Phenotypic and functional evaluation of CD3$^+$CD4$^-$CD8$^-$ T cells in human CD8 immunodeficiency

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ABSTRACT

Background

Human CD8 immunodeficiency is characterized by undetectable CD8$^+$ lymphocytes and an increased population of CD4$^-$CD8$^-$ (double negative) T lymphocytes.

Design and Methods

We hypothesized that the double negative subset corresponds to the cellular population that should express CD8 and is committed to the cytotoxic T lymphocyte lineage. To assess this, we determined the phenotype and function of peripheral blood mononuclear cells and/or magnetically isolated double negative T lymphocytes from two CD8-deficient patients. To analyze the expression and co-localization with different organelles, 293T cells were transfected with plasmids bearing wild-type or mutated CD8α.

Results

CD8α mutated protein was retained in the cytoplasm of transfected cells. The percentages of double negative cells in patients were lower than the percentages of CD8$^+$ T cells in healthy controls. Double negative cells mostly had an effector or effector memory phenotype whereas naïve T cells were under-represented. A low concentration of T-cell receptor excision circles together with a skewed T-cell receptor-Vβ repertoire were observed in the double negative population. These data suggest that, in the absence of CD8 co-receptor, the thymic positive selection functions suboptimally and a limited number of mature T-cell clones would emerge from the thymus. *In vitro*, the double negative cells showed a mild defect in cytotoxic function and decreased proliferative capacity.

Conclusions

It is possible that the double negative cells are major histocompatibility complex class-I restricted T cells with cytolytic function. These results show for the first time in humans that the presence of the CD8 co-receptor is dispensable for cytotoxic ability, but that it affects the generation of thymic precursors committed to the cytotoxic T lymphocyte lineage and the proliferation of mature cytotoxic T cells.

Key words: CD8 immunodeficiency, double negative T lympho cytes, MHC class-I, cytotoxic T lymphocyte proliferation.

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The online version of this article has a Supplementary Appendix.

Introduction

$CD8^+$ cytotoxic T lymphocytes recognize short peptides presented by major histocompatibility complex class I (MHC-I) molecules on the surface of target cells. This recognition enables cytotoxic T lymphocytes to eliminate infected and tumor cells and allogeneic grafts. Antigen specificity is conferred by T-cell receptors (TCR),[1] but the peptide-MHC-I complex also interacts with the T-cell surface glycoprotein CD8, which binds to invariable regions of the MHC-I molecule.[2] CD8 is thought to act as a co-receptor to TCR function; the CD8-MHC-I contact stabilizes the TCR/peptide-MHC-I interaction and increases the TCR triggering rate for low affinity peptide-MHC-I ligands.[3] CD8 cytosolic domains bind to the scr tyrosine protein kinase $p56^{lck}$ leading to the rapid activation of TCR through the CD3ζ chain tyrosine phosphorylation pathway.[4] Acquisition of cytolytic capability implies expression of effector molecules such as perforin, granzymes and granulysin. CD8 is also critical for the progression of cytotoxic T lymphocytes through the process of positive selection during differentiation in the thymus.[5]

CD8 molecules are expressed on the cell surface either as an αα homodimer or as an αβ heterodimer. Surface expression of CD8β is dependent on the expression of CD8α, given that in the absence of CD8α expression CD8β polypeptides are retained in the endoplasmic reticulum and degraded.[6] Both α and β chains are formed of a single extracellular immunoglobulin-like domain, a membrane-proximal hinge region, a transmembrane domain and a cytoplasmic tail. CD8αα is expressed on αβ-T cells (although CD8αβ is predominant) and exclusively on natural killer cells and γδ-T cells.[7] The CD8 α chain has been shown to be a more effective T-cell activator than its heterologous counterpart.[8] More recent data have alluded to a role as an immune modulator due to the ability of two CD8 α chains to bind non-classical MHC molecules.[9]

Within the population of human peripheral $CD8^+$ T lymphocytes, T $CD8^+$ naïve lymphocytes ($CD45RA^+$ $CD27^{bright}CD28^+$) can be distinguished from memory T cells ($CD45RA^-CD27^+CD28^+$).[10,11] These latter can be further subdivided, according to their capacity to enter secondary lymphoid organs, into central memory T cells (T_{CM}), expressing lymph node homing receptors (e.g., CD62L, CCR7), and effector memory T cells (T_{EM}), without homing receptors.[12] $CD8^+$ T_{EM} down-regulate surface expression of T-cell co-receptor molecules, CD45RO, CD28 and CD27, during further differentiation toward an effector T-cell phenotype. Homeostatic proliferation of naïve and memory $CD8^+$ T cells is promoted by the cytokine interleukin-7.[13]

Two patients with CD8 deficiency due to a Gly111Ser mutation in the CD8α chain (CD8αmut) have been previously reported in the literature.[14,15] Neither patient has a detectable $CD8^+$ lymphocyte population, but both have an increased population of $CD4^-CD8^-TCR\alpha\beta^+$ (double negative, DN) T lymphocytes. We hypothesized that the DN subset corresponds to the cellular population that should express the CD8 molecule and is committed to the cytotoxic T lymphocyte lineage. To assess this, we determined the phenotype and function of peripheral blood mononuclear cells and/or magnetically isolated DN T lymphocytes from the two CD8-deficient patients.

