Comparison of Primary Human Cytotoxic T Cell and Natural Killer Cell Responses
Reveal Similar Molecular Requirements for Lytic Granule Exocytosis but Differences in
Cytokine Production

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Abstract

Cytotoxic lymphocytes, encompassing cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, kill pathogen-infected, neoplastic, or certain hematopoietic cells through release of perforin-containing lytic granules. Here, we first performed probability state modeling of differentiation and lytic granule markers on CD8⁺ T cells to enable the comparison of bona fide CTL with NK cells. Analysis identified CD57bright-expression as a reliable phenotype of granule marker-containing CTL. We then compared CD3⁺CD8⁺CD57bright CTL with NK cells. Healthy adult peripheral blood CD3⁺CD8⁺CD57bright CTL expressed more granzyme B but less perforin than CD3⁺CD56dim NK cells. Upon stimulation, such CTL degranulated more readily than other T cell subsets, but had a similar propensity to degranulate as NK cells. Remarkably, the CTL produced cytokines more rapidly and with greater frequency than NK cells. In patients with biallelic mutations in UNCI3D, STX11, or STXBP2 associated with familial hemophagocytic lymphohistocytosis (FHL), CTL and NK cell degranulation was similarly impaired. Thus, cytotoxic lymphocyte subsets have similar requirements for Munc13-4, syntaxin-11, and Munc18-2 in lytic granule exocytosis. The present results provide a detailed comparison of human CD3⁺CD8⁺CD57bright CTL and NK cells, and suggest that analysis of CD57bright CTL-function may prove useful in the diagnosis of primary immunodeficiencies including FHL.
INTRODUCTION

Subsets of T cells and natural killer (NK) cells have the capacity to kill pathogen-infected, neoplastic, as well as certain hematopoietic cells through targeted release of perforin and granzymes from lytic granules. Upon release, perforin forms pores in the target cell membrane, facilitating entry of apoptosis-inducing granzymes.\(^1,2\) Most perforin-expressing, cytotoxic T lymphocytes (CTL) recognize target cells using somatically rearranged, clonally distributed T cell receptors (TCR) that bind specific MHC class I/peptide complexes on target cells.\(^3\) Accordingly, such CTL express the CD8 co-receptor for MHC class I. Complementing T cell-mediated immunity, NK cells recognize target cells using numerous germline-encoded activation receptors, with such recognition being potentiated by the loss of MHC class I expression on target cells.\(^4,5\) Besides cytotoxicity, another important function of cytotoxic lymphocytes is the production of chemokines and cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\), which promote immunity against intracellular pathogens. The signaling cascades controlling the variety of effector functions are perceived to be similar between CTL and NK cells.\(^6\) However, in the mouse, \textit{in vitro} and \textit{in vivo} imaging studies have noted differences in how CTL and NK cells recognize and eliminate target cells.\(^7,8\) Whereas CTL form stable contacts with target cells, NK cells are more tentative and form transient contacts inducing less profound Ca\(^{2+}\)-mobilization and cytoskeletal polarization. These observations may reflect differences in signaling and regulation. Thus, meticulous comparisons of human CTL and NK cells may provide insights into shared \textit{versus} distinct mechanisms regulating their cytolytic and cytokine-mediated effector functions.

Defects in lymphocyte cytotoxic function are associated with often-fatal diseases. Individuals with biallelic nonsense mutations in the gene encoding perforin, \textit{PRF1}, typically develop hemophagocytic lymphohistiocytosis (HLH) in infancy.\(^9\) HLH is a hyperinflammatory disorder characterized by unremitting fever, splenomegaly,
hyperferritinemia, cytopenia, and sometimes hemophagocytosis. \textsuperscript{10-12} HLH caused by \textit{PRF1} mutations is termed familial HLH type 2 (FHL2). In addition, milder missense mutations in \textit{PRF1} may not cause HLH, but are associated with hematological malignancies later in life. \textsuperscript{13,14} Besides \textit{PRF1}, biallelic mutations in \textit{UNC13D}, \textit{STX11}, and \textit{STXBP2} are associated with FHL3, 4, and 5, respectively. \textsuperscript{15-18} These genes encode the Munc13-4, syntaxin-11, and Munc18-2 proteins, respectively, which are required for critical steps in lytic granule exocytosis. \textsuperscript{15,17-19} Accordingly, defective NK cell cytotoxicity is a central criterion among the eight diagnostic criteria for HLH (of which at least five must be fulfilled). \textsuperscript{20} A rapid diagnosis is imperative for effective treatment of FHL. \textsuperscript{20} In a clinical setting, assays quantifying NK cell function have been useful for the diagnosis and discrimination of different FHL subtypes. \textsuperscript{19,21,22} In certain settings, such as patients with low frequencies of peripheral blood NK cells, assays that could quantify primary human T cell degranulation would be useful.

To enable use of T cell-based functional analyses for clinical diagnostic purposes, a more detailed knowledge of CTL biology is required. Furthermore, through comparisons of primary human CTL and NK cells, insights into molecular similarities and differences in cytotoxic lymphocyte subsets can also be gained. For such comparisons to be made, markers for the identification of \textit{bona fide} CTL, here defined as differentiated, perforin-containing CD8\textsuperscript{+} T cells, are required. Highly cytotoxic, perforin-containing NK cells are typically defined as CD3\textsuperscript{-}CD56\textsuperscript{dim} cells and constitute approximately 10\% of peripheral blood mononuclear cells (PBMC) in adults. \textsuperscript{23} Many different subsets of CD8\textsuperscript{+} T cells circulate in peripheral blood, representing distinct differentiation states. \textsuperscript{24,25} Several markers have been used to distinguish these subsets. Among CD8\textsuperscript{+} T cells, naïve cells are often defined as CD8\textsuperscript{+}CD27\textsuperscript{-}CD45RA\textsuperscript{+}, memory cells as CD8\textsuperscript{+}CD45RO\textsuperscript{+}, and effector cells as CD8\textsuperscript{+}CD27\textsuperscript{-}CD45RA\textsuperscript{+}. \textsuperscript{26,27} Besides these defined subsets, the molecules CD56, CD57, and CD62L have
been suggested as good markers for identifying CTL.\textsuperscript{26,28,29} However, no consensus has been reached regarding the best phenotypic definition of CTL.\textsuperscript{30}

