMRA CD8 T cells in CMV-seropositive individuals with low frequencies of NKG2C^{pos} NK cells, ranging from 10 to 15% NKG2C^{pos} NK cells and CMV-seropositive individuals with high frequencies of NKG2C^{pos} NK cells of over 30% NKG2C^{pos} NK cells. Interestingly, individuals with high frequencies of NKG2C^{pos} NK cells had significantly lower frequencies of TEMRA CD8 T cells than individuals with low NKG2C expansion (Fig. 2D, $p = 0.0146$). This effect was not observed for naive, CM, or EM CD8 cells or any CD4 T cell subset (data not shown).

Next, it was investigated whether HLA-E expression is altered upon CD8 T cell activation. For this purpose, PBMCs were stimulated with CD3/CD28 bearing beads, and the expression of HLA-E on CD8 T cells was analyzed after 3 d. CD8 T cells from bead-stimulated cultures showed a significantly increased expression of HLA-E (Fig. 3A, $p = 0.0014$) compared with unstimulated controls. Because HLA-E expression was upregulated on activated CD8 T cells, we hypothesized that activated HLA-E^{high}–expressing CD8 T cells might become targets for NKG2C^{pos} NK cells. To address this hypothesis, we compared the HLA-E expression levels of CMV-specific CD8 T cells upon activation with the cognate Ag in the presence and absence of K562-activated NK cells. Representative results of CMV-specific CD8 T cells are shown in Fig. 3C. After peptide stimulation, HLA-E

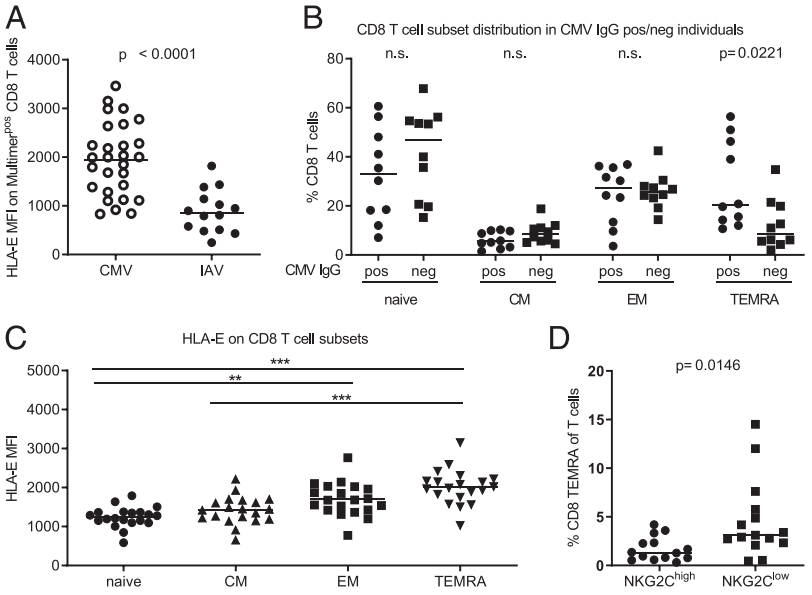

FIGURE 2. HLA-E expression on CD8 T cell subsets. (**A**) HLA-E expression on CMV- and IAV-specific CD8 T cells in CMV-seropositive individuals is shown. CMV-specific CD8 T cells expressed significantly more HLA-E than IAV-specific CD8 T cells in the same individuals (CMV-specific CD8 T cells, $n = 28$; IAV-specific CD8 T cells, $n = 14$; t test). (**B**) The frequencies of naive, CM, EM, and TEMRA CD8 T cells were analyzed in 10 CMV-seropositive and 10 CMV-seronegative individuals. CMV-seropositive individuals had significantly higher frequencies of TEMRA CD8 T cells than CMV-seronegative individuals (t test; n.s., not significant) (**C**) HLA-E expression on naive, CM, EM, and TEMRA CD8 T cells of 20 individuals was analyzed. HLA-E expression increased with the differentiation stage (Kruskal–Wallis test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). (**D**) The frequencies of TEMRA CD8 T cells of CMV-seropositive individuals with high frequencies (>30%) and low frequencies (10–17%) of NKG2C^{pos} NK cells were analyzed. Individuals with high frequencies of NKG2C^{pos} NK cells had significantly fewer TEMRA CD8 T cells than individuals with low frequencies of NKG2C^{pos} NK cells (t test).