Design and Methods

CD8 deficient patients

This study was carried out with approval from the Ethics Committee of 12 de Octubre University Hospital and the informed consent of the patients. We evaluated two unrelated subjects with CD8 deficiency belonging to the only two familial cases of CD8 deficiency described in the literature.[16] Both patients are homozygous carriers of p.Gly111Ser mutation in the *CD8A* gene and are from consanguineous Spanish Roma families (MIM#608957). The patients' main clinical manifestations are recurrent infection of the respiratory tract. Patient 1 (P1) is a 31-year old woman, sister of the deceased, first reported case of CD8 deficiency.[14] Patient 2 (P2) is an 18-year old woman, the second reported case of CD8 deficiency.[15]

Magnetic cell sorting

Control $CD8^+$ and $CD4^+$ T cells were isolated from peripheral blood mononuclear cells using first anti-CD8-coated magnetic beads (Miltenyi Biotec); secondly, $CD3^+$ cells were isolated from the $CD8^+$ and $CD8^-$ cells with anti-CD3-coated magnetic beads. Patients' $CD4^-CD8^-CD3^+$ (DN) and $CD4^+$ T cells were isolated from peripheral blood mononuclear cells using first anti-CD4-coated magnetic beads; secondly, $CD3^+$ cells were isolated from the $CD4^-$ and $CD4^+$ cells by using anti-CD3-coated magnetic beads.

Highly enriched $CD8^+$ T cells (>90%) (controls) and DN T cells (>90%) (patients) were used for flow cytometric analysis and quantification of T-cell receptor excision circles (TREC). TCRγδ cells in the total isolated DN population from patients were evaluated ($CD3^+TCR\gamma\delta$ cells: 1% in P1 and 8% in P2) and were comparable to amounts of TCRγδ $CD8^+$ cells present in the isolated $CD8^+$ T cells from healthy controls (3-5%). The quantification of signal joint (sj) TREC gives a measure of the recombination of the α locus and the presence of TCRγδ cells in the starting sample does not influence the final result. Isolated $CD4^+$ T cells were used for TREC quantification.

Flow cytometric analysis

Whole blood lymphocyte subsets were identified using CYTO-STAT tetraCHROME CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5, CYTO-STAT tetraCHROME CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5 and the monoclonal antibodies CD3-ECD (Beckman-Coulter), TCRαβ-FITC, TCRγδ-PE, CD45RO-PE and CD45RA-FITC (BD Bioscience).

Magnetically sorted DN T cells from patients and $CD3^+CD4^-$ $CD8^+$ from control subjects were analyzed using the following antibodies: CD27-PE, CD28-FITC, CD11a-PE (Immunotech), CD45RA-ECD, CD11b-PE (Beckman-Coulter), CCR7-FITC (R&D systems), CD56-PE, CD57-FITC, CCR5-PECy5, perforin-FITC, granzyme A-PE and granzyme B-Alexa Fluor 647 (BD Bioscience). For intracellular staining cells were made permeable with Perm-2 solution (BD Bioscience).

TCRVβ families were analyzed in control $CD3^+CD4^+$ and $CD3^+CD8^+$ cells and patients' $CD3^+CD4^+$ and DN T cells, using TCR Vβ antibodies corresponding to 24 different specificities (about 70% coverage of the normal human TCR Vβ repertoire) (Beckman-Coulter).

Samples were acquired using a flow cytometer (Cytomics FC 500, Beckman-Coulter).

Quantification of cytomegalovirus-specific T cells

Peptide-specific T cells from patients (HLA-A*0201) were quantified using the HLA-A*0201-NLV (NLVPMVATV, HCMV pp65 495–504) MHC class I pentamers (ProImmune). Pentamer binding

was visualized using anti-Pentamer PE and cells were co-stained with CD3-FITC (clone SK7, BD Bioscience) and CD4-APC (clone SK3, BD Bioscience). Viral-specific HLA-I-restricted responses were analyzed in $CD3^+CD4^-$ cells.

Quantification of signal joint T-cell receptor excision circles

TREC levels in $CD3^+CD4^+$ T populations from controls and patients, $CD3^+CD8^+$ T cells from controls and DN T cells from patients were analyzed by real-time quantitative PCR (qPCR) in a LightCycler system (Roche Diagnostic GmbH). A standard curve was constructed using a plasmid that includes a 375 base pair fragment of the sjTREC sequence.[17] All samples were analyzed in duplicate and the mean values were used for data analysis.

Proliferation assays

Total peripheral blood mononuclear cells from P1 and three normal controls were obtained by density gradient centrifugation. Cells were labeled with 1 μM CFSE (Invitrogen) and cultured with phytohemagglutinin at 5 and 10 μg/mL (Sigma Aldrich), phytohemagglutinin 5 μg/mL and interleukin-2 at 30 U/mL (Roche) and ConA at 10 μg/mL (Sigma Aldrich) for 5 days. Proliferation was analyzed by flow cytometric measurement of CFSE dilution in gated $CD4^+$ T cells and $CD8^+$ or DN T cells using CD3-PerCP, CD4-APC and CD8-PCy7 monoclonal antibodies (BD Bioscience). Samples were acquired in a FACSCanto II flow cytometer (BD Bioscience).

Generation of alloantigen-specific cytotoxic T lymphocytes and cytotoxicity assays

Peripheral blood mononuclear cells were cultured at a density of 2×10^6 cells/mL in RPMI 1640 plus 10% fetal calf serum, 2 mM L-glutamine and 50 U/mL penicillin. Alloantigen-specific cytotoxic T lymphocytes were generated by co-culturing those peripheral blood mononuclear cells with mitomycin-treated (2 h at 25 μg/mL) R69-LCL cells at a ratio 1:0.7 for 6 days in the presence of 30 IU/mL of interleukin-2. The HLA antigens expressed by the R69 LCL[18] are not shared by patients or controls, leading to the generation of a comparable anti-allogenic cytotoxic T lymphocyte response in every case. R69 cells are not sensitive to anti-Fas monoclonal antibody-induced apoptosis, so cytotoxicity should be due to the perforin/granzymes system. $CD8^+$ or DN cells were isolated by negative selection in a MACS. Cytotoxic function was tested on [H]-thymidine-labeled R69 cells.[19] Effector:target mixtures were cultured for 6 h at ratios ranging from 25:1 to 1:1. The specificity of the anti-allogenic cytotoxic T lymphocyte response was confirmed by the absence of lysis of the murine cell line L1210 or human erythroleukemia K562. Cells bound to the columns were also obtained, and natural killer cell cytotoxicity was tested on K562 cells using the same method. The percentage of specific lysis was calculated in all cases by the formula: % specific lysis = [(experimental release – spontaneous release) / (maximum release - spontaneous release)] x 100.

