Natural killer cell receptor expression reflects the role of human cytomegalovirus in the pathogenesis of a subset of CD4⁺ T-cell large granular lymphocytosis

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A B S T R A C T
A high frequency of CD4⁺ T-cell large granular lymphocyte (T-LGL) lymphocytosis occurs in human leukocyte antigen (HLA)–DRB1*0701 individuals displaying monoclonal expansions of Vβ13.1⁺ CD4⁺ T-cell clones, which specifically respond to human cytomegalovirus (HCMV) antigens. We previously reported the expression of natural killer (NK)–cell associated receptors (NKR) by HCMV-specific cytolytic CD4⁺ T cells from healthy donors. In the present study a high expression of different NKR (i.e., NKG2D, killer Ig-like receptors (KIR), CD94, ILT2) was observed in CD4⁺ T cells from both Vβ13.1⁻ and Vβ13.1⁺ CD4⁺ T-LGL cases. Remarkably, elevated numbers of CD94/NKG2C⁺ NK cells, previously shown to expand in association to HCMV infection, were preferentially found in Vβ13.1⁺ T-LGL, further supporting its role in the pathogenesis of a subset of CD4⁺ T-LGL.

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1. Introduction
T-cell large granular lymphocyte (T-LGL) lymphocytosis are characterized by the expansion of monoclonal cytolytic T cells in peripheral blood. CD8⁺ T-LGL are more frequent than CD4⁺ T-LGL and often display a memory/effector phenotype (CD45RA mary antigenic stimulus driving Vβ (NKR), also detectable in subsets of peripheral blood CD8⁺ T cells [4] in healthy individuals. By contrast, NKR peripheral blood. CD8 characterize by the expansion of monoclonal cytolytic T cells in rare cases [3]. Altogether, these results strongly suggest that HCMV provides a primary antigenic stimulus driving Vβ13.1⁺CD4⁺ T-LGL lymphocytosis.

CD8⁺ T-LGL were reported to express NK-cell–associated receptors (NKR), also detectable in subsets of peripheral blood CD8⁺ T cells [4] in healthy individuals. By contrast, NKR⁺ CD4⁺ T cells are rarely found in healthy donors and have been associated with pathologic conditions such as rheumatoid arthritis, cancer, and celiac disease. We reported that HCMV-specific CD4⁺ T cells from healthy donors express different NKR (i.e., NKG2D, ILT2 and killer Ig-like receptors (KIR)) [5].

In the present study, we assessed the expression of NKR in Vβ13.1⁺ (n = 5) and Vβ13 – (n = 4) CD4⁺ T-LGL cases, analyzing in parallel the frequency of CD94/NKG2C⁺ NK cells, a subset reported to expand in association to HCMV infection.

2. Subjects and methods
2.1. Subjects
Peripheral blood mononuclear cells (PBMC) from CD4⁺ T-LGL patients (n = 9) were obtained as previously described [5] from the Haematology Service of the Hospital Universitario Virgen de las...
Nieves (Granada, Spain), and from the Department of Immunology of the Erasmus Medical Center (Rotterdam, the Netherlands). Peripheral blood samples from healthy donors were obtained at Institut Municipal d’Investigació Mèdica-IMIM (Barcelona, Spain). Written informed consent was obtained from every donor, as approved by the corresponding Ethics Committees.

2.2. Immunofluorescence analysis and polymerase chain reaction

The panel of monoclonal antibodies (mAbs) against the different NKR has been previously reported [5,6]. KIR expression was assessed using a mixture of anti-KIR2DL1/S1/S3, anti-KIR3DL1, anti-KIR2DL2/S2/L3, anti-KIR3DL1/L2, and KIR2DS4 antibodies. Indirect immunofluorescence analysis was carried out with allophycocyanin-labeled goat anti-mouse Ig (BD Biosciences Pharmingen). Anti-NKG2C (MAb1381) and anti-NKG2C-PE mAb were from R&D Systems (Minneapolis, MN). Anti-CD4-FITC, anti-CD3-PerCP, and anti-CD56-allophycocyanin were from BD Bioscience Pharmingen. Anti-Vβ13.1-PE was from Immunotech. Immunophenotypic analysis was performed using samples of whole fresh blood or cryopreserved PBMC as described [7]. Immunofluorescence staining and flow cytometry analysis were performed according to the protocols previously described [5].

The NKG2C gene sequence between intron 1 and exon 3 was amplified from total genomic DNA as previously described [7].