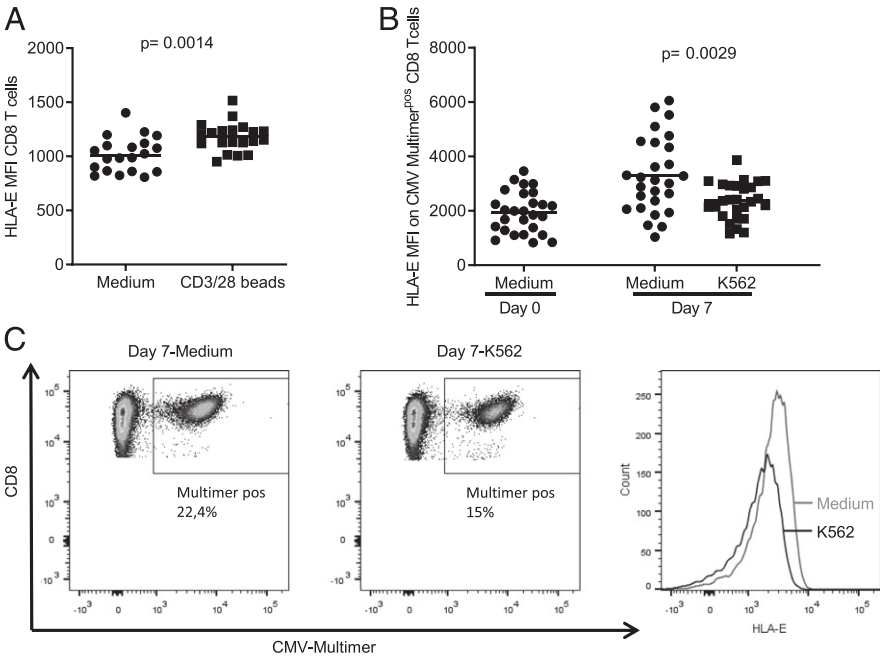

FIGURE 3. HLA-E expression on activated CD8 T cells. (**A**) PBMCs of 20 individuals were stimulated with CD3/CD28-coated beads. HLA-E expression was significantly upregulated on stimulated CD8 T cells after 3 d (*t* test). (**B**) CMV-specific CD8 T cells were expanded in the presence or absence of irradiated K562 cells, and HLA-E expression on CMV-specific CD8 T cells was analyzed on day 0, 7, and 10. A representative staining of CMV-specific CD8 T cells and the expression of HLA-E after 7 d in the presence or absence of K562 cells is shown. (**C**) On day 7, CMV-specific CD8 T cells (n = 28) expressed significantly less HLA-E in the presence of K562 cells compared with in the absence of K562 cells (*t* test).

was upregulated on CMV-specific CD8 T cells by day 7. Importantly, in the presence of K562-activated NK cells, the HLA-E expression level on CMV-specific CD8 T cells was significantly lower compared with controls in the absence of activated NK cells (Fig. 3B, $p = 0.0029$), which is consistent with selective inhibition of HLA-E^{high} CD8 T cells.

