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Elevated Numbers of FcγRIIIA⁺ (CD16⁺) Effector CD8 T Cells with NK Cell-Like Function in Chronic Hepatitis C Virus Infection


CTL are crucial in the defense against viral infections. In the course of investigating peripheral blood and intrahepatic CD8 T cells in patients with chronic hepatitis C virus (HCV) infection, we observed a significant population of CD8 T cells expressing the FcγRIIIA (CD16) receptor. This observation led us to characterize these cells with respect to their phenotype and function in a cohort of patients with chronic HCV infection as well as in healthy blood donors. On average, 10% of peripheral blood CD8 T cells from HCV-infected patients expressed CD16 compared with only a few percent in healthy donors. CD16⁺ CD8 T cells displayed a late-stage effector phenotype with high levels of perforin. These cells exhibited a restricted TCR profile suggesting underlying clonal expansion. Stimulation of CD16 on CD8 T cells evoked a vigorous response similar to that of CD16 stimulation in NK cells. Our data suggest that CD8 T cells, during chronic HCV infection in humans, continue to differentiate beyond defined stages of terminal effector cells, acquiring CD16 and NK cell-like functional properties. The Journal of Immunology, 2008, 181: 4219–4228.

More than 120 million people are estimated to be infected with hepatitis C virus (HCV) worldwide (1). Acute HCV infection can, in some cases, spontaneously be cleared by a robust multi-specific CD8 T cell response (2–4). However, chronic disease frequently develops following HCV infection with high morbidity and mortality, strongly associated with development of liver cirrhosis and hepatocellular carcinoma (5). Failure to clear the infection is at least in part associated with impaired CD8 T cell function, including defective maturation and/or cellular exhaustion, as well as viral escape (6, 7).

In a characterization of CD8 T cells from patients with chronic HCV infection, we observed a significant proportion (often up to 10%) of TCRαβ CD8 T cells expressing FcγRIIIA (CD16). CD16 is a low-affinity FcR for IgG. This receptor is commonly expressed on many NK cells as well as on several other immune cells including neutrophils and monocytes (8). CD16 is best characterized for its role in Ab-dependent cellular cytotoxicity (ADCC) (8, 9). Stimulation of CD16 on NK cells also results in production of several cytokines including IFN-γ and TNF-α (10).

The observation, per se, of CD16 expression on a small number of T cells in healthy peripheral blood donors is not new (11). Already in 1985, Lanier et al. (12) provided the first functional analysis of such cells in humans. At this time, however, no distinction was made between TCRαβ and TCRγδ expression, and the phenotypic and functional characterization was limited to the methods available at that time. Since then, CD16 expression on TCRγδ T cells has been analyzed quite extensively (11, 13–15). Few studies in the literature have, however, characterized human CD16⁺ TCRαβ CD8 T cells in healthy individuals and in chronic infection with respect to phenotype and functionality.

Our results reveal that a subset of phenotypically terminally differentiated effector CD16⁺ CD8 T cells are present in elevated numbers in patients with chronic untreated HCV infection and at low but clearly detectable numbers in healthy controls. CD16⁺ CD8 T cells were found not only in peripheral blood but also in the liver. The high numbers of CD16⁺ TCRαβ CD8 T cells observed here in individuals chronically infected with HCV prompted us to characterize this subset of cells in detail with respect to maturational stage, tissue distribution, TCR specificity, and functionality.

Materials and Methods

Patient material

Peripheral blood was obtained from 53 patients chronically infected with HCV (genotype 1, n = 16; genotype 2, n = 18; genotype 3, n = 19). All patients were treatment naive for their HCV infection and HIV-1 negative. Median age for the HCV cohort was 54 years. Buffy coats were collected from 34 healthy blood donors. Median age for the healthy blood donors was 45 years. Liver tissue and whole blood were also acquired from five HCV-infected patients and one patient with HCV-related hepatocellular carcinoma undergoing liver transplantation. This study was approved by

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Abbreviations used in this paper: HCV, hepatitis C virus; ADCC, Ab-dependent cellular cytotoxicity; IHL, intrahepatic lymphocyte; KIR, killer cell Ig-like receptor; NKR, NK cell receptor.

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the regional ethics committee (approval nos. 03-541, 2006/971-31/1, and 2006/229-31/3). Written informed consent was obtained from all patients.