Quantification of interleukin-7 in plasma samples

Plasma interleukin-7 levels were measured in P2 and healthy controls using a high sensitivity enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D System).

Plasmid constructs and transfection in 293T cells

Human *CD8A* cDNA (CD8Awt) was PCR-amplified from a fetal thymus cDNA library using primers containing EcoRI and BamHI restriction sites. The purified PCR product was digested and ligated in pEYFP-N1 (also digested with EcoR1 and BamH1) with T4 DNA-ligase (New England Biolabs). Correct insertion of CD8Awt was checked by direct sequencing. CD8Amut was generated by site-directed mutagenesis (GeneTailor Site Directed Mutagenesis System, Invitrogen) in 100 ng of plasmid DNA carrying CD8Awt. The transformation reaction was performed in competent *E. coli* and CD8Amut was directly sequenced to check for correct mutagenesis. Human cell lines 293T or Hep-2 were transfected with pEYFP-CD8Awt or pEYFP-CD8Amut plasmids with FuGENE (Roche Diagnostic GmbH). The constructs were also co-transfected into cell lines together with plasmids expressing specific proteins for Golgi (pECFP-Golgi), endoplasmic reticulum (pEYFP-ER) and endosomes (pEGFP-ENDO) (Clontech). Expression was observed by fluorescent microscope and co-localization was analyzed using Image J software (NIH).

Statistical analysis

The Student's t test was used to compare differences between mean values of patients and controls. Statistical computations were performed with SPSS 12.0 software.

Results

Patients showed a high percentage of $CD4^-CD8^-TCR\alpha\beta^+$ T cells and did not have CD8 expression detectable by flow cytometry

Flow cytometric analysis in both patients (P1 and P2) showed a complete lack of $CD8^+$ T cells in peripheral blood, as previously reported (Figure 1A). Furthermore, the mean fluorescence intensity of CD8α expression on the cell surface was diminished in parents and siblings heterozygous for the Gly111Ser mutation. $CD4^-CD8^-$ cells were enumerated in the $TCR\alpha\beta^+$ T-cell population to exclude contamination by $TCR\gamma\delta^+$ T cells, which are mainly $CD3^+CD4^-CD8^-$. Both patients showed a high percentage of $CD4^-CD8^-TCR\alpha\beta^+$ T cells (DN T cells) (P1= 7-10%, P2=10-16%; the reference range for DN T cells in healthy controls is 0.1-2%; the reference range for CD8 T cells in healthy controls is 13-31%). Other subpopulations of peripheral blood lymphocytes from P1 and P2 showed no essential differences from those from healthy controls.

CD8αmut protein is retained in the cytoplasm of transfected cells

We speculated that the CD8αmut protein could be expressed in an abnormal conformation that impairs expression on the cell surface. We transfected 293T or Hep-2 cells with CD8Awt-pEYFP and CD8Amut-pEYFP plasmid constructs, and analyzed them by fluorescence microscopy. Whereas a diffuse, cytoplasmatic pattern was observed in cells transfected with CD8Awt-pEYFP, the fluorescence accumulated in clusters in most of the CD8Amut-pEYFP-transfected cells (Figure 1B). Diffuse fluorescence in CD8Awt-pEYFP-transfected cells allowed the visualization of multiple cytoplasmic prolongations, whereas these were barely visible in cells transfected with CD8Amut-pEYFP. This suggests that the mutation may cause a conformational change that alters protein trafficking through the cytoplasm.

To test whether the mutated protein was preferentially retained in endoplasmic reticulum, Golgi apparatus or endosomes, we co-transfected Hep2 cells with a mixture of either CD8Awt-pERFP or CD8Amut-pERFP plasmids together with plasmids carrying fluorescent probes for the different organelles. We did not observe a specific co-local-

ization of the mutated protein together with any of the mentioned subcellular structures (Figure 1C).

The predominant immunophenotype found in double negative T cells is suggestive of effector and effector memory cytotoxic cells

Analysis of the phenotype of patients' DN T cells revealed that the naïve subset (defined as $CD45RA^{+}$ $CD27^{bright}CD28^{+}$ and $CD27^{bright}CD45RA^{+}CCR7^{+}$) was profoundly decreased as compared to $CD8^{+}$ T cells from controls [P1=8.5±0.7 (mean±SD), P2=4.5±0.7, controls = 39.3±13.6] (Figure 2 and Table 1). The DN T cells predominantly showed an effector phenotype ($CD45RA^{+/-}$ $CD27^{low/-}CD28^{-}$) in P1, and an effector memory phenotype ($CD27^{+}CD45RA^{-}CCR7^{-}$) in P2.

We measured the expression of other co-stimulatory molecules (CD56 and CD57), adhesion molecules (CD11a and CD11b) and chemokine receptors (CD62L and CCR5) in the DN T population. The results obtained confirmed the phenotype. Effector DN T cells in P1 were predominantly $CD62L^{-}CCR5^{+/-}CD57^{+/-}CD11a^{high}CD11b^{+}$, while memory effector DN T cells in P2 were predominantly $CCR5^{+}$ $CD57^{-}$ $CD11a^{high}CD11b^{+/-}$ (*Online Supplementary Figure S1*).