In this study, to obtain an unbiased view of CD8\textsuperscript{+} T cell lineage marker relationships, we performed probability state modeling of primary human cells with respect to expression of cytotoxic granule constituents and various lineage and differentiation markers. Results identify bright expression of CD57 as a useful marker for detection of CTL in healthy individuals. Remarkably, functional analyses revealed that CD3\textsuperscript{+}CD8\textsuperscript{+}CD57\textsuperscript{bright} CTL display a similar propensity to degranulate and kill target cells as CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells. However, the subsets differed in the composition of their lytic granule content and in their propensity for production of cytokines. Besides delineating similarities and differences in the function of primary human CTL and NK cells, our findings also provide a reproducible means for rapidly quantifying CTL degranulation. This may prove useful for the diagnosis of FHL and other primary immunodeficiencies.
PATIENTS and METHODS

Patients and controls

The study was approved by The Regional Ethical Review Board in Stockholm. Healthy controls were recruited from the Karolinska University Hospital blood bank. Written consent was obtained from families of patients with confirmed biallelic mutations in PRF1, UNC13D, STX11, or STXBP2, in accordance with the Declaration of Helsinki.

Cells

Peripheral blood from healthy controls and patients was collected in heparin tubes and processed within 24 hours of venipuncture. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Lymphoprep, Axis-Shield) and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin; all Hyclone). In some experiments, PBMC were stimulated with 500 IU/ml IL-2 (Proleukin, Novartis) for 36-48 hours. For cytotoxicity assays, CD3⁺CD8⁺ T cell subsets or CD3⁻CD56⁺ NK cells were isolated from PBMC by negative selection using magnetic beads. CD8⁺ T cell subsets were isolated using a CD8⁺ T cell isolation kit, then supplemented with anti-CD45RO and anti-CD57-coupled beads for naïve T cell isolation, with anti-CD45RA and anti-CD57 coupled beads for memory CD8⁺ T cell isolation, and with an anti-CD28 depletion kit for effector CD8⁺ T cell isolation (all Miltenyi Biotec). NK cells were isolated using a NK cell isolation kit (Miltenyi Biotec). Purity of the isolated cell populations was checked by flow cytometry ( naïve T cells were 89±5% CD3⁺CD8⁺CD45RA⁺CD45RO⁻CD57⁻ cells, memory T cells were 85±9% CD3⁺CD8⁺CD45RA⁻CD45RO⁺CD57⁻ cells, effector T cells were 82±16% CD3⁺CD8⁺CD45RA⁺CD45RO⁻CD57⁺ cells, and NK cells were 96±2% CD3⁻CD56⁺ cells). Isolated cells were resuspended in complete media and used in assays the next day. The
human erythroleukemia cell line K562 and mouse mastocytoma cell line P815 (both ATCC) were maintained in complete medium.

**Antibodies**

For cell staining and flow cytometry, fluorochrome-conjugated anti-CD3 (clone S4.1, Invitrogen), anti-CD4 (S3.5, Invitrogen), anti-CD8 (RPA-T8, Biolegend), anti-CD14 (M5E2, BD Bioscience), anti-CD19 (HIB19, BD Bioscience), anti-CD27 (M-T271, BD Bioscience), anti-CD28 (CD28.2, Biolegend), anti-CD45RA (MEM-56, Invitrogen), anti-CD45RO (UCHL1, Beckman Coulter), anti-CD56 (NCAM16.2, BD Bioscience), anti-CD57 (HCD57, Biolegend), anti-CD62L (Dreg 56, BD Bioscience), anti-CD107a (H4A3, BD Bioscience), anti-perforin (δG9, BD Bioscience), anti-granzyme A (CB9, BD Bioscience), anti-granzyme B (Gb11, BD Bioscience), anti-granzyme K (GM6C3, Santa Cruz), anti-MIP-1β (D21-1351, BD Bioscience), anti-TNF-α (MAb11, BD Bioscience), and anti-IFN-γ (4S.B3, Biolegend) were used. Fluorochrome-conjugated IgG1 and IgG2b (MOPC-21 and 27-35, BD Bioscience) isotype control antibodies were also used. For stimulation, anti-CD16 (3G8, BD) or anti-CD3 (S4.1, Invitrogen) were used.

**Flow cytometry**

For intracellular staining of perforin and other granule constituents, freshly isolated PBMC were surface stained with fluorochrome-conjugated antibodies as indicated, as well as a fixable dead cell stain (Live/Dead, Invitrogen). Cells were then fixed (Cytofix, BD) followed by intracellular staining as indicated in permeabilization solution (Cytoperm, BD). As controls for the internal stainings, a set of isotype control antibodies were used. For assessment of functional responses, freshly isolated PBMC or PBMC stimulated with IL-2 were incubated alone, with K562 cells, P815 cells, or P815 cells supplemented with either
anti-CD16 or anti-CD3 antibody. Following stimulation, the lymphocytes were surface
stained with antibodies as indicated, as well as a fixable dead cell stain (Live/Dead,
Invitrogen). In some experiments, GolgiPlug (BD Bioscience) was added after the first hour
of stimulation. Thereafter, cells were fixed, permeabilized, and stained internally with anti-
MIP-1β, anti-TNF-α, anti-IFN-γ, and anti-CD69. All flow cytometry data were acquired on
an LSR Fortessa instrument (BD) and the resulting data analyzed with Flow Jo (v9.4, Tree
Star), Spice (v5.2b),31 and Graphpad Prism (v5.0, GraphPad). Probability state modeling was
performed with GemStone software (v1.0.63, Verity Software), and is explained in further
detail in the Supplemental Methods, Results and Figures.