**Preparation of PBMC and intrahepatic lymphocytes (IHL)**

For isolation of PBMC, whole blood from patients and buffy coats from healthy donors were separated by Lymphoprep gradient centrifugation (Axis-Shift). PBMC were analyzed immediately after isolation or frozen in 10% DMSO (Sigma-Aldrich) and 90% FCS (Life Technologies) and stored in liquid nitrogen for later analysis. Liver tissues were mechanically disrupted into 1-mm³ fragments, incubated in RPMI 1640 supplemented with 10% FCS and 0.02% RNase I (Roche) with gentle agitation for 30 min at 37°C, then filtered through a 100-μm nylon mesh, washed twice, and separated by Lymphoprep gradient centrifugation for isolation of IHL. IHL were stained and analyzed immediately after isolation.

**mAbs and tetramers**

Anti-CD3 Pacific Blue and anti-CD3 Cascade Yellow were obtained from DakoCytomation. Anti-CD56 PE-Cy7, anti-CD16 Alexa Fluor 647, anti-CD28 PE-Cy7, anti-CCR7 PE-Cy7, anti-CD28 allophycocyanin, anti-CD28 FITC, anti-CD27 FITC, anti-CD27 allophycocyanin-Cy7, anti-TCRβ FITC, anti-TCRVγ6 FITC, anti-perforin FITC, anti-CD3 PE, anti-CD3 allophycocyanin, anti-CD3 PE-Cy7, anti-granzyme B Alexa Fluor 647, anti-NKG2D PE, anti-KIR3DL1 FITC, anti-CD38 allophycocyanin, and anti-CD57 allophycocyanin were purchased from BD Biosciences. Anti-CD56 PE-Cy5, anti-CD58 PE-Cy5, anti-KIR2DL2/3/5 PE, anti-NKG2A PE, anti-2B4 PE, and anti-CD57 FITC were obtained from Beckman Coulter. Anti-KIR2DL1 allophycocyanin and anti-NKG2C PE were purchased from R&D Systems. Anti-CD7 PE was obtained from Coulter ImmunoTech. Anti-CD45RA Pacific Blue was from Invitrogen. Anti-CD27 allophycocyanin-Cy7 was from Biolegend. IgG1, IgG2b, and IgM isotypes were from BD Biosciences. Intracellular cytokines were visualized with anti-IFN-γ PE (BD Biosciences) and anti-TNF-α Alexa Fluor 647 from eBioscience. The TCR Vβ FITC/PE was from Beckman Coulter. Polystyrene beads (BD Biosciences) were used for compensation purposes.

Subjects were evaluated for HLA-A2 expression by staining with anti-HLA-A2 (BD Biosciences). CD8 T cells specific for viruses were identified and enumerated using allophycocyanin-conjugated HLA-A2 tetrameric complexes refolded with the CMV pp65 epitope NLVPMVATV, HCV NS3 1073 epitope CIMVGCWTV, and the HCV NS3 1406 epitope KLVALGINAV (Coulter Immunotech).

**Flow cytometry**

For cell surface staining, purified PBMC or IHL were incubated in PBS with 5% FCS on 20 min on ice in the dark with saturating amounts of the desired mAb combination, washed twice, and fixed in PBS containing 1% formaldehyde. Isotype control mAbs were used at corresponding concentrations. When intracellular staining was conducted, cells were permeabilized with Cytofix/Cytoperm (BD Biosciences), washed in permeabilization buffer, and then stained with intracellular Abs for 20 min on ice and finally washed twice. Data were acquired on a CyAn ADP nine-color flow cytometer (Beckman Coulter) and analyzed with FlowJo software version 8 (Tree Star) as previously described (16).

**Absolute T cell counts**

MultiTEST CD3 FITC, CD8 PE, CD45 PerCP, and CD4 allophycocyanin reagent (BD Biosciences) and heparinized whole blood were mixed in a TrueCount tube (BD Biosciences) and incubated for 20 min at room temperature in the dark. Next, FACS lysing solution (BD Biosciences) was added and the samples were incubated for another 30 min at room temperature in the dark. The samples were immediately acquired on a FACSCalibur four-color flow cytometer (BD Biosciences) and analyzed with CellQuest software.