![Fig. 1](image-url)

**Fig. 1.** NKR expression in Vβ13.1+ and Vβ13.1-CD4+ T LGL. (A and B) PBMC from healthy donors and patients undergoing CD4+ T LGL lymphocytosis were stained for NKG2D, ILT2, KIR and CD94 in combination with CD4 mAb. (A) NKR expression on CD4+ T cells from representative examples of a patient and healthy control are compared. CD4+ CD94+ T cells were in every case NKG2A– NKG2C+. (B) NKR expression in individual CD4+ T LGL cases. (C) NKR distribution was assessed in Vβ13.1+ T-LGL cases gating on the Vβ13.1+ and Vβ13.1-CD4+ T cells. A representative example corresponding to patient 5 is shown. (D) NKG2C expression in CD3-CD56+ NK cells from Vβ13.1+ and Vβ13.1-CD4+ T LGL.
3. Results and discussion

Monoclonal expansions of CD4+ T LGL have been reported to display a common phenotype (TCRβ+, CD4+, CD8−/dim, CD57+), with cytotoxic capacity (perforin and granzyme B+) and effector/memory markers (CD2+ bright, CD7+ dim, CD11a+ bright, CD28−, CD62L−, HLA-DR+) [1,8]. We analyzed the expression of NGK2D, ILT2, KIR and CD94 in peripheral blood CD4+ T cells of patients diagnosed with CD4+ T-LGL lymphoproliferation (age range, 51–68 years; mean ± SD 59.1 ± 6.4 years) and healthy donors (n = 15, age range 23–61 years; mean ± SD = 44.1 ± 10.5 years). Five cases were HLA-DRB1*0701+ and presented an expansion of Vβ13.1+CD4+ T cells, shown to specifically respond to a peptide from the gB HCMV antigen presented by HLA-DR*0701 [2,3] (Table 1). Expression of CD56 was also studied, as it has been reported to be often associated to CD4+ T-cell expansions in these patients [8]. Consistent with previous reports [5,9–11], samples from healthy donors contained low numbers of NKR+ CD4+ T cells (mean ± SD, range): NGK2D+ (0.9 ± 0.6, 0.1–1.9%); ILT2+ (1.6 ± 1.8, 0.1–5.6%); KIR+ (0.4 ± 0.5, 0.1–1.9%); and CD94+ (0.3 ± 0.4, 0.1–1%). By contrast, eight of the nine patients showed increased proportions of NK+ CD4+ T cells (Figs. 1A, 1B). The expression pattern of NGK2D, ILT2 and KIR varied between CD4+ LGL cases, as previously reported for in vitro expanded HCMV-specific CD4+ T cells from healthy donors [5]. Moreover, the dissociated distribution of the different KIR indicated that their expression in CD4+ T cells may be differentially regulated by still undefined mechanisms or, alternatively, may be stochastically acquired during differentiation. Whether some activating and/or inhibitory KIR might be preferentially displayed by T-LGL, particularly by the Vβ13+ subset, deserves attention. Yet, considering the genomic diversity of KIR haplotypes, gathering a much larger cohort of patients would be required to reliably address that question, as well as to assess the eventual clinical relevance of the KIR phenotype in CD4+ T-LGL.

NKR were detected in samples from both Vβ13.1+ and Vβ13.1-CD4+ T-LGL cases (Fig. 1B) and were preferentially associated with CD56 expression (not shown). Moreover, NKR were mainly detected on the Vβ13.1+ CD4+ T-cell subset (Fig. 1C), reported to specifically recognize the gB antigen. Although the specificity of Vβ13.1-CD4+ T LGL has not been defined, it is conceivable that they might recognize different antigens from HCMV or other pathogens. Increased proportions of CD49/NGK2C+ cells in peripheral blood have been associated with a positive serology for HCMV in healthy individuals [7], and human immunodeficiency virus-1 (HIV-1)-infected patients [12,13]. Moreover, NGK2C+ NK cells were shown to expand upon co-culture of PBMC with HCMV-infected fibroblasts [14]. Recently, a patient with a selective T-cell immunodeficiency was reported to display an NGK2C+ NK cell lymphoproliferosis in response to an acute HCMV infection [15]. Considering the specificity of Vβ13.1+ T cells for the gB HCMV antigen, it is conceivable that Vβ13.1+ T-cell expansions in T-LGL patients may be associated to an increased HCMV reactivation rate in these individuals. On that basis, the expression of NGK2C on NK cells was assessed in T-LGL patients. As shown in Fig. 1D), higher proportions of NGK2C+ NK cells were detected in samples from Vβ13.1+ patients compared with those from most Vβ13.1− cases (Fig. 1D) and HCMV+ healthy donors (n = 132, age range 35–79 years; mean ± SD: 55.4 ± 11.9 years). In healthy HCMV+ individuals, the mean proportion of NGK2C+ NK cells was (mean ± SD) 13.5% ± 14.75%, and only ~5% presented ≥45% NGK2C+ NK cells. Remarkably, three of four Vβ13.1+ patients studied presented ≥45% NGK2C+ NK cells (Fig. 1D); the fifth patient (patient 6) had a homozygous deletion of the NGK2C gene evidenced by polymerase chain reaction of genomic DNA (data not shown), found in ~4% healthy individuals [16]. The increased proportions of NGK2C+ NK cells are consistent with the pressure of HCMV infection underlying the development of Vβ13.1+ CD4+ T-LGL lymphoproliferosis. It is of note that a Vβ13.1-CD4+ T LGL case (patient 2) also displayed high numbers of peripheral blood NGK2C+ NK cells, suggesting that HCMV infection may have driven the CD4+ T-cell proliferation in that patient. Further studies in a larger cohort of CD4+ T LGL cases are required to assess whether an expansion of NGK2C+ NK cells may constitute a marker of the involvement of HCMV in the development of this lymphoproliferative disorder.

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