**Cytotoxicity assays**

For assessment of cytotoxicity by PBMC or isolated lymphocyte subsets, effector cells were
mixed with $5 \times 10^3$ $^{51}$Cr-labeled K562 cells, P815 cells, P815 cells supplemented with 0.5
µg/ml anti-CD16 mAb or with anti-CD3 mAb, and incubated in triplicate for 4 hours at 37°C.
Effector-to-target cell ratios ranged from 10 to 0.3 in 200 µl medium in 96-well V-bottom
plates. The supernatants were measured for $^{51}$Cr-release on a gamma-counter (Cobra Gamma
Counter, Packard Instruments). Cytotoxic activity in patient samples was measured as
previously described.32

**DNA extraction, amplification, and sequence analysis**

Genomic DNA was isolated from peripheral blood according to standard procedures. **PRF1,**
**UNC13D, STXI1,** and **STXB2** were sequenced as previously described.32 Primers, PCR
conditions, and sequencing reaction conditions are available upon request.
RESULTS

CD57\textsuperscript{bright} expression marks perforin-expressing T cell subsets

To systematically determine the relationships between markers associated with lytic granule content in CD8\textsuperscript{+} T cells, high-dimensional flow cytometric analysis coupled to multiparametric probability state modeling was performed.\textsuperscript{33} PBMC from healthy human adult blood donors were surface stained with fluorochrome-conjugated antibodies to lineage and differentiation markers, including CD3, CD4, CD8, CD14, CD19, CD27, CD28, CD45RA, CD45RO, CD57, CD62L, and a dead cell marker. Thereafter, cells were fixed, permeabilized and stained intracellularly with fluorochrome-conjugated antibodies to perforin and granzyme B, followed by flow cytometric analysis. Data on 30,000 randomly selected CD3\textsuperscript{−}CD14\textsuperscript{−}CD19\textsuperscript{−}CD4\textsuperscript{−}CD8\textsuperscript{+} living lymphocytes (CD8\textsuperscript{+} T cells) from each of 12 adult donors were concatenated and subjected to probability state modeling (Figure 1A). The parameters to be monitored were added to the model in the following order: granzyme B, perforin, CD28, CD57, CD27, and finally CD62L, which was branched due to observed heterogeneity relative to the other parameters. CD45RA and CD45RO were not included as parameters in the model due to variability relative to other markers. Intracellular perforin and granzyme B expression was closely linked and was identifiable in 25±13\% and 27±14\% of CD8\textsuperscript{+} T cells, respectively. Strikingly, the analyses revealed a strong positive correlation of intracellular perforin and granzyme B expression with surface expression of CD57 (Figure 1A, B). In contrast, perforin and granzyme B expression was negatively correlated with CD27 and CD28 expression on CD8\textsuperscript{+} T cells (Figure 1A, 1B). According to the analysis, granzyme B was more widely expressed than CD57, whereas perforin was mostly restricted to CD57-expressing cells. Moreover, the majority of perforin and granzyme B-expressing cells expressed CD45RA, but not CD45RO or CD62L. Probability state modeling of individual healthy donors corroborated the overall phenotype of perforin- and granzyme B-expressing
cells, although the size of the subsets varied from less than 5% to 35% (Supplemental Figure S1). Of note, probability state modeling of donor #10 revealed a disparate pattern of CD57-expressing cells relative to other donors. In this donor, 44% of CD57 cells expressed CD45RO (average 30±13%) and 50% CD45RA (average 68±14%), explaining the unique outcome of the model in this individual.

In agreement with the probability state modeling analyses, plots of CD57 versus perforin expression in CD8+ T cells revealed that high CD57 (CD57bright) expression was associated with high levels of intracellular perforin expression (Figure 1C). Notably, CD8+ T cells expressing low CD57 (CD57dim) levels were mostly negative for perforin, as were CD8−CD57− T cells. Similarly, CD57bright expression was associated with intracellular perforin expression in CD4+ T cells as well as double-negative (DN) CD4−CD8− T cells (Figure 1C). In contrast to T cells, CD57bright expression was not strictly associated with perforin expression in CD3−CD56dim NK cells, although high CD57 expression correlated with somewhat increased expression of perforin, as previously described.29,34 CD3−CD56bright NK cells expressed no CD57 and relatively little perforin (Figure 1C).23 Thus, our data corroborate reports describing a correlation between surface CD57 and intracellular perforin and granzyme B expression in CD8+ T cells from healthy adults.26,29 In view of the data, and as Chattopadhyay and colleagues recently suggested,29 CD57bright-expression may represent a useful marker of effector CD8+ T cells, or bona fide CTL, which are more commonly defined as CD8+CD27−CD45RA+ T cells.26,27

In the T cell population as a whole, CD57-expressing cells were predominately CD8+ T cells, but substantial populations of CD4+ and DN CD4−CD8− T cells also expressed CD57 in healthy adults (Supplemental Figure S2). To obtain insight into the heterogeneity of CD57-expressing T cells and how this relates to expression of granule proteins, the phenotype of CD57-expressing T cells was examined in further detail in data concatenated from equal
numbers of CD3+CD4+CD8−, CD3+CD4−CD8+ or DN CD3+CD4−CD8− cells from seven healthy individuals (Figure 1D). Notably, CD8+CD57+ T cells consisted primarily of CD57brightCD27−CD45RA+ cells and CD57dimCD27+CD45RA+/− cells. The former population expressed abundant levels of perforin, whereas as the latter, in addition to CD57CD27−CD45RA+/− cells, expressed low levels of perforin. Among CD4+ T cells, perforin expression was confined to a CD57brightCD27− cell subset, many of which expressed CD45RO (Figure 1D), as previously described.35 Among DN CD4−CD8− T cells, perforin expression was, similar to CD8+ T cells, mostly confined to a CD57brightCD27−CD45RA+ cell subset (Figure 1D). Thus, substantial differences exist in the differentiation stage of various perforin-expressing T cell subsets.