**Functional CD16 assay**

PBMC were maintained in complete medium (RPMI 1640 supplemented with 100 μg/ml l-glutamine, 10% heat-inactivated FCS, 100 μM penicillin, 100 μg/ml streptomycin). For Ab coating, P815 cells (American Type Culture Collection) propagated in complete medium were incubated at 1 × 10⁶ cells/ml for 30 min at room temperature with 10 μg/ml of anti-CD16 mAb or the corresponding isotype control and then washed twice. PBMC were then mixed with P815 cells at a ratio of 10:1 in round-bottom 96-well plates in a final volume of 200 μl. Anti-CD16/70a FITC and a corresponding IgG1 isotype control were added at the beginning of the assay and then cells were incubated at 37°C and 5% CO₂ for 6 h. Monensin (GolgiSTOP; BD Biosciences), 1/50 dilution, and brefeldin A (Golgi-PLUG; BD Biosciences), 1/250 dilution, were included after 1 h of cultivation. Following incubation, the cells were washed and surface Ag staining was performed. Following this step, cells were washed twice, fixed, and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences). Finally, the cells were washed twice in the permeabilization buffer and stained for intracellular cytokines for 20 min on ice and then washed twice. Data were immediately acquired after completion of the staining procedure.

**Long-term culture assay**

PBMC were isolated as previously described, washed twice, and enriched for CD8 T cells by negative selection using a CD8 T cell isolation kit 2 from Miltenyi Biotec. Next, the enriched CD8 T cells were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, anti-CD27, and anti-CD16 and then sorted using a FACSaria instrument (BD Biosciences). In brief, 1 × 10⁶ of each purified CD8 T cell subset, naïve T cells (CD16⁻ CD8⁺ CD27⁺ CD45RA⁺), central memory T cells (CD16⁺ CD27⁺ CD45RA⁺), and effector memory T cells (CD16⁺ CD27⁻ CD45RA⁺) were cultured in RPMI 1640 medium supplemented with 10% FCS and 250 IU/ml rIL-2 (Chiron) for 3 or 7 days in 96-well round-bottom plates at 37°C and 5% CO₂. At the end of the culture, the cells were stained for CD3, CD8, CD16, and the nucleic acid stain 7-aminoactinomycin D, counted, and analyzed for CD16 expression on the CyAn ADP instrument.

**Results**

Elevated numbers of CD16⁺ CD8 T cells in patients with chronic HCV infection

In the course of analysis of CD8 T cells in patients with chronic HCV infection, we observed that a large proportion of the CD8 T cell pool expressed CD16 (Fig. 1A). This finding prompted us to study the relative frequencies and absolute numbers of CD16⁺ CD8 T cells in a larger cohort encompassing 53 patients chronically infected with HCV and 34 healthy controls. These studies revealed a marked increase in frequency of CD16⁺ CD8 T cells in HCV-infected individuals compared with healthy controls (mean, 10.1 ± 9.5% CD16⁺ CD8 T cells in HCV-infected individuals; mean, 3.8 ± 4.6% CD16⁺ CD8 T cells in healthy controls; p < 0.0001) (Fig. 1B).

Since the percentage out of total CD8 T cells is a relative measurement, we also determined absolute numbers of CD16⁺ CD8 T cells in peripheral blood in patients with chronic HCV infection and healthy controls (Fig. 1C). A 5-fold increase in absolute numbers of CD16⁺ CD8 T cells were observed in patients with chronic HCV infection compared with healthy controls (mean, 29.2 ± 38.8 cells/μl CD16⁺ CD8 T cells in HCV-infected individuals; mean, 5.8 ± 3.8 cells/μl CD16⁺ CD8 T cells in healthy controls; p < 0.01). When comparing expression levels of CD16 in HCV-infected patients and healthy donors, there was a trend toward higher levels of CD16 present on CD8 T cells identified exclusively expression on the CyAn ADP instrument.

Taken together, these results demonstrate the presence of a major CD16⁺ CD8 T cell population in HCV-infected individuals, representing ~10% of the total CD8 T cell pool. This observation led us to characterize CD16⁺ CD8 T cells in more detail with regard to phenotype and function.
assess the maturational status of the CD16<sup>+</sup> CD8 T cell subset, we stained this population for CCR7, CD45RA, CD27, CD28, and CD7. CD16<sup>+</sup> CD8 T cells displayed a uniform differentiation profile when compared with CD16<sup>-</sup> CD8 T cells (Fig. 2B). Eighty-four percent of the CD16<sup>+</sup> CD8 T cells were CCR7<sup>-</sup>CD45RA<sup>-</sup> compared with 27% among the CD16<sup>-</sup> CD8 T cells (p < 0.001). Only 24% of the CD16<sup>+</sup> CD8 T cells expressed the costimulatory receptors CD27 and CD28, whereas 85% of the CD16<sup>-</sup> CD8 T cells expressed CD27 or CD28 (p < 0.001). Furthermore, CD16<sup>+</sup> CD8 T cells expressed low levels of CD7 on their surface (Fig. 2, B and C).