Consistent with these findings, a considerable number of P1 DN T cells expressed substantial amounts of the cytotoxic mediators perforin and granzymes (47.5% of DN cells were positive for granzyme A, 23.6% for granzyme B and 16.2% for perforin), whereas the cellular population negative for the three cytolytic-effector molecules ($GraA^{-}GraB^{-}Per^{-}$) was the predominant one in control CD8 T cells (76%). The expressions of these cytotoxic mediators in DN P2 cells were similar to those in controls (15.6% of DN cells were positive for granzyme A, 12.2% were positive for granzyme B and 11.7% were positive for perforin).

Defects in the expansion and cytotoxicity of double negative cells in patients

We observed that while control $CD3^{+}CD4^{-}CD8^{+}$ T cells expanded during 6 days of culture in the presence of interleukin-2 and mitomycin-treated R69 cells, the patients' $CD3^{+}CD4^{-}CD8^{-}$ T cells did not expand, and rather retracted. The proliferative capacity of $CD4^{-}CD8^{-}$ T cells was formally tested in a CFSE-dilution experiment. Results obtained confirmed the previous observation: after 5 days

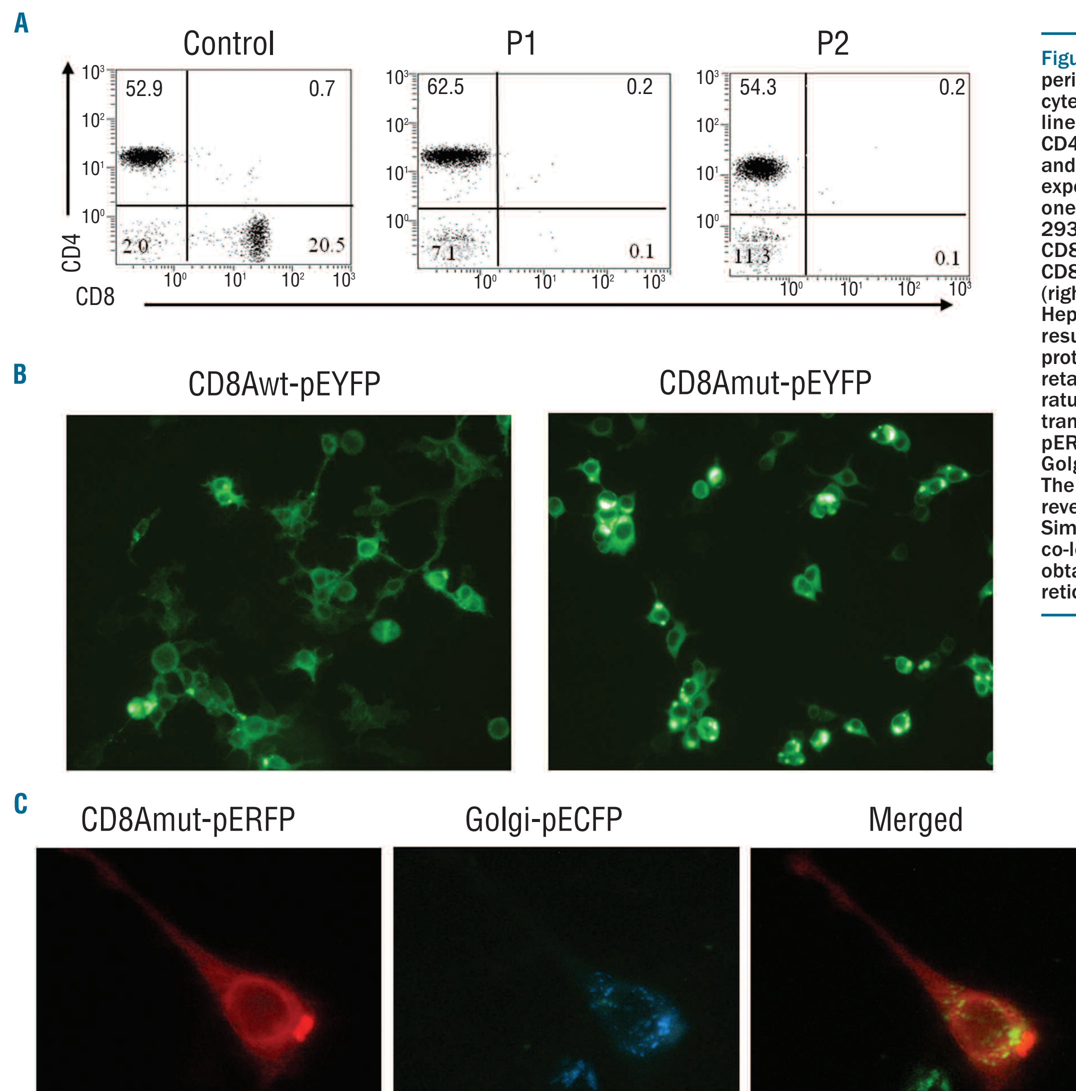
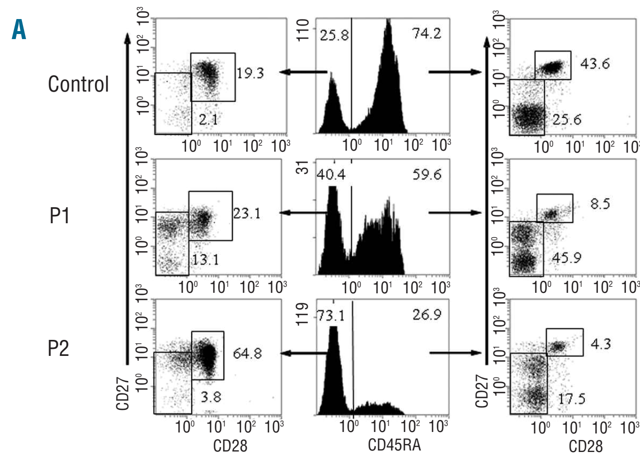
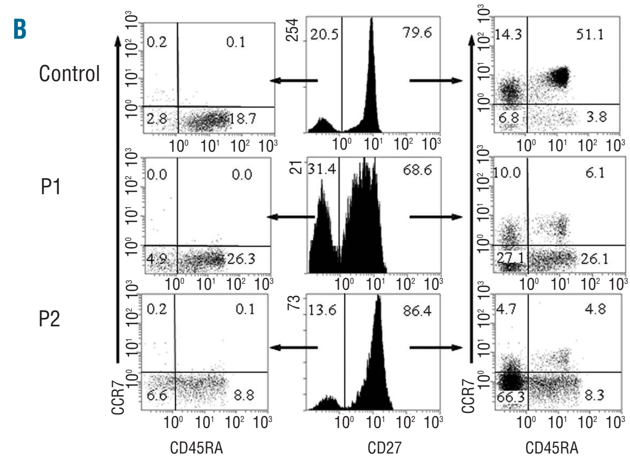