Comparison of granule content in cytotoxic lymphocyte subsets

The distribution of perforin-expressing cytotoxic lymphocyte subsets, and the respective level of perforin expressed, was assessed in healthy adults relative to CD57 expression. On average, the majority of perforin-expressing cells were either CD8+CD57bright T cells or CD3−CD56dim NK cells (25±17% and 43±16%, respectively; mean±SD; n = 12; Figure 2A). DN CD4−CD8−CD57bright T cells constituted the second major population of perforin-expressing T cells (8±6%). CD4+CD57bright T cells represented only a minor subset of perforin-positive T cells (2±3%). Remarkably, perforin-expression was significantly lower in CD8+CD57bright and DN CD4−CD8−CD57bright T cells as compared to CD3−CD56dim NK cells (3.1 and 3.2-fold, respectively). Furthermore, perforin expression was lower in CD4+CD57bright T cells as compared to CD3−CD56dim NK cells (6.1-fold). Intracellular expression of perforin was generally very low in CD57dim and CD57+ T cell subsets.

Quantification of other granule constituents, including granzyme A, granzyme B, and granzyme K, in addition to CD107a (a marker of lysosomal membranes), by intracellular
staining and flow cytometry also revealed differences. In addition to perforin, surface CD57\textsuperscript{bright} expression correlated with high intracellular expression of granzyme A and granzyme B in all T cell subsets (Figure 2B). In contrast, granzyme K did not correlate with CD57\textsuperscript{bright} expression (Figure 2B). Similar to perforin, granzyme A expression was lower in CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells as compared to in CD3\textsuperscript{+}CD56\textsuperscript{dim} NK cells (1.8-fold). In contrast, granzyme B expression was higher in CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells as compared to CD3\textsuperscript{+}CD56\textsuperscript{dim} NK cells (1.5-fold). Granzyme K displayed a distinct expression pattern, with highest expression in CD8\textsuperscript{+}CD57\textsuperscript{dim} T cells, DN CD4\textsuperscript{+}CD8\textsuperscript{+}CD57\textsuperscript{-/dim} T cells, and CD3\textsuperscript{+}CD56\textsuperscript{bright} NK cells. Intracellular CD107a expression followed the expression of cytotoxic granule constituents. In summary, although CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells and CD3\textsuperscript{+}CD56\textsuperscript{dim} NK cells both express abundant perforin, the composition of other granule constituents varies among the cytotoxic lymphocyte subsets.

\textit{Comparison of degranulative and cytotoxic capacity of cytotoxic lymphocyte subsets}

Having established CD57\textsuperscript{bright} as a useful marker of T cell subsets with high lytic granule content, we next compared the degranulation-capacity of the different T cell and NK cell subsets. PBMC were incubated alone or with different target cells for two hours at 37°C. Thereafter, cells were stained with fluorochrome-conjugated antibodies to lineage and differentiation markers, extracellular CD107a and a dead cell marker and analyzed by flow cytometry. All examined T cell subsets degranulated in response to P815 target cells with anti-CD3, but not to P815 cells alone, P815 cells with anti-CD16, or K562 cells (Figure 3A, B). The CD8\textsuperscript{+}CD57\textsuperscript{bright} T cell subset displayed the highest frequency of degranulation (57±11%; n = 14; Figure 3B), closely followed by CD8\textsuperscript{+}CD57\textsuperscript{dim} T cells (52±12%). By comparison, CD4\textsuperscript{+} and DN CD4\textsuperscript{-}CD8\textsuperscript{-} T cell subsets degranulated less than the CD8\textsuperscript{+}CD57\textsuperscript{+} T cell subset, but also in these subsets degranulation was most prominent in CD57\textsuperscript{bright} T cells.
In contrast to T cells, CD3^-CD56^{dim} NK cells responded vigorously to P815 cells with anti-CD16 as well as to K562 cells (42±15% and 20±11%, respectively; n = 14), but not to P815 cells alone or P815 cells with anti-CD3, as expected.\textsuperscript{19} Degranulation by CD3^-CD56^{bright} NK cells was considerably lower. In summary, all T cell subsets degranulated in response to TCR-engagement. Notably, CD8^+CD57^{bright} and CD8^+CD57^{dim} T cells degranulated with higher frequency and with greater intensity than other T cell subsets. Their degranulation was comparable to that of CD3^-CD56^{dim} NK cells triggered by CD16-engagement. Thus, for sensitive evaluation of the capacity for CD8^+ T cells to degranulate, the CD8^+CD57^+ T cell subset can be assessed.

In regards to cellular cytotoxicity, purified naïve (CD45RO and CD57-depleted), memory (CD45RA and CD57-depleted), and effector (CD28 and CD45RO-depleted) T cell subsets were isolated and mixed with different \textsuperscript{51}Cr-labeled target cells. Cytotoxicity by T cell subsets was compared to that of total PBMC as well as purified NK cells. Specific lysis was calculated after 4 hours of incubation. Whereas PBMC populations lysed P815 cells in the presence of anti-CD3 or anti-CD16, as well as K562 cells, naïve and memory T cells did not lyse any of the target cells (Figure 3C). In contrast, effector T cells efficiently lysed P815 cells with anti-CD3 and also demonstrated some lysis of P815 cells with anti-CD16, but no lysis of K562 cells (Figure 3C). Lysis of P815 cells with anti-CD3 by effector T cells, that were more than 74±14\% CD8^+CD57^+ T cells, was at least 20-fold more efficient than that of naïve T cells. As expected, NK cells efficiently lysed P815 cells with anti-CD16 as well as K562 cells, but did not lyse P815 cells alone or with anti-CD3 (Figure 3C). In summary, naïve and memory CD8 T cell subsets did not lyse P815 cells upon antigen receptor engagement, whereas effector CD8^+ T cells triggered by TCR-engagement and NK cells triggered by Fc receptor-engagement lysed P815 target cells to a similar extent. Hence, the
results formally demonstrate that CD8⁺CD57⁺ T cells, enriched for perforin and with high degranulative ability, exhibit strong cytotoxic capacity similar to that of NK cells.