NKG2C^{pos} NK cells release high levels of Granzyme B in response to an HLA-E–expressing T cell line and lead to upregulation of caspase 3/7-activity

We hypothesized that HLA-E upregulation on activated T cells causes NKG2C^{pos} NK cells to release cytotoxic granules. Because activated CD8 T cells differ from resting T cells beyond the level of HLA-E expression, we chose a T cell line to test our hypothesis. For this purpose, we selectively stabilized HLA-E on the wild-type T cell line Jurkat by the exogenous addition of an HLA-E–stabilizing peptide (VMAPRTLFL) derived from CMV UL-40. Similar to T cells, Jurkat cells per se express HLA-E, and HLA-E stabilization with the peptide leads to a further increase of HLA-E expression comparable with that on activated T cells (Fig. 4A). PBMCs of 10 individuals with frequencies of NKG2C^{pos} NK cells ranging from 22.1 to 60% were stimulated with untreated Jurkat cells (HLA-E^{low}) or Jurkat cells with peptide-stabilized HLA-E (HLA-E^{high}). The expression of IFN-γ, TNF-α, CD107a, Perforin, and Granzyme B on NKG2C^{neg}CD56^{dim} NK cells and NKG2C^{pos}CD56^{dim} NK cells was then analyzed. There was no significant difference in the expression of IFN-γ, TNF-α, CD107a, or Perforin with the tested conditions, although there was a tendency that NKG2C^{pos} NK cells produced more IFN-γ and TNF-α (Supplemental Fig. 1C, 1D). However, resting NKG2C^{pos}CD56^{dim} NK cells showed higher levels of Granzyme B than NKG2C^{neg}CD56^{dim} NK cells (Fig. 4B, $p = 0.0010$). Stimulation with HLA-E^{high}– and HLA-E^{low}–expressing Jurkat cells led to a decrease in Granzyme B levels in NK cells. Released Granzyme B was defined as the difference in Granzyme B levels between the medium control and the stimulated cells. NKG2C^{neg}CD56^{dim} NK cells released similar levels of Granzyme B whether stimulated with HLA-E^{high}– or HLA-E^{low}–expressing Jurkat cells (Fig. 4C). In contrast, NKG2C^{pos}CD56^{dim} NK cells released higher levels of Granzyme B, and the release of Granzyme B was highest by NKG2C^{pos}CD56^{dim} NK cells stimulated with HLA-E^{high}–expressing Jurkat cells (Fig. 4C). Because NK cells are confronted with both resting and activated T cells during an infection, we aimed to analyze whether the HLA-E shift we observed on activated T cells was sufficient to selectively induce apoptosis. Activated caspase 3 and 7 were selected as markers for apoptosis because they are activated in the Granzyme B apoptosis pathway. We stimulated 10^6 PBMCs of 10 individuals with frequencies of NKG2C^{pos} NK cells ranging from 22.1 to 60% simultaneously with 100,000 HLA-E^{high} and 100,000 HLA-E^{low} Jurkat cells stained with two different cell tracers and analyzed the expression of activated caspase 3/7 after 24 h (Fig. 4D). In the presence of PBMCs, HLA-E^{high}–expressing Jurkat cells showed on average 19% more active caspase 3/7 than their HLA-E^{low} counterparts (Fig. 4E, median HLA-E^{low} = 9.5% active caspase, median HLA-E^{high} = 11.35% active caspase, $p < 0.0001$), indicating that apoptosis can be selectively induced in HLA-E^{high}–expressing activated T cells.

Discussion

NKG2C^{pos} NK cells have long been attributed an important role in immune control of CMV infection (9–13). In this study, we propose an additional role for NKG2C-expressing NK cells in shaping CD8 T cell responses. Although others have reported a direct correlation between NKG2C^{pos} CD57^{pos} NK cells and CMV-specific T cell response ex vivo (27), we have not observed a correlation between the frequency of NKG2C^{pos} NK cells and the frequency of spot-forming cells after overnight stimulation with overlapping peptides of the CMV proteins pp65 or IE-1 (Fig. 1E). Simultaneous activation of CD8 T cells with a CMV-derived peptide and activation of NK cell with K562 cells leads to decreased frequencies of CMV-specific CD8 T cells after 10 d of expansion, indicating that activated NK cells and T cells interact. However, NK cell activation by K562 cells probably represents a super physiological activation and may not be relatable to the activation status of NK cells in vivo in the context of CMV infection. Therefore, to further analyze the influence of NK cells in general and NKG2C^{pos} NK cells in particular, we depleted NK cells or NKG2C^{pos} NK cells prior to expansion of CMV-specific