Figure 1. CD8 expression in peripheral blood T lymphocytes and transfected cell lines. (A) Representative CD4, CD8 evaluation in P1 and P2 (one out of four experiments is shown) and one healthy control. (B) 293T cells transfected with CD8Awt-pEYFP (left) or CD8Amut-pEYFP construct (right). Transfection of the Hep-2 cell line gave similar results. (C) The mutated protein (CD8Amut) is not retained in the Golgi apparatus. Hep2 cells were co-transfected with CD8Amut-pERFP (left) and pECFP-Golgi construct (central). The merge results (right) revealed no colocalization. Similar images showing no co-localization were obtained for endoplasmic reticulum or endosomes.





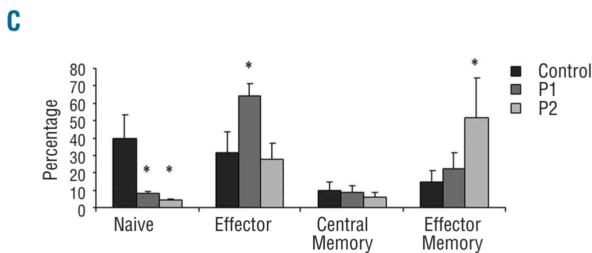


Figure 2. Flow cytometry analysis of isolated DN T cells from patients *versus* control CD8$^+$ T cells. (A) CD27/CD28 expression, in gated CD45RA$^+$ and CD45RA$^-$ cells. (B) CD45RA/CCR7 expression in gated CD27$^+$ and CD27$^-$ cells. (C) Significant differences between control CD8$^+$ *versus* and patients DN T cell subpopulations. Subpopulations defined as: naive (CD45RA$^+$CD27$^+$CD28$^+$), effectors (CD45RA$^{+/-}$CD27$^{low/-}$CD28$^-$), central memory (CD27$^+$CD45RA$^-$CCR7$^+$) and effector memory (CD27$^+$CD45RA$^-$CCR7$^-$). Results are the mean ± SD of three analyses.

of culture, patients' DN cells accounted for 2% of all enumerated cells, while control CD8$^+$ T cells accounted for 30-36%. P1 DN cells proliferated less than P1 CD4$^+$ T cells (48% *versus* 86%) and less than control CD8$^+$ T cells (80-90%) in a culture stimulated with phytohemogglutinin 5 μg/mL (Figure 3A). Similar results were obtained for other mitogens used.

After 6 days of culture, negatively isolated CD8$^+$ T cells were analyzed for CD3 and CD8 expression by flow cytometry. Almost 99% of this isolated population was CD3$^+$CD8$^+$ in controls, while it was CD3$^+$CD8$^-$ in patients, demonstrating that CD8 expression was not induced in patients' T cells during the *in vitro* culture. Although the number of cytotoxic T lymphocytes was clearly diminished in the peripheral blood mononuclear cell culture obtained from the patients, these isolated DN cytotoxic T lymphocytes were tested on R69 LCL at the same effector to target ratio as that for CD8$^+$ cytotoxic T lymphocytes obtained from controls. P1 cytotoxicity analyzed at 25:1 and 10:1 effector:target ratios was high and very similar to control levels, with cytotoxicity still not titrating down. Because of sample limitations, such ratios could not be tested in P2. However, at effector:target ratios equal to or lower than 5:1 for P1 and of 2:1 and 0.5:1 for P2, the cytotoxicity was significantly lower in patients than in controls, suggesting that in limiting conditions, the cytotoxicity is affected by the absence of CD8 (Figure 3B).

Similar amounts of natural killer cells were obtained after culture from both controls and patients (around 20% of CD56$^+$CD3$^-$ cells). When these populations were tested against the classical human natural killer cell target K562, at an effector:target ratio of 150:1, the cytotoxicity was very similar in all cases, correlating with a similar and high percentage of intracellular perforin expression in CD56$^+$ cells.

Table 1. Phenotypic characteristics in control CD8$^+$ T cells and in patients' DN T cells.