Defective degranulation by Munc13-4-, syntaxin-11-, and Munc18-2-deficient cytotoxic T cells

To obtain insights into the degree to which CTL and NK cells share requirements for lytic granule exocytosis, as well as evaluate the potential use of assays quantifying CD8⁺ T degranulation for the diagnosis of FHL patients, PBMC from healthy donors as well as patients with confirmed biallelic mutations in genes associated with FHL (Table 1) were incubated alone or with different target cells for two hours at 37°C. Thereafter, the PBMC were stained with fluorochrome-conjugated antibodies to lineage and differentiation markers, CD107a, as well as a dead cell marker, and analyzed by flow cytometry. Whereas CD8⁺CD57⁺ T cells from healthy controls and FHL2 patients with perforin-deficiency degranulated in response to P815 target cells with anti-CD3, cells from FHL3, FHL4, and FHL5 patients did not (Figure 4A, B). In the FHL2 patients, as in healthy controls, CD8⁺CD57⁺ T cells degranulated more than CD8⁺CD57⁻ T cells (Figure 4A). Relative to healthy controls, cytotoxic lymphocytes from two FHL2 patients displayed somewhat low degranulation, as previously observed in NK cells from a larger cohort of FHL2 patients (Figure 4B).²² Comparison of CD8⁺CD57⁺ T cell and CD3⁺CD56dim NK cell degranulation ex vivo, and following IL-2 stimulation, revealed similar degrees of impairment in individual FHL3, FHL4, and FHL5 patients carrying mutations in the genes encoding for Munc13-4, syntaxin-11, and Munc18-2, respectively (Figure 4B, C). Generally, T cells and NK cells from patients with UNC13D, STX11, or STXBP2 mutations associated with late-onset HLH both displayed somewhat more degranulation than those cells from patients with early-onset HLH. Patient FHL3-D:1 represented an exception to this. If anything, a little more
degranulation was observed in resting CTL as compared to NK cells from these patients. In summary, CTL from Munc13-4-, syntaxin-11-, and Munc18-2-deficient patients display defective degranulation. Thus, evaluation of CD8⁺CD57⁺ T cell degranulation may replace analysis of NK cells in patients with too few NK cells in circulation, potentially caused by specific defects in NK cell development or through consumption by ongoing infections, and may in addition complement analyses of NK cell degranulation for the diagnosis of novel primary immunodeficiency syndromes differentially affecting lymphocyte cytotoxicity.

Although hardly any perforin or CD57 expression is detectable in T cells at birth,¹⁹,³⁶ some infant patients displayed high frequencies of CD57-expressing CD8⁺ T cells (Supplemental Figure S3A). The frequencies of CD57-expressing CD3⁻CD56⁺ NK cells were low in infants, but generally higher than the frequencies of CD57-expressing CD8⁺ T cells (Supplemental Figure S3B-D). Importantly, only one out of 20 patients studied was excluded due to our inability to detect sufficient CD8⁺CD57⁺ T cells for assessment of T cell degranulation. As Figure 5A depicts, analysis of patient FHL5-M:1 with a Munc18-2-deficiency, whose sample was obtained prior to administration of immunosuppressive treatment, expressed a high perforin level also in CD8⁺CD57⁻ T cells (Figure 5A). Remarkably, these cells had the phenotype of CD8⁺CD27⁺CD28⁺CD45RA⁻CD45RO⁺CD57⁻ memory T cells (Figure 5B). Thus, a subset of CD8⁺CD57⁺ T cells is detected in most infant FHL patients and surface CD57 expression may not be a prerequisite for perforin expression in T cells.

Comparison of cytokine production by cytotoxic lymphocytes subsets

Similar to NK cells, CD8⁺ T cells are important sources of pro-inflammatory cytokines such as TNF-α and IFN-γ.²⁶,³⁷ Noteworthy, CD3⁻CD56bright NK cell excel for production of cytokines following stimulation with exogenous cytokines, whereas CD3⁻CD56dim NK cell
are better at producing cytokines upon target cell recognition.\textsuperscript{37} To the best of our knowledge, cytotoxic T cells and NK cells have not been directly compared in regards to their potential for cytokine production. In a time-course experiment, PBMC were incubated alone or with different target cells at 37°C. Thereafter, cells were surface stained with lineage and differentiation markers, CD107a, and a dead cell marker, fixed, permeabilized and stained intracellularly with fluorochrome-conjugated antibodies to MIP-1β, TNF-α, IFN-γ, and CD69, followed by flow cytometric analysis. CD8\textsuperscript{+} T cell subsets and CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cell responded rapidly to strong activation stimuli such as anti-CD3 and anti-CD16 antibodies, respectively, with expression of CD69 in more than 80% of cells. Similarly, MIP-1β was expressed in more than 70% of CD8\textsuperscript{+} T cell subsets and CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cell following strong activation stimuli. In contrast, CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells produced the highest levels of TNF-α and IFN-γ upon stimulation as compared to other CD8\textsuperscript{+} T cell subsets (Figure 6, CD8\textsuperscript{+}CD57\textsuperscript{bright} versus CD8\textsuperscript{+}CD57\textsuperscript{dim} T cells after 2 hours of stimulation, p < 0.05, Wilcoxon signed rank test). Unexpectedly, by comparison, CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells responded more rapidly and vigorously in terms of MIP-1β, TNF-α, and IFN-γ production to anti-CD3 antibodies than did CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells to anti-CD16 antibodies or coincubation with K562 cells (Figure 6; CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells versus CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells after 2 hours of stimulation, p < 0.05, Wilcoxon signed rank test). Of note, CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells and CD8\textsuperscript{+}CD57\textsuperscript{dim} T cells degranulated as rapidly in response to anti-CD3 antibodies as CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cell did in response to anti-CD16 antibodies or coincubation with K562 cells (Figure 6A). Two hours of stimulation, yielded many more multifunctional CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells following TCR-engagement than CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells following Fc receptor-engagement (Figure 6B). Together, the data demonstrate that, although CTL and NK cells have similar propensities for undergoing degranulation and for inducing CD69 and MIP-1β
production upon receptor stimulation, CTL are better poised for production of TNF-α and IFN-γ.
DISCUSSION