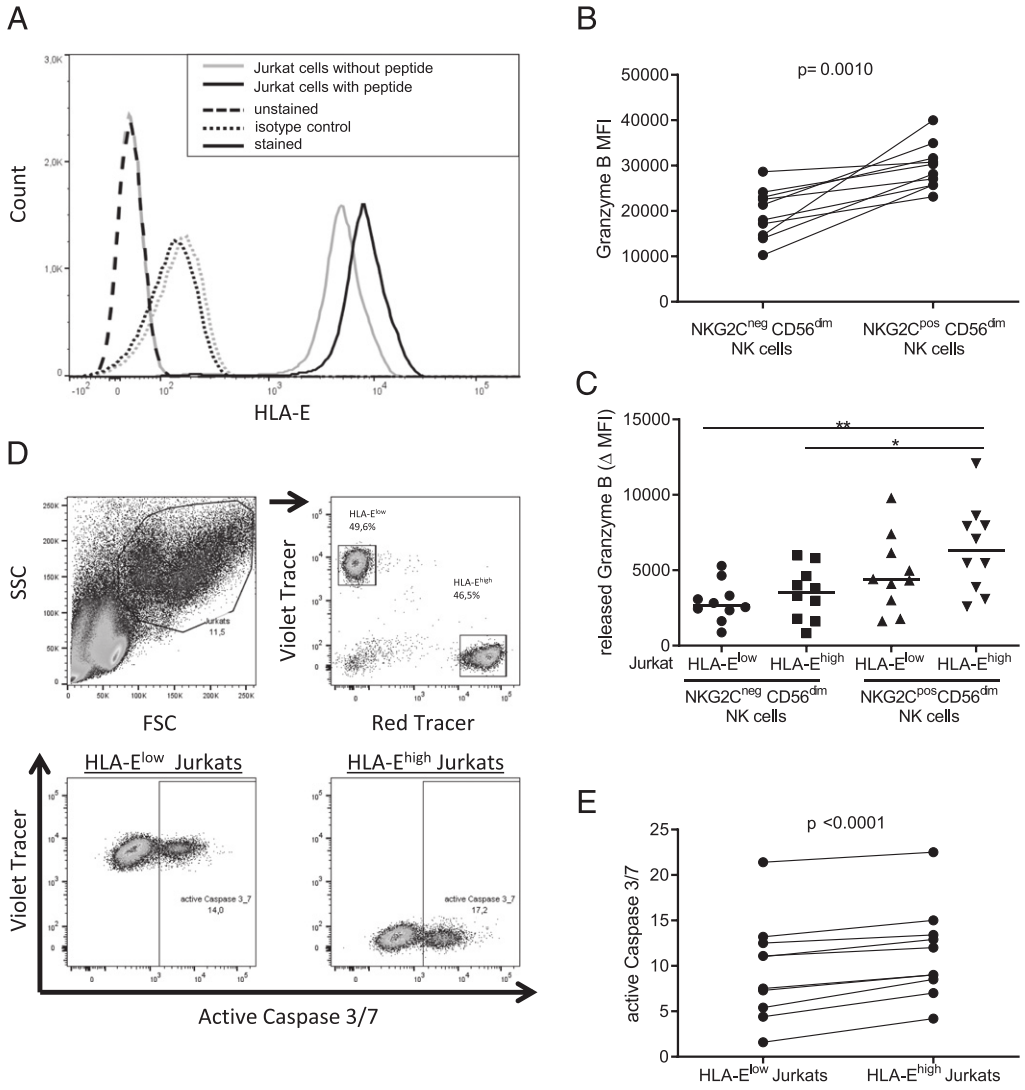

FIGURE 4. Functional analysis of NKG2C^{neg}CD56^{dim} and NKG2C^{pos}CD56^{dim} NK cells after stimulation with HLA-E^{low} and HLA-E^{high} Jurkat cells. (**A**) Jurkat cells were incubated in the presence (blue line) or absence (red line) of 100 μM peptide (VMAPRTLFL) overnight at 26˚C. The peptide-stabilized HLA-E on Jurkat cells. (**B**) Basal level of intracellular Granzyme B was analyzed in NKG2C^{neg}CD56^{dim} and NKG2C^{pos}CD56^{dim} NK cells. The Granzyme B level was significantly higher in NKG2C^{pos}CD56^{dim} NK cells compared with NKG2C^{neg}CD56^{dim} NK cells (paired *t* test). (**C**) PBMCs were incubated with Jurkat HLA-E^{low} or Jurkat HLA-E^{high} cells for 24 h at 26˚C. NKG2C^{neg}CD56^{dim} and NKG2C^{pos}CD56^{dim} NK cells were identified by flow cytometry, and released Granzyme B was defined as the difference between Granzyme B levels in the medium control and after stimulation (Kruskal–Wallis test, $*p \leq 0.05$, $**p \leq 0.01$). (**D** and **E**) 10^6 PBMCs were incubated for 24 h at 26˚C with 100,000 HLA-E^{low}– and 100,000 HLA-E^{high}–expressing Jurkat cells that were either traced with a red or violet cell tracer. Subsequently, caspase 3/7 activity was analyzed on the Jurkat cells. Caspase 3/7 displayed significantly more activity in HLA-E^{high}–expressing Jurkat cells (paired *t* test).

CD8 T cells. Depletion of NKG2C^{pos} NK cells alone was sufficient to improve the expansion of CMV-specific CD8 T cells. In accordance with this, we found an increased expression of HLA-E on CMV-specific CD8 T cells compared with IAV-specific CD8 T cells in the same individuals. Activation of CMV-specific CD8 T cells with a CMV-derived peptide led to a further increase of HLA-E