Subset	Control (n=4)	Patient 1	p1*[1]	Patient 2	p2*[2]
Naïve CD45RA$^+$CD27$^+$CD28$^+$	39.3±13.6	8.5±0.7	0.02	4.5±0.7	0.015
Effector CD45RA$^{+/-}$CD27$^{low/-}$CD28$^-$	31.2±12.5	63.9±7.4	0.02	28.1±9.5	0.48
Central memory CD27$^+$ CD45RA$^-$CCR7$^+$	9.8±5.1	8.7±3.7	0.55	6.3±2.6	0.33
Effector memory CD27$^+$ CD45RA$^-$CCR7$^-$	14.3±7.2	22.5±9.1	0.40	52.1±22.3	0.02

[1]p1: Significance for patient 1 versus *controls.* *[2]p2: Significance for patient 2* versus *controls*

We then explored the presence of antigen-specific MHC class I-restricted T cells *ex vivo* in the patients. As both P1 and P2 showed elevated antibody titers against cytomegalovirus and bear the HLA-A*0201 allele, a fluorescent HLA-A*0201 pentamer binding a cytomegalovirus peptide was used for the assessment of viral-specific CD3$^+$DN T cells. Cell frequencies were compared to those of positive controls by using peripheral blood mononuclear cells from HLA-A*0201 healthy individuals with high titers of antibodies against cytomegalovirus. Because CD8 could not be detected in the patients, peripheral blood mononuclear cells from patients and controls were stained with CD3 and CD4 monoclonal antibodies in combination with the pentamers. In controls, 1.4±0.8% (mean±SD) of CD3$^+$CD4$^-$ cells were found to be positive for cytomegalovirus-pentamer staining, whereas pentamer-positive cells represented 1.1% and 0.4% of CD3$^+$CD4$^-$ cells in P1 and P2, respectively (Figure 3C).

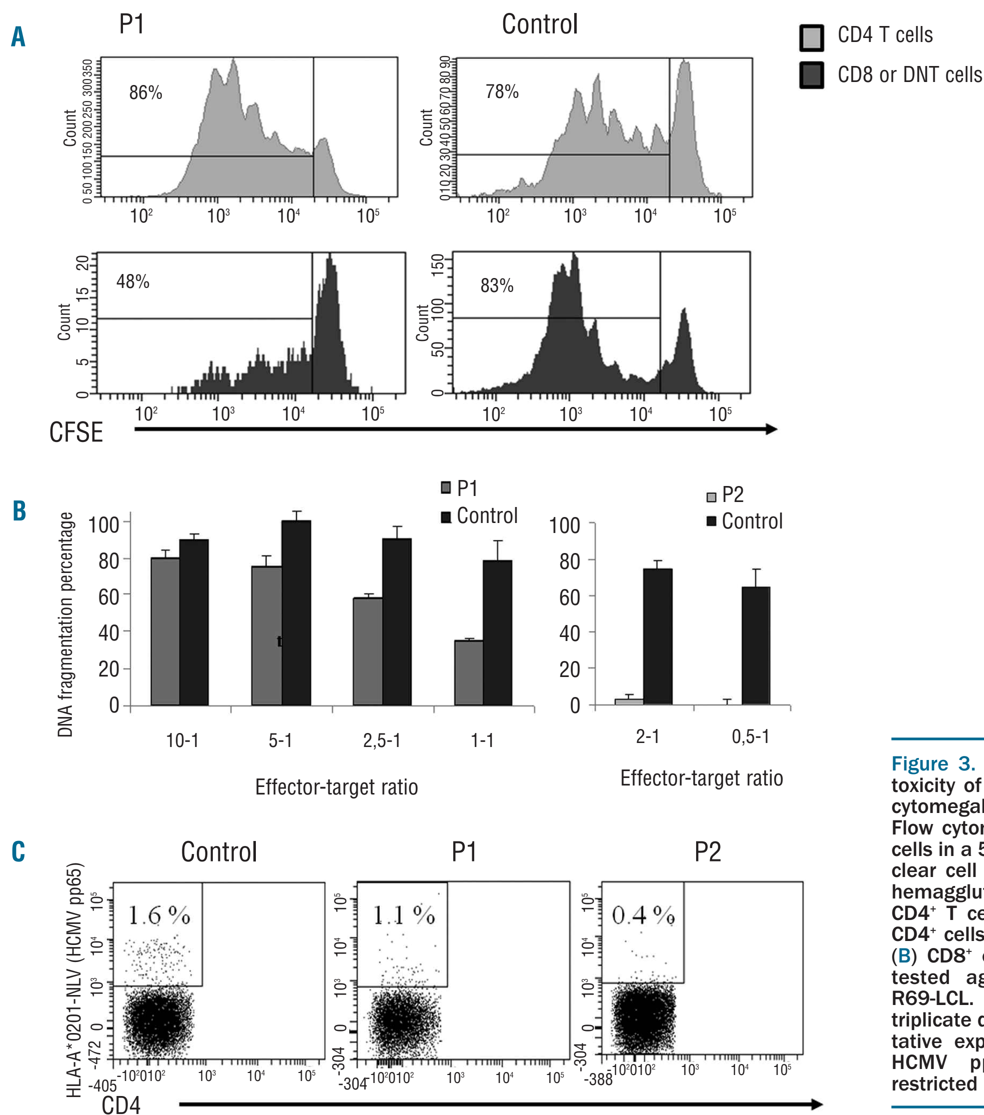


Figure 3. *In vitro* **proliferation and cytotoxicity of patients' DN cells and** *ex vivo* **cytomegalovirus specific response. (A) Flow cytometry analysis of CFSE-labeled cells in a 5 day peripheral blood mononuclear cell culture stimulated with phytohemagglutin 5 μg/mL. P1 DN cells and CD4⁺ T cells** *versus* **CD8⁺ T cells and T CD4⁺ cells from a representative control. (B) CD8⁺ or DN T-cell cytotoxic capacity tested against ^{3}[H] thymidine-labeled R69-LCL. Results are the mean±SD of triplicate determinations in one representative experiment of three. (C)** *Ex vivo* **HCMV pp65 antigen-specific MHC-I restricted response.**