The complexity of the immune system represents a challenge for understanding of human immunity and implementing of immunological insights into clinical practice. In the present study, the probability state modeling of lineage and differentiation markers on human CD8+ T cells revealed that CD57<sub>bright</sub>-expression is a useful marker of perforin-containing CTL, extending former findings by Chattopadhyay and colleagues. That is, by using CD57<sub>bright</sub>-expression as a marker and comparing the granule content and function of cytotoxic lymphocyte subsets in human peripheral blood, we identified unexpected differences in granule content, but similar propensities to degranulate. Moreover, cytotoxic lymphocyte degranulation was similarly impaired by a deficiency in Munc13-4, syntaxin-11, or Munc18-2. Notably, CTL excelled in cytokine production induced upon target cell recognition as compared to NK cells.

Methodological innovations that facilitate increasingly high-dimensional flow cytometric analyses are advancing translational approaches to further understanding of the human immune system. In defined cell populations, protein concentrations in individual cells are stochastically distributed, since they are subject to fluctuations due to transcriptional regulation, turnover, and environmental perturbations. Although signal-to-noise ratios vary with time, flow cytometry captures a snapshot of such variations. When interrogating aspects of cellular populations and networks by such steady-state measurements of individual components, efficient methods to simulate, compute, and visualize high-dimensional distributions are needed. Here, we have demonstrated the efficacy of probability state modeling on CD8<sup>+</sup> T cell differentiation, illustrating a strong correlation between perforin and CD57 expression.

In 1981, Abo and Balch first described the CD57 antigen as a differentiation antigen associated with cytotoxic activity. Interestingly, the expression of CD57 is almost absent in
newborns, but increases with age.\textsuperscript{42} Cytomegalovirus infection, as well as other viral infections, promotes the development of CD57-expressing T cells.\textsuperscript{42} Analysis of CD8\textsuperscript{+} T cell subsets suggested a sequential acquisition of granzymes, CD57, and perforin. This was not the case for NK cells, in which perforin expression was also present in CD57\textsuperscript{−} cells. Moreover, analysis of a patient with ongoing HLH, \textit{i.e.}, a systemic inflammatory state, suggested that perforin expression may not necessarily be coupled to CD57 also in T cells. \textit{In vitro} experiments indicated that perforin can be rapidly up-regulated upon TCR-engagement.\textsuperscript{43} Thus, CD57\textsuperscript{bright}-expression does not appear to be a prerequisite for intracellular perforin expression, although CD57\textsuperscript{bright}-expression generally marks perforin-expressing T cells. Expression of distinct granzymes is regulated upon CD8\textsuperscript{+} T cell differentiation.\textsuperscript{44,45} How TCR and cytokines signals epigenetically regulate effector molecule and CD57 expression during T cell differentiation is of interest for future studies.

We compared the phenotype and granule content of distinct CD57-expressing lymphocyte subsets \textit{ex vivo}. Interestingly, those analyses revealed a variable content of cytotoxic granule constituents. By comparison, CD3\textsuperscript{−}CD56\textsuperscript{dim} NK cells expressed more perforin and granzyme A than CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells, whereas the CD8\textsuperscript{+}CD57\textsuperscript{bright} T cells expressed more granzyme B. Granzyme B is a potent inducer of apoptosis through caspase-dependent and -independent mechanisms.\textsuperscript{46} Granzyme A is considered a weak mediator of caspase-independent cell death.\textsuperscript{46} In addition, mounting evidence suggests a role for granzyme A in promoting inflammation through proteolysis of cytokines.\textsuperscript{47} Although in our analyses, provided appropriate activation signals, CTL and NK cells displayed similar capacities to kill target cells, it is tempting to speculate that differences in their granule content might differentially impact cell death and inflammation.

NK cells are often considered to be important early mediators of immune responses in terms of cytokine production. Such cytokine production is induced by target cell engagement
in CD3^−CD56^{dim} NK cells, whereas CD3^−CD56^{bright} NK cells respond more vigorously albeit slower to exogenous cytokines.\(^{37}\) In fact, in our experiments, CD8^+CD57^{bright} T cells produced TNF-\(\alpha\) and IFN-\(\gamma\) more rapidly and with greater frequency than CD3^−CD56^{dim} NK cells. The differences demonstrate a greater propensity for effector T cells to produce proinflammatory cytokines upon activation. This ability to more rapidly respond to stimuli might reflect epigenetic differences at the cytokine gene loci during T cell differentiation that do not occur in NK cells. Thus, further comparisons of T cell and NK cell subsets in regard to epigenetic regulation of cytokine genes are of interest.