expression, which peaked after 7 d of expansion and decreased by day 10. In this setting, the presence of activated NK cells led to a loss of HLA-E^{high}–expressing CD8 T cells after 7 d of expansion and to a decreased frequency of CMV-specific CD8 T cells after 10 d. In combination with the finding that individuals with high frequencies of NKG2C^{pos} NK cells exhibit decreased frequencies of TEMRA CD8 T cells, the T cell subset with the highest HLA-E expression levels, we hypothesized that NKG2C^{pos} NK cells may selectively target HLA-E^{high}–expressing CD8 T cells. To test this hypothesis, we stimulated PBMCs with Jurkat cells expressing high or low levels of HLA-E. NKG2C^{pos} NK cells stimulated with HLA-E^{high}–expressing Jurkat cells released high levels of Granzyme B. In addition, the activation of caspase 3 and 7, both part of the Granzyme B apoptosis pathway, was stimulated in HLA-E^{high}–expressing Jurkat cells when both HLA-E^{high}– and HLA-E^{low}–expressing Jurkat cells were present.

In combination, our data suggest the following hypothesis: in the absence of CMV viremia, there is no direct interaction between NKG2C^{pos} NK cells and resting CMV-specific CD8 T cells. However, this changes when CMV-specific CD8 T cells are activated. Activation leads to an expansion of CMV-specific CD8 T cells, which is accompanied by an increase of HLA-E expression. The high HLA-E expression level on terminally differentiated resting CMV-specific CD8 T cells in combination with this additional induction of HLA-E probably marks activated CMV-specific CD8 T cells as targets for NKG2C^{pos} NK cells. This suggests that NKG2C^{pos} NK cells are not only involved in the killing of CMV-infected cells but might also contribute to limiting