Double negative cells from patients show low levels of T-cell receptor excision circles and a skewed Vβ repertoire

Quantification of TREC in T cells is considered a measure of recent thymic emigrants, and consequently, naïve T cells show higher TREC contents.[20] Because most DN cells from patients showed an effector or memory-effector phenotype with low numbers of naïve T cells, we hypothesized that the CD8α Gly111Ser mutation mainly affects the generation of naïve cells and the DN population would present with a lower TREC content than that of normal CD8⁺ cells. We measured TREC concentrations in the DN cell populations from patients and compared them to TREC levels in the patients' CD4⁺ cells and the control subjects' CD8⁺ cells. TREC numbers in patients' CD4⁺ T cells were similar to those in their control counterparts. However, consistent with the phenotype profile, the number of TREC was lower in DN T cells than in control CD8⁺ T cells (Figure 4A).

Next, analysis of the TCR Vβ repertoire was performed independently in the CD4⁺ and DN cells from both patients. The distribution of the 24 evaluated Vβ families was similar in CD3⁺CD4⁺ lymphocytes of patients and normal controls (Figure 4B). However, the analysis of the TCR Vβ repertoire in DN T cells from both patients showed different expansions and contractions *versus* the TCR Vβ repertoire in control CD8⁺ T cells (Figure 4C). Some Vβ families were virtually undetectable (Vβ-2, Vβ-4, Vβ-5.1, Vβ-5.2, Vβ-7.1, Vβ-13.6, Vβ-17, Vβ–22 and Vβ-23 in P1, and Vβ-5.2, Vβ-13.1, Vβ-18, Vβ-22 in P2), whereas others were over-represented, such as Vβ–14 in P1 and Vβ-3 in P2. These observations are consistent with a skewed repertoire of TCR Vβ families in the DN T-cell population from patients.

Finally, quantification of serum interleukin-7 revealed significantly higher levels in patient P2 (25.9 pg/mL) than in normal controls [2.2 pg/mL; 0.66-9.24 pg/mL (mean; range)].

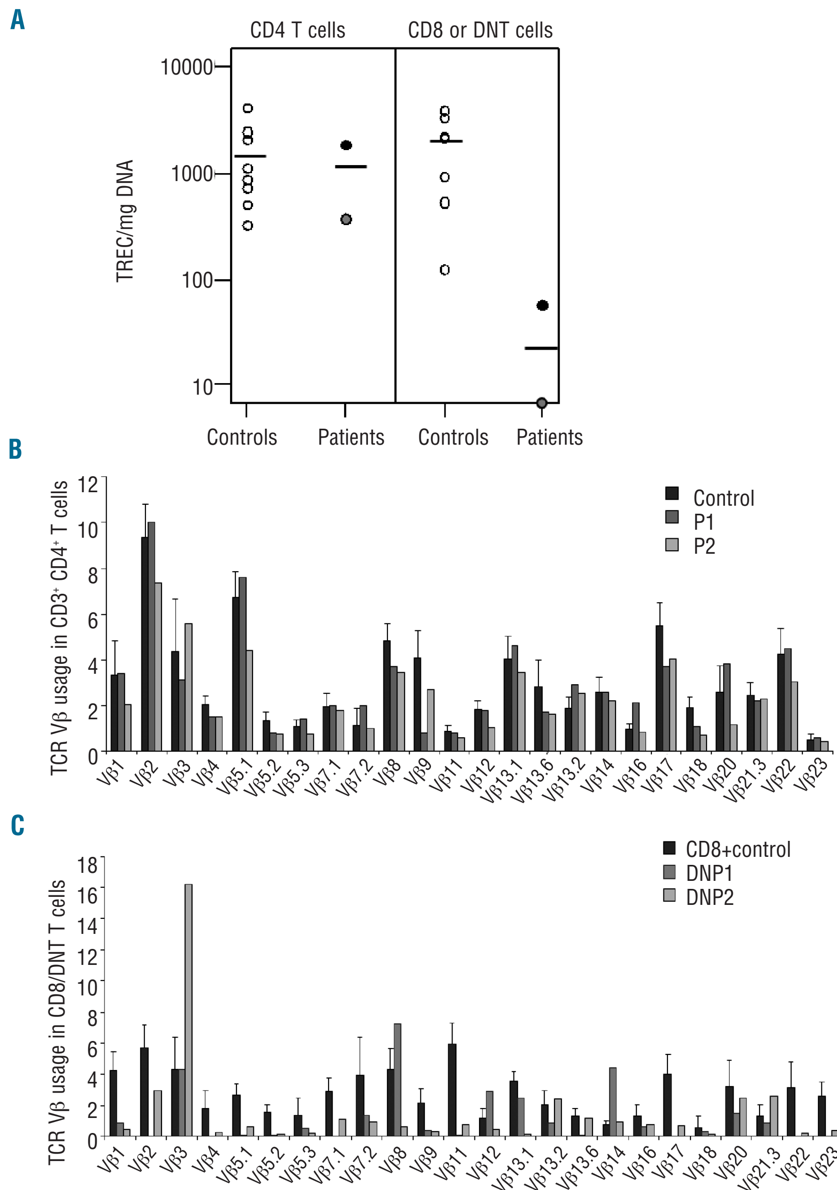


Figure 4. Analysis of TREC and TCR Vβ repertoire in patients' DN T cells. (A) Patients CD4+ and DN T cells and control CD4+ and CD8+ T cells were magnetically sorted, and TRECs levels were analyzed by qPCR. (B) TCR Vβ families were analyzed by flow cytometry in gated CD3+CD4+ T cells from controls and patients. (C) TCR Vβ families were analyzed by flow cytometry in gated CD3+CD8+ T cells from controls or CD3+CD4-CD8- DN T cells from patients.