Effector T cells characteristically express intracellular effector molecules, including perforin and granzymes. A majority of the CD16<sup>+</sup> CD8 T cells expressed perforin and virtually all CD16<sup>-</sup> CD8 T cells contained granzyme B, as detected by intracellular staining (Fig. 2, D and E). Significantly higher frequencies of perforin and granzyme B<sup>+</sup> cells were found among the CD16<sup>+</sup> CD8 T cells compared with CD16<sup>-</sup> CD8 T cells (perforin expression: 68.6 ± 19.6% vs 23.7 ± 16.9%, p < 0.0001; granzyme B expression: 92.7 ± 5.5% vs 33.2 ± 14.9%, p < 0.005). CD16<sup>+</sup> CD8 T cells from HCV-infected patients displayed a profile similar to that of CD16<sup>+</sup> CD8 T cells obtained from healthy blood donors (data not shown).

To further elucidate the phenotypic profile of CD16<sup>+</sup> CD8 T cells, different subsets of CD16<sup>-</sup> CD8 T cells were sorted on the basis of differentiation status and put in cultures supplemented with a low dose of IL-2 to support cellular survival. Terminally differentiated (CD45RA<sup>-</sup>CD27<sup>-</sup>) CD16<sup>-</sup> CD8 T cells showed evidence of CD16 surface expression after 3 days in culture and levels of CD16 expression were further elevated at day 7 (Fig. 2F). In contrast, neither naive (CD45RA<sup>+</sup>CD27<sup>+</sup>) nor central memory (CD45RA<sup>-</sup>CD27<sup>+</sup>) CD16<sup>-</sup> CD8 T cells up-regulated CD16 upon culture.

We also assessed whether the increased frequency of CD16<sup>+</sup> CD8 T cells found in patients with HCV infection correlated with markers of T cell activation. CD8 T cells from healthy and HCV-infected patients were investigated for CD38 expression. CD8 T cells from HCV-infected patients expressed significantly higher frequencies of CD38 compared with healthy (mean, 5.74 ± 4.7% vs 2.5 ± 1.63%, p < 0.05). CD38 expression correlated positively

**FIGURE 1.** CD16<sup>+</sup> CD8 T cells are expanded in patients with chronic HCV infection. A, Flow cytometric identification of CD16<sup>+</sup> CD8 T cells excluding doublet events (FSC Lin vs FSC Area) and CD14<sup>+</sup> cells in a HCV-infected patient. B, Frequency (percent) of CD16<sup>+</sup> CD8 T cells among total CD8 T cells in healthy and HCV-infected individuals. C, Absolute numbers (cells/µL) of CD16<sup>+</sup> CD8 T cells in healthy and HCV-infected patients. D, Expression of CD16 on CD8 T cells from one representative HCV-infected patient and one representative healthy control. E, Expression of CD16 and CD3 on CD56<sup>-</sup>CD3-NK cells (blue contour plot) and CD8 T cells (red contour plot) from one HCV-infected patient. An unpaired t test was used for statistical analysis in B and the Mann-Whitney U test in C; ***, p < 0.01 and ***, p < 0.0001. FSC, Forward scatter.
FIGURE 2. CD16⁺ CD8 T cells are late-stage effector T cells. A, Expression of TCR on CD16⁻ and CD16⁺ CD8 T cells of one representative donor. B, Expression of CD45RA, CCR7, CD27, CD28, and CD7 on CD16⁻ (black density plot background) and CD16⁺ (red contour plot overlay) CD8 T cells. C, Compilation of expression patterns of CD45RA and CCR7, CD27 and CD28, and CD27 and CD7 on CD16⁻ and CD16⁺ CD8 T cells from six donors (two-way ANOVA with Bonferroni post tests for statistical evaluation, *p < 0.05 and ***p < 0.001). D, Expression of perforin and granzyme B on CD16⁻ (black density plot background) and CD16⁺ (red contour plot overlay) CD8 T cells. E, Compilation of expression patterns of perforin and granzyme B. Paired t test was used when n ≥ 15 and Wilcoxon matched pairs test when n < 15; **p < 0.01 and ***p < 0.001. F, Expression of CD16 on sorted naive (CD45RA⁺ CD27⁻), central memory (CD45RA⁺ CD27⁺), and terminally differentiated (CD45RA⁻ CD27⁻) CD16-negative CD8 T cells after 3 or 7 days in culture. One representative experiment of three independent experiments is shown. G, Compilation of expression of CD38 compared with CD16 on CD8 T cells from 18 healthy donors. H, Compilation of expression of CD38 compared with CD16 on CD8 T cells from 10 HCV-infected patients. Linear regression was used for statistical evaluation (G and H).
with CD16 expression in both healthy and HCV-infected individuals (Fig. 2, G and H).