Assays assessing NK cell degranulation provide a sensitive means for the diagnosis of FHL.\(^{19,21,22}\) In this setting, quantification of the frequency of cells with induced CD107a surface expression as well as the intensity of CD107a staining of such degranulating cells is informative.\(^{48,49}\) In a clinical setting, sensitive and reproducible assays for the quantification of other major cytotoxic lymphocyte subsets would be useful for several reasons. First, some patients have very few circulating NK cells. Thus, in these patients, assays quantifying degranulation in other cytotoxic lymphocyte subsets could provide a means of diagnosis. Second, quantification of degranulation in additional cytotoxic subsets could strengthen suspicion of defects in patients with abnormal or borderline NK cell degranulation. Third, differences in CTL and NK cell function may exist and be clinically relevant for the diagnosis of novel primary immune deficiency (PID) disease. Notably, assays quantifying CTL degranulation using phytohemagglutinin and IL-2-stimulated blasts have been reported.\(^{22,50}\) However, in our hands, these assays have been difficult to interpret due to high assay variability and donor-to-donor variation. Moreover, they require at least 48 hours of pre-stimulation. Gating on CD8^-CD57^+ CTL, we observed robust responses with relatively low donor-to-donor variability in freshly isolated cells. In fact, degranulative responses by CD8^-CD57^+ T cells upon CD3-stimulation were greater in magnitude and displayed
considerably less standard deviation than those by NK cells upon K562 stimulation. Thus, assays quantifying degranulation by CD8^+CD57^+ T cells may offer a more sensitive and rapid means of detecting defects in degranulation. The use of multiple stimulations with better signal-to-noise ratios may be particularly useful for identifying patients with milder defects associated with later clinical presentations. Importantly, a limitation of such assays is that differentiated, CD57-expressing T cells are absent at birth, but rather develop in response to infections. However, for the most part, we were able to detect and assess perforin-expressing CD8^+CD57^+ T cells from infants as young as one month. This suggests that such effector CD8^+CD57^+ T cells rapidly develop in infants, possibly driven by infections that may also act as triggers of HLH. Of note, if parameters are limited due to flow cytometer constraints, analysis of CD107a expression on CD3^+CD57^+ T cells can provide a better indication of the capacity of T cells to degranulate than analysis on CD3^+CD8^+ T cells (Supplemental Figure S4).

Taken together, the current work, together with that of Chattopadhyay and colleagues, provides a rational to use CD57^bright-expression as a marker of CTL in humans and further highlights similarities and differences in cytotoxic lymphocyte subset phenotype and function. From a primary immunodeficiency disease perspective, evaluation of CD57^bright T cell numbers might aid the diagnosis of syndromes with impaired CTL development. Assays quantifying both CTL and NK cell degranulation may provide a better means for diagnosis of patients with late-onset FHL associated with more subtle defects in cytotoxic lymphocyte degranulation. Moreover, such approaches may unravel novel primary immunodeficiency diseases associated with selective defects in CTL or NK cell function. The efficacy of assays quantifying degranulation by CD8^+CD57^+ T cells for the diagnosis of defects in lymphocyte cytotoxicity will be assessed in future studies.
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REFERENCES


FIGURE LEGENDS

Figure 1. CD57 expression marks perforin-expressing T cells. (A-D) Freshly isolated, resting PBMC from 12 healthy adult volunteers were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD14, CD19, CD27, CD28, CD45RA, CD45RO, CD56, CD57, and CD62L, followed by fixation, permeabilization, and intracellular staining with isotype control antibodies or antibodies to perforin and granzyme B, then analyzed by flow cytometry. Lymphocytes were gated on forward scatter / side scatter plots. (A, B) Concatenated data for 30,000 randomly selected CD3⁺CD14⁻CD19⁻CD4⁻CD8⁺ living lymphocytes (CD8⁺ T cells) from each of 12 healthy adult donors. (A) Probability state model of CD8⁺ T cells. The parameters were added to the model in the following order: granzyme B, perforin, CD28, CD57, CD27, and finally CD62L that was branched due to observed heterogeneity relative to the other parameters. CD45RA and CD45RO were not included as parameters in the model, but are displayed in the final analysis. (B) Plots show the distribution of differentiation marker expression on individual cells, as indicated, according to the probability state model. (C) Plots show CD57 versus isotype or perforin-staining on T cell and NK cell subsets, as indicated, from one representative donor. (D) Plots show CD57 versus CD27 staining on T cell subsets, as indicated, on data concatenated from seven healthy donors. Histograms display the expression of perforin, granzyme B, CD28, CD45RA, CD45RO, and CD62L in CD27⁺CD57⁺, CD27⁺CD57⁻, CD27⁻CD57⁺, CD27⁻CD57⁻ T cell subsets, as indicated.

Figure 2. Quantification of perforin and other granule constituents in T cell and NK cell subsets. (A, B) Freshly isolated, resting PBMC from healthy adult volunteers were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD14, CD19, CD56, and CD57, followed by fixation, permeabilization, and intracellular staining with isotype
control antibodies or antibodies to perforin, CD107a, granzyme A, granzyme B, or granzyme K, and analyzed by flow cytometry. Lymphocytes were gated on forward scatter / side scatter plots. (A) Perforin-expressing cells were assessed relative to expression of the lineage markers CD3, CD4, CD8, CD56, as well as CD57. The pie chart depicts the relative distribution of the lymphocyte subsets, as indicated. Shading reflects the relative mean fluorescence intensity (R-MFI) of perforin staining in each subset and the frequency of each subset is indicated. Results are derived from 12 donors. Numbers indicate mean±SD frequencies of indicated subsets relative to the total perforin-expressing lymphocyte population. (B) Values represent R-MFI, where the MFI values of indicated staining have been subtracted from MFI values of isotype control antibodies. Values represent the mean±SD of 12 donors. Numbers indicate mean±SD frequencies of indicated subsets relative to the total lymphocyte population. Statistical analyses were performed using Wilcoxon signed rank matched pairs test. * denotes P < 0.05, ** denotes P < 0.01, and *** denotes P < 0.001.