CD8 T cell inflation in CMV-seropositive individuals. It is well established that CMV-specific T cells have a TEMRA phenotype and that TEMRA CD8 T cells are expanded in CMV-seropositive individuals (reviewed in Refs. 6 and 26). Liu et al. (16) reported that CMV-seropositive individuals with a homozygous deletion for the NKG2C gene have significantly higher frequencies of TEMRA CD8 T cells at an age <50 y than the age-matched group carrying the NKG2C gene. In line with these findings, we report in this study that individuals with high frequencies of NKG2C^{pos} NK cells have even reduced frequencies of TEMRA CD8 T cells. These findings support the idea that NKG2C^{pos} NK cells are involved in regulating HLA-E^{high}–expressing CD8 T cells. In turn, HLA-E expression on CMV-infected cells has been reported to stimulate the expansion of NKG2C^{pos} NK cells (28, 29) in a peptide-dependent manner (30). Therefore, it can even be speculated that higher expression levels of HLA-E on terminally differentiated CMV-specific CD8 T cells represent an additional trigger that promotes the expansion of NKG2C^{pos} NK cells in seropositive individuals. Interestingly, Costa-García et al. (24) recently reported that NKG2C^{pos} adaptive NK cells can serve as APC for CD4 T cells. These APCs preferentially activated CD4 T cells with a TEMRA phenotype. Although we observe decreased frequencies of CD8 TEMRA cells in individuals with high frequencies of NKG2C^{pos} NK cells, we do not observe decreased frequencies of CD4 TEMRA cells. This is probably due to the fact that CD4 TEMRA cells express significantly lower levels of HLA-E than CD8 TEMRA cells (data not shown). Taken together, these data indicate that the interaction between NKG2C^{pos} NK cells and T cells is very complex and strongly depends on the differentiation and activation status of T cells.

In the context of NK cells, HLA-E is usually perceived as an inhibitory ligand because binding to the NKG2A receptor mediates inhibitory signals. The binding affinity of HLA-E to NKG2A and the frequency of NK cells expressing NKG2A are higher compared with NKG2C (31). In particular, NKG2C^{pos} NK cells are typically negative for NKG2A, and homeostasis of NKG2C^{pos} NK cells in HCMV infection is at least partially controlled by the coexpression of cognate inhibitory KIRs (25). In CMV-seronegative individuals, HLA-E upregulation may indeed result in protection from NK cell killing. In patients with multiple sclerosis, increased HLA-E expression on CD4 T cells was reported to block T cell inhibition by CD56^{bright} NK cells (32). In the LCMV infection mouse model, we recently showed that the absence of the mouse HLA-E homologue Qa-1b resulted in an increased NK cell–mediated negative regulation of antiviral T cells following viral infection (33). Notably, CD8 T cells were protected from NK cell–mediated killing when ligands for inhibitory NK cell receptors were upregulated in response to type I IFNs on T cells (21, 22). Xu et al. (22) reported that Qa-1b was upregulated in response to treatment with anti-CD3 and IFN4a. However, whereas HLA-E in humans can activate and inhibit NK cells by either binding to NKG2C or NKG2A, NKG2C is basically absent from murine NK cells (34). Accordingly, inhibition of HLA-E–expressing T cells by NK cells may be a hallmark of human CMV infection and may be absent in LCMV-infected mice, in which it may even result in increased T cell immunity.

Finally, our findings raise the question whether NKG2C^{pos} NK cells also influence unrelated T cell responses. In the past, CMV infection has been associated with immunosenescence and impaired immunity in the elderly (35). In the analysis of IAV-specific CD8 T cells in elderly individuals after vaccination, CD8 T cells of CMV-seropositive individuals expressed less Granzyme B (36) or IL-2R (CD25) (37) than CD8 T cells of the CMV-seronegative control group. In contrast to the situation in the elderly, in young mice (38–40) and humans (41), CMV seropositivity was even associated with improved immune responses to unrelated pathogens. However, this effect was only transient (38, 40) and was associated with increased levels of circulating IFN-γ observed in both young mice and humans (40).

Taken together, our data suggest that NKG2C^{pos} NK cells interact with HLA-E–expressing CD8 T cells in CMV-seropositive individuals and thereby negatively regulate the expansion of CMV-specific CD8 T cells upon activation.

Disclosures

The authors have no financial conflicts of interest.

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