In summary, CD16+ CD8 T cells exhibited a homogeneous differentiation profile with the majority of the cells being CCR7-CD45RA+CD27-CD28-CD7low/neg. Furthermore, CD16+ CD8 T cells contained granules with effector molecules. Collectively, these data strongly suggest that CD16+ CD8 T cells found in peripheral blood are terminally differentiated effector CD8 T cells.

**Distinct phenotype of intrahepatic CD16+ CD8 T cells**

HCV exhibits strong tropism for hepatocytes. Therefore, we investigated whether CD16+ CD8 T cells also resided in the liver of patients with chronic HCV infection. For this purpose, liver samples and peripheral blood were obtained from HCV-infected patients undergoing liver transplantation due to HCV-related liver disease. Similar frequencies of CD16+ CD8 T cells were found in the liver and peripheral blood in these patients (6.8 ± 3.3% vs 7.5 ± 5.4%; Fig. 3A). In contrast to peripheral blood, the major phenotype of intrahepatic CD16+ CD8 T cells was CCR7+CD27-CD28-perforin+ while the CD16+ CD8 T cells from peripheral blood in the same patients exhibited a terminally differentiated phenotype (CCR7-CD27-CD28-perforin+; Fig. 3B). These data suggest that a similar high number of CD16+ CD8 T cells found in peripheral blood of HCV-infected individuals is found also at the site of active HCV replication. However, intrahepatic CD16+ CD8 T cells exhibit a unique phenotypical profile expressing both costimulatory and effector molecules as well as a receptor associated with entry into secondary lymphoid tissues.

**TCR Vβ repertoire analysis of CD16+ CD8 T cells discloses a restricted repertoire**

A key step in the eradication of viral infections is clonal expansion of Ag-specific CD8 T cells. To compare the relative levels of clonality among CD16+ and CD16- CD8 T cell subsets, we assessed the TCR Vβ distribution in these populations. The TCR composition of different CD8 T cell subsets was determined using an array of 24 Abs specific for different TCR Vβ. The repertoire of CD16+ CD8 T cells was, in the majority of donors studied, dominated by a single (or a few) TCR Vβ (Fig. 4A). This was in contrast to the broad TCR Vβ repertoires of naive
(CD16⁺ CD45RA⁺ CCR7⁺) and effector memory/terminally differentiated (CD16⁻ CD45RA⁻ CCR7⁻) CD8 T cells. The dominant TCR Vβ in the CD16⁺ CD8 T cell subset differed between donors (dominant Vβ13.1, in 3 of 11 donors; dominant Vβ3, in 3 of 11 donors; dominant Vβ2, -5.1, -7.2, -13.2, and -17, in 1 of 11 donors). The frequency of the dominant Vβ ranged from 8 to 59%, with a mean of 32 ± 19% in the CD16⁺ CD8 T cells compared with 9.3 ± 4.9% for CD16⁻ effector memory/terminally differentiated and 5.0 ± 1.2% for CD16⁻ naive CD8 T cells (CD16⁺ CD8 T cells vs CD16⁻ naive CD8 T cells, p = 0.001; CD16⁺ CD8 T cells vs CD16⁻ effector CD8 T cells, p = 0.002; Fig. 4B).

HCV-specific CD8 T cells are notoriously hard to identify in peripheral blood of infected individuals using specific tetramers (18). In the present study, we failed to detect HCV-specific CD8 T cells using HCV-specific HLA-A2 tetramers in HLA-A2 patients.

Taken together, the data presented suggest that CD16⁺ CD8 T cells have a restricted TCR repertoire compared with CD16⁻ effector memory and terminally differentiated CD8 T cells. This indicates that a substantial degree of clonal expansion have taken place within the CD16⁺ CD8 T cell subset.