Discussion

Individuals with absence of CD8+ lymphocytes due to the Gly111Ser mutation in CD8α have an increased population of peripheral αβ DN T cells. Increased frequencies of TCRαβ+CD4-CD8- DN cells are a hallmark of autoimmune lymphoproliferative syndrome. The origin of DN cells in this syndrome is controversial as they are claimed to be clonally related to CD8+ T cells or unrelated to CD4+ or CD8+ single positive cells. DN cells in patients with autoimmune lymphoproliferative syndrome are highly proliferative, reaching up to 40% of peripheral blood T cells, and exhibit an unusual phenotype that includes the expression of the CD45RA isoform.[21-23] We hypothesized that αβ DN T cells in CD8-deficient patients correspond to the "normal" CD8+ population if CD8 were expressed and that they are committed to the cytotoxic T lymphocyte lineage. This is supported by several observations. First, the DN T-cell population has a phenotype associated with cytotoxic function: effector (CD45RA+/- CD27low/- CD28-) with high expression of cytotoxic mediators in P1, and memory effector (CD27+ CD45RA- CCR7-) in P2. Secondly, we have demonstrated that, in an anti-allogeneic response, cytotoxicity is mostly conserved, and the absence of CD8 expression in patients impairs the cytotoxic capacity of the DN T cells to a moderate extent. This is in agreement with the normal cytotoxicity responses observed in CD8 knockout mice[24-26] and is shown in humans for the first time in the present work. Third, the high antibody titers to several viral infections in the patients[15] demonstrate that they have been in contact with those viruses and have been immunocompetent enough to beat the infections.

Another important observation in CD8-deficient patients is that the DN T-cell population is in a cytopenic state in comparison to the CD8+ T cells of healthy subjects, as the number and percentage of DN T cells in peripheral blood of patients are lower than those of CD8+ T cells in controls. In addition, analysis of the DN T-cell phenotype showed a scarcity of naïve cells in comparison

to controls. Studies in CD8α-chain deficient mice have shown that the population of DN T cells is small and about 10-fold reduced compared with the corresponding population of DN T cells in CD4-deficient mice. These results indicate that the overall efficiency of positive selection in the absence of CD4 or CD8 is asymmetric and heavily biased toward the helper phenotype.[27] Data from *CD8B* or *CD8A* knock-out mice indicate that the TCR repertoire of the DN cells is somewhat more limited than in the normal CD8+ population because of altered thymic development.[27,28] In our patients, analysis of Vβ families showed a limited and skewed TCR repertoire in DN T cells in comparison to that in patients' CD4+ cells or control CD8+ cells. This may suggest that, in the absence of CD8 co-receptor, the thymic positive selection functions suboptimally and only a limited number of mature T cell clones would emerge from the thymus. Once in the periphery, the clones would expand under the effect of homeostatic cytokines and antigenic stimulation and would acquire an effector or memory effector phenotype. On the other hand, the circulating DN cellular populations observed might be a consequence of peripheral selection favoring a restricted repertoire of effector or memory effector DN T cells. The low number of TREC recorded in patients' DN T cells in comparison with the number in patients' CD4+ cells or control CD8+ T cells would further support the proliferation of that population in the periphery together with a relative inefficiency of the thymus to refill the naïve compartment.[20] The large amounts of circulating interleukin-7 found in P2 would reflect a compensatory attempt to boost the expansion of the T cells and the thymic regenerative capacity to overcome the depletion of peripheral T cells.

A fraction of the CD3+CD4- T cells from the patients was found to be positive for cytomegalovirus-pentamer staining. HLA class-I multimers detect T cells directed against viral, tumor, and transplantation antigens with exquisite sensitivity. These results show that cytomegalovirus-specific T cells could be generated in the absence of CD8, although at a lower grade compared with in healthy individuals.

The present results also demonstrate that although cytotoxicity is mostly preserved in the anti-allogeneic response, the absence of CD8 expression in the mutant circulating DN cells affects the *in vitro* expansion of this population. This indicates that the main role of the CD8 module in the experimental setting designed is to transmit signals leading to proliferation. These data are in agreement with previous results indicating that the proliferation of naïve CD8 T cells is associated with signal transduction through the CD8/lck module.[29] It has been shown that lck deficiency induced in naïve mice abrogates the antigen-specific activation and clonal expansion of CD8+ T cells during a primary response to acute viral infections.[30] Moreover, the magnitude of primary CD8 T-cell expansion depended on the duration of lck-dependent TCR signaling. However, lck was dispensable for enhanced functional avidity, maintenance, and reactivation of memory CD8+ T cells *in vitro* and *in vivo*.[31] Alternatively, the absence of *in vitro* proliferation of the DN cells may suggest senescence of this population because of previous *in vivo* expansions driven by natural contact with antigenic stimuli.

CD8 chains are first expressed and used by T cells early during their intrathymic development to increase the likelihood of being rescued in the event that their TCR has low affinity for MHC-I. CD8α is also expressed by human monocytes and enhances FcγR-dependent responses.[32] However, in CD8 deficiency T-cell clones that fulfill the functional requirements for intrathymic survival display aberrant phenotypes in the periphery (DN T cells), but seem to be functional. It is, therefore, possible that in human CD8 immunodeficiency the DN cells are in fact MHC class I-restricted, with cytolytic function.

Authorship and Disclosures

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