Figure 3. Degranulation and cytotoxicity by T cell and NK cell subsets. (A, B) Freshly isolated, resting PBMC from healthy adult volunteers were incubated alone or mixed with target cells and mAbs, as indicated, at 37°C for two hours. Thereafter, cells were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD56, CD57, and CD107a. (A) Contour plots of CD57 versus CD107a staining on CD8+ T cell and CD3−CD56+ NK cell subsets, following of indicated target cell stimulations. Plots with concatenated data from six representative donors are shown. Numbers indicate percentages of degranulating CD57−, CD57dim, and CD57bright cells. (B) Induced CD107a surface (ΔCD107a+) expression on T cell and NK cell subsets after two hours of target cell stimulations, as indicated. Values represent the mean±SD of 14 donors. (C) CD3+CD8+ T cell subsets, as specified, or CD3−
CD56⁺ NK cells were isolated by negative selection. Effector cells were thereafter incubated with target cells, as indicated, for four hours. Specific lysis was calculated. Values represent the mean±SD of at least 4 donors. Statistical analyses were performed using Wilcoxon signed rank matched pairs test. * denotes P < 0.05, ** denotes P < 0.01, and *** denotes P < 0.001.

**Figure 4. Degranulation and cytotoxicity by T cell and NK cell subsets from patients with perforin, Munc13-4, syntaxin-11, and Munc18-2-deficiencies.** (A, B) Freshly isolated, resting or (C) IL-2-stimulated PBMC from healthy adult volunteers or patients with biallelic mutations in FHL-associated genes were incubated alone or mixed with target cells and mAbs, as indicated, at 37°C. Thereafter, cells were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD56, CD57, and CD107a. (A) Contour plots of CD57 versus CD107a staining on CD8⁺ T cell subsets, following two hours of indicated target cell stimulations. Plots of representative donors and patients are shown. Numbers indicate percentages of degranulating CD57⁻ and CD57⁺ cells. (B, C) Induced CD107a surface (ΔCD107a⁺) expression on (B) freshly isolated or (C) IL-2-stimulated CD8⁺CD57⁺ T cell and CD3⁺CD56⁺ NK cell subsets after two hours of target cell stimulations, as indicated. For healthy donors, bars indicate the mean±SD of 34 donors. For patients, which are color-coded according to age at onset, boxes indicate values for each patient and are linked with lines.

**Figure 5. Perforin expression in T cell subsets from a Munc18-2-deficient patient with ongoing HLH.** Freshly isolated PBMC from healthy adult volunteers as well as an untreated Munc18-2-deficient patient with HLH were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD14, CD19, CD27, CD28, CD45RA, CD45RO, CD56, and CD57 followed by fixation, permeabilization, and intracellular staining with isotype control
or anti-perforin and anti-granzyme B antibodies or antibodies to perforin and granzyme B, then analyzed by flow cytometry. (A) Plots show CD57 versus isotype or perforin-staining on T cell and NK cell subsets, as indicated. (B) Plots show CD57 versus CD27 staining on T cell subsets, as indicated. Histograms display the expression of perforin, granzyme B, CD28, CD45RA, CD45RO, and CD62L in CD27^+CD57^+ and CD27^+CD57^- T cell subsets, as indicated.

**Figure 6. Time course analysis of degranulation and cytokine responses by different T cell and NK cell subsets from healthy adults.** Freshly isolated, resting PBMC from healthy adult volunteers were incubated alone or mixed with target cells and mAbs, as indicated, at 37°C. Thereafter, cells were surface stained with fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD56, CD57, and CD107a, followed by fixation, permeabilization and intracellular staining with antibodies to MIP-1β, TNF-α, IFN-γ, and CD69. (A) Time course of functional responses on CD8^+ T cell subsets and CD3^-CD56^+ NK cells following target cell stimulations, as indicated. Values represent the mean±SD of six donors from one of three independent experiments. (B) Indicated lymphocyte cells were gated, and a Boolean gating strategy was used for analysis. Pie-charts represent the frequency of cells positive for the given number of measured responses (CD107a, MIP-1β, TNF-α, and IFN-γ). Thus, cells can be categorized into the number of responses they display. Arcs depict the relative frequency of cells specifically positive for CD107a, MIP-1β, TNF-α, and/or IFN-γ, staining, as indicated. Values represent the mean of six different donors from one of three independent experiments.
Figure 1. Chiang, Theorell et al.
Distribution of perforin+ lymphocytes

CD4–CD8+CD57dim T cells (3.0±2.1%)
CD4+CD8–CD57dim T cells (0.2±0.3%)
CD4+CD8–CD57– T cells (0.6±0.5%)
CD4–CD8–CD57bright DN T cells (8.0±5.5%)
CD4–CD8–CD57– DN T cells (6.0±7.1%)
CD4–CD8–CD57dim NK cells (1.8±2.4%)
CD4+CD8–CD57bright T cells (2.1±2.8%)
CD4–CD8+CD57– T cells (5.7±4.7%)
CD4–CD8+CD57bright T cells (24.9±16.7%)
CD3–CD56bright NK cells (1.8±2.4%)
CD3–CD56- cells (0.9±0.5%)
CD4+CD8+ T cells (1.2±2.0%)
CD4+CD8 CD57 T cells (0.6±0.5%)
CD4+CD8 CD57dim T cells (0.2±0.3%)
CD4+CD8 CD57bright T cells (2.1±2.8%)
CD4+CD8 CD57 T cells (5.7±4.7%)
CD4+CD8 CD57dim T cells (3.0±2.1%)

B

Granule content (R-MFI)

CD3-CD56+ T cells
CD4-CD8-CD57dim T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
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CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells
CD3-CD56+ T cells
CD4-CD8-CD57+ T cells

CD4+CD8+ T cells (1.2±2.0%)
CD4+CD8 CD57 T cells (0.6±0.5%)
CD4+CD8 CD57dim T cells (0.2±0.3%)
CD4+CD8 CD57bright T cells (2.1±2.8%)
CD4+CD8 CD57 T cells (5.7±4.7%)
CD4+CD8 CD57dim T cells (3.0±2.1%)

Figure 2. Chiang, Theorell et al.
Figure 3. Chiang, Theorell et al.
Figure 4. Chiang, Theorell et al.
Figure 5. Chiang, Theorell et al.
Figure 6. Chiang, Theorell et al.
Comparison of primary human cytotoxic T cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production


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