**CD16 stimulation triggers a multifunctional response in CD16⁺ CD8 T cells**

Following the assessment of frequencies and phenotypic characteristics of CD16⁺ CD8 T cells, we set out to investigate the response evoked upon specific triggering of CD16 on such cells. For this purpose, CD16⁺ CD8 T cells from healthy donors were used. P815 cells coated with anti-CD16 mAb were used and a multifunctional readout system simultaneously monitoring degranulation (CD107a up-regulation), IFN-γ, and TNF-α expression was designed (Fig. 5A, top row). The responses were compared with
responses by donor-matched NK cells stimulated in a similar fashion. CD16$^+$ CD8 T cells readily responded with degranulation (CD107a$^+$ cells, 34.9 ± 19.4%) and cytokine production (IFN-γ$^+$ cells, 21.0 ± 14.5%; TNF-α$^+$ cells, 19.7 ± 13.4%) after 6 h of stimulation. No statistically significant differences with respect to frequencies of responding cells were observed when compared with NK cells (Fig. 5B). There was, however, a tendency toward less degranulation in CD16$^+$ CD8 T cells as compared with NK cells.

Robust multifunctional T cell responses have recently been associated with slower disease progression in chronic infections in humans and with protection in vaccination models (19, 20). We therefore asked whether CD16$^+$ CD8 T cells have the capacity to respond to two or more functions simultaneously. Indeed, ~33% of the CD16$^+$ CD8 T cells responded with concurrent CD107a up-regulation, IFN-γ, and TNF-α production or any two combinations of these three functions (Fig. 5C). No significant differences in multifunctionality existed between CD16$^+$ CD8 T cells and NK cells upon CD16 triggering. Production of IFN-γ and TNF-α without simultaneous degranulation was more common among the CD16$^+$ CD8 T cells than in NK cells (Fig. 5D).

These results demonstrate that stimulation through CD16, in the absence of TCR triggering, is sufficient for induction of cytokine production and degranulation with similar levels of multifunctionality as compared with NK cells. This also illustrates that the levels of CD16 expressed on CD8 T cells are sufficient for eliciting biological responses.

**NK receptor expression profile of CD16$^+$ CD8 T cells**

Since CD16$^+$ CD8 T cells responded to CD16 triggering independently of the TCR with a profile similar to that of NK cells, we finally set out to determine the expression profile of major activating and inhibitory NK cell receptors on the CD16$^+$ CD8 T cells in HCV-infected patients. All CD16$^+$ CD8 T cells showed evidence of 2B4 and NKG2D surface expression (Fig. 6A). Interestingly, 2B4 was more highly expressed on CD16$^+$ CD8 T cells compared with CD16$^-$ CD8 T cells (CD16$^-$: mean, 40.2 ± 8.3; CD16$^+$: 24.7 ± 7.6; n = 18, p < 0.001). On the contrary, CD16$^+$ CD8 T cells expressed lower levels of NKG2D compared with CD16$^-$ CD8 T cells (CD16$^-$: mean, 78.1 ± 14.8; CD16$^+$: 87.7 ± 10.7; n = 18, p < 0.01). Subsets of CD16$^+$ CD8 T cells were positive for inhibitory killer cell Ig-like receptors (KIRs) (Fig. 6B). A Boolean platform analysis was performed simultaneously assessing KIR2DL1, KIR2DL2/3, and KIR3DL1 expression on CD16$^+$ CD8 T cells in 25 HCV-infected patients. With the exception of KIR2DL2/3$^+$ KIR2DL1$^-$ KIR3DL1$^-$ cells (mean, 27.8 ± 25.6%), none of the other six subsets represented >5% of the CD16$^+$ CD8 T cells. On average, 60% of the CD16$^+$ CD8 T cells were negative for KIRs.

In line with a terminally differentiated phenotype, roughly 80% of the CD16$^+$ CD8 T cells expressed the senescent marker CD57 (Fig. 6C) (21). CD57-positive cells were significantly more frequent within CD16$^+$ CD8 T cells compared with CD16$^-$ CD8 T cells (CD16$^+$: mean, 76.8 ± 13.5%; CD16$^-$: mean, 24.9 ± 13.2%; n = 25, p < 0.001). A characteristic of activated T cells is surface up-regulation of CD56 along with the acquisition of NK cell-like properties (22). We therefore evaluated CD56 expression on the CD16$^+$ CD8 T cells from HCV-infected patients and healthy controls (Fig. 6C). Roughly 40% of the CD16$^+$ CD8 T cells from HCV-infected patients expressed CD56. CD56 expression was more frequent on CD16$^+$ CD8 T cells from HCV-infected patients compared with healthy individuals (HCV: n = 35, mean 41.0 ± 20.3%; healthy n = 27, mean 30.8 ± 19.8%; p < 0.05). Furthermore, CD56 expression was much lower on CD16$^-$ CD8 T cells in both cohorts (HCV: n = 35, mean 12.6 ± 8.4%, healthy: n = 27, mean 7.4 ± 7.5%). Finally, minor populations within the CD16$^+$ CD8 T cell subset in HCV-infected patients expressed inhibitory CD94/NKG2A (mean, 9.6 ± 10.9%) and activating CD94/NKG2C (mean, 11.8 ± 12.7%) receptors (Fig. 6D).

These data suggest that CD16$^+$ CD8 T cells in HCV-infected individuals express a wide array of different NK cell-associated activating and inhibitory receptors. The expression of these receptors is in most, but not all cases, more prominent on CD16$^+$ CD8 T cells compared with CD16$^-$ CD8 T cells. Future studies will
have to address the importance of the clustering of NK cell-associated receptors on CD16+ CD8 T cells in patients with HCV infection.

**Discussion**

In the course of studying CD8 T cells in patients with chronic HCV infection, we discovered a sizable fraction of FcγRIIIA (CD16)-expressing CD8 T cells. This unique population of CD8 T cells was significantly expanded both in relative frequencies (percentages) and absolute numbers in peripheral blood of HCV-infected patients as compared with healthy subjects. CD16+ CD8 T cells exhibited a terminally differentiated phenotype and had a highly restricted TCR Vβ repertoire. Moreover, CD16 functioned independently of the TCR and yielded a prominent multifunctional response consisting of both effector cytokine production and degranulation. Taken together, the findings indicate that an effector CD8 T cell population with NK cell-like properties can persist and expand in chronic HCV infection, despite that this infection is known to be associated with T cell exhaustion (23).

The function of CD16 has been best studied on NK cells. These cells are regulated by the integrated input from a wide array of activating and inhibitory NK cell receptors (NKR) (24, 25). The combined input from at least two activating receptors is necessary for granular polarization and release with subsequent cytotoxicity in resting NK cells. CD16 is the only activating receptor on resting NK cells that can autonomously trigger these events (25). In human NK cells, CD16 associates with FcγR or CD3ζ homodimers for signal transduction (26, 27). CD3ζ is the major subunit utilized by the TCR complex for signal transduction. FcγR-containing TCR complexes have been reported in rare subsets of T cells such as NKT cells, activated γδ T cells, and double-negative T cells, but not in CD8+ TCRαβ+ T cells (28). Therefore, TCR-independent CD16 signaling in CD16+ CD8 T cells is likely to occur through CD3ζ.

Many NKR are not restricted in their expression to NK cells but can also be found on CD8 T cells (22, 29–31). In humans, this includes KIR CD94/NKG2A, 2B4, and NKG2D and, more recently, in the context of HCV infection CD161 (32). Some NKR are uniformly induced on CD8 T cells responding to Ag, whereas others are restricted to subsets of differentiated CD8 T cells. NKR on CD8 T cells are in most situations dependent on TCR signaling for their function and works as sentinels modulating T cell responses (22, 29, 32–34). In contrast, CD16 seems to be functional on its own in CD8 T cells evoking prominent cytokine production and high levels of degranulation. This indicates that CD16 on T cells works in a similar fashion as CD16 expressed by NK cells, i.e., without the necessity for combined input from multiple activating receptors. Furthermore, it is interesting to note that the magnitude of responses evoked by CD16+ CD8 T cells was very similar to those of NK cells.

It has been suggested that the stepwise transformation of CTL into more “innate-like” cells with NK cell properties is one underlying mechanism for immunopathology in chronic inflammatory diseases (35). Some evidence for this comes from the reprogramming occurring within intraepithelial CTL in patients suffering from celiac disease. Multiple activating NKR, including NKG2C, NKG2D, NKP44, and NKP46, appear to be strongly upregulated on oligoclonally expanded IFN-γ-producing effector T cells (35, 36). Dysregulated IL-15 expression was suggested to govern this transformation into NK-like lymphokine-activated killer cells (36). A hallmark of chronic progressing HCV infection is hepatic inflammation yielding fibrosis development and eventually liver cirrhosis. Similar to reprogrammed CD8 T cells in celiac disease, CD16+ CD8 T cells expanded in chronic HCV infection show evidence for clonal expansion as well as TCR-independent effector functions. In line with the up-regulation of multiple activating NKR on intraepithelial CTL in patients with celiac disease, we were able to detect expression and accumulation of activating NKR on CD16+ CD8 T cells from HCV-infected patients. Interestingly, we could also find expression of some inhibitory NKR but at low frequencies. When intrahepatic IL-15 levels were investigated, we could not detect a difference between HCV-infected and healthy individuals (N.K.B., unpublished observations). It is thus less likely that dysregulated IL-15 expression is responsible for the expansion of the CD16+ CD8 T cell subset.

CD8 T lymphocytes have a central role in the resolution of viral infections. Naive CD8 T cells encounter Ag in secondary lymphoid organs and activation by Ag induces proliferation and differentiation into diverse effector and memory stages. Complex expression patterns of cell surface maturation markers have been used to describe and identify different maturational stages within the CD8 T cell pool (17, 37). Naive and central memory CD8 T cells expresses CCR7 and CD62L, both necessary for homing to lymph nodes, whereas effector memory and terminally differentiated effector CD8 T cells lack expression of these markers (37). A second model for CD8 T cell differentiation employs the presence and subsequent stepwise down-regulation of the costimulatory molecules CD27, CD28, and CD7 along with CD45RA to define naive (CD27+CD45RA+CD7low/CD45RA+), memory (CD27+CD45RA+CD7high/low), and effector (CD27−CD45RA−CD28low/negative) CD8 T cells (38, 39). In the present study, we used five surface markers to define in detail the maturational status of CD16+ CD8 T cells. Interestingly, CD16+ CD8 T cells exhibited a distinct and homogeneous profile. The combination of the two models presented above strongly suggests that the CD16+ CD8 T cells are terminally differentiated. This observation is further strengthened by the significant intracellular accumulation of cytolytic effector molecules that was found in the CD16+ CD8 T cell subset.

HCV exhibits strong tropism for hepatocytes. Consequently, HCV-related morbidity is usually manifested as a progressive liver inflammation with fibrosis development finally leading to end-stage liver disease. In an attempt to address the clinical significance of elevated numbers of CD16+ CD8 T cells in HCV infection, HCV viral load and serum alanine aminotransferase levels were compared and correlated to frequencies of CD16+ CD8 T cells in the HCV-infected patients. Neither HCV viral load nor serum alanine aminotransferase correlated with higher frequencies of CD16+ CD8 T cells found in HCV-infected individuals (data not shown). However, we were able to detect similar levels of CD16+ CD8 T cells in the liver as compared with peripheral blood. It has previously been shown that intrahepatic and peripheral blood HCV-specific CD8 T cells differ in phenotype, with a predominance of effector memory (CCR7+CD45RA−) CD8 T cells in the liver and a more diverse profile found in peripheral blood (18). Furthermore, defective maturation of HCV-specific CD8 T cells may be associated with imperfect down-regulation of CD27 and CD28 (40). Interestingly, the ambiguous profile of intrahepatic CD16+ CD8 T cells with both immature markers (CD27 and CD28) and effector molecules is more similar to that of HCV-specific CD8 T cells. We have not had the possibility to functionally characterize intrahepatic CD16+ CD8 T cells due to scarcity of primary human liver material. Possibly, costimulation received through CD27 or CD28 would further elevate the already strong functional response evoked upon specific CD16 triggering. Because CD16+ CD8 T cells found in peripheral blood to a large extent lack costimulatory molecules, we have not been able to address this hypothesis using peripheral blood-derived cells.
The phenomenon of T cell exhaustion has been extensively studied in two major human chronic viral infections, HIV-1 and HCV. One characteristic affecting CD8 T cells is the hierarchical loss of T cell functions. The ability to produce IL-2 and convey cytotoxicity are lost early in the process followed by deficient TNF-α expression, whereas IFN-γ production is the function remaining intact for the longest time (23). On the other hand, patients who control (HIV-1) or clear (HCV) their chronic infection exhibit highly multifunctional responses much similar to those of T cells that respond and clear acute infections or suppress pathogens into latency (19). In this context, we evaluated the functional T cells that respond and clear acute infections or suppress pathogen maintaining intact for the longest time (23). On the other hand, patients with chronic viral infections might lead to the differentiation of terminal effector CD8 T cells beyond defined stages, acquiring NK cell-like properties.

Disclosures

The authors have no financial conflict of interest.

References


