Clinicobiological, Immunophenotypic, and Molecular Characteristics of Monoclonal CD56\(^{-}\)/+\(^{\text{dim}}\) Chronic Natural Killer Cell Large Granular Lymphocytosis

Indolent natural killer (NK) cell lymphoproliferative disorders include a heterogeneous group of patients in whom persistent expansions of mature, typically CD56\(^{+}\), NK cells in the absence of any clonal marker are present in the peripheral blood. In the present study we report on the clinical, hematological, immunophenotypic, serological, and molecular features of a series of 26 patients with chronic large granular NK cell lymphocytosis, whose NK cells were either CD56\(^{-}\) or expressed very low levels of CD56 (CD56\(^{-}\)/+\(^{\text{dim}}\) NK cells), in the context of an aberrant activation-related phenotype. In that concerning the expression of killer receptors, CD56\(^{-}\)/+\(^{\text{dim}}\) NK cells showed bright and homogeneous CD94 expression, and dim and heterogeneous reactivity for CD161, whereas CD158a and NKB1 expression was variable. From the functional point of view, CD56\(^{-}\)/+\(^{\text{dim}}\) showed a typical Th1 pattern of cytokine production (interferon-\(\gamma\), tumor necrosis factor-\(\alpha\)). From the clinical point of view, these patients usually had an indolent clinical course, progression into a massive lymphocytosis with lung infiltration leading to death being observed in only one case. Despite this, they frequently had associated cytopenias as well as neoplastic diseases and/or viral infections. In summary, we describe a unique and homogeneous group of monoclonal chronic large granular NK cell lymphocytosis with an aberrant activation-related CD56\(^{-}\)/+\(^{\text{dim}}\)/CD11b\(^{-}\)/+\(^{\text{dim}}\) phenotype and an indolent clinical course, whose main clinical features are related to concomitant diseases.

Indolent natural killer (NK) cell lymphoproliferative disorders (LPDs) are usually designated as chronic NK cell lymphocytosis (CNKCL) or chronic NK-large granular lymphocytes (LGL) LPD.\(^{1-6}\) CNKCLs are heterogeneous and they most probably include reactive chronic NK cell lymphocytosis, true chronic NK cell large granular lymphocyte (LGL) leukemia, and even some aggressive NK cell lymphoma/leukemia cases.\(^{7-9}\) because of difficulties in establishing the clonal nature of the NK cell proliferation.

Previous studies have shown that in most CNKCL cases the expanded population of NK cells co-expresses CD56, CD16, and CD94 and displays NK activity. Such phenotypic and functional features are similar to those...
observed in neoplastic NK cells from aggressive NK cell leukemia/lymphoma, nasal, and nasal-type lymphomas.10 Despite this, few cases of CNKCL and LGL leukemias of CD56−/+/dim NK cells have been sporadically described10−13 or used to establish NK cell lines.14 NK cells showing decreased or no expression of CD56 have not been reported either in healthy individuals or in disease conditions associated to either a transient or persistent increase in the number of NK cells in the peripheral blood (PB),15 in such cases, the expanded cell population constantly shows co-expression of CD16 and CD56 frequently in association with acute or chronic NK cell activation features. These findings may suggest that a CD56−/+/dim NK cell phenotype could be aberrant and reflect underlying clonal genetic abnormalities. However, to the best of our knowledge no study has been reported so far in which this unique group of CNKCL is studied in detail.

In contrast to both CD8+/TCRαβ+ and CD4+/TCRαβ+ T-cell LGL leukemias, in which the most characteristic clinical manifestations of the disease—such as its association with cytopenias and arthritis or second neoplasias—are well known,16−18 the clinical features associated with CNKCL including NK cell LGL leukemia, are far away from being well established. Previous studies have suggested a possible association of CNKCL with cytopenias, vasculitis, neutropenic or nonneutropenic fevers, BM granulomas,1,3,19,20 and even Epstein-Barr virus (EBV) infection in endemic areas.21,22

In the present study we report on the clinical, hematological, immunophenotypical, functional, serological, and molecular features of 26 patients with CNKCL in whom the expanded PB NK cells were either CD56− or expressed very low levels of surface CD56 (CD56−/+/dim) CNKCL in the context of an aberrant immunophenotype and a mature LGL morphological appearance.

Materials and Methods

Patients

A total of 26 consecutive patients with CNKCL whose LGL appearing NK cells were CD56−/+/dim, were studied at diagnosis in two different centers. These centers received cases referred for the diagnosis of T/NK chronic LPD from other hospitals in Portugal (Cytometry Unit of the Hematology Service at the Hospital Geral de Santo António, Porto, Portugal) and in Spain (Cytometry Service, University and University Hospital of Salamanca, Salamanca, Spain). Altogether, these CD56−/+/dim patients represented 20% of the total group of 130 CNKCL cases and 1% of a total of 2368 mature chronic LPDs diagnosed in both institutions between September 1997 and December 2002, from which 64% were B-cell chronic lymphocytic leukemias. Results obtained were compared to those observed on PB CD56+ NK cells from 12 age- and sex-matched healthy individuals, 15 patients with acute viral infection, and 15 patients with CD56+ chronic NK cell lymphocytosis associated with tumors or chronic infections whose immunophenotypic features have been previously published in detail.15,23

Immunophenotypic Studies

In all cases cell surface immunophenotypic studies were performed as previously described in detail.10 Stainings were performed using an ethylenediaminetetraacetic acid-anti-coagulated whole blood stain-lyse-and-then-wash method and a four-color panel of monoclonal antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, phycoerythrin-cyanine 5, or peridin chlorophyll protein and allophycocyanine directed against the following antigens: TCRαβ, TCRγδ, CD8, CD4, CD3, CD28, CD2, CD7, CD5, CD57, CD11c, CD38, CD11b, CD45RA, CD45RO, CD122, CD25, CD11a, HLA-DR, CD158a, CD161, CD16, NKBl, and CD94.23 In addition, the expression of cytoplasmic granzyme B was evaluated using the Fix and Perm reagent kit (Caltag Laboratories, Burlingame, CA), according to the manufacturer’s instructions.

Functional analysis of cytokine production by the expanded NK cells was also performed as previously described.18 Briefly, 500 μl of heparinized blood were placed into a tube containing 500 μl of RPMI 1640 culture medium (BioWhittaker, Walkersville, MD) supplemented with 2 μmol/L of L-glutamine. Cells were cultured for 4 hours at 37°C in a 5% CO2 and 95% humidity sterile environment in the presence of 25 ng/ml of phorbol-12-myristate 13-acetate, 1 μg/ml of ionomycin, and 10 μg/ml of brefeldin A (stimulated samples) or only brefeldin A (nonstimulated samples). Once this incubation period was completed, each sample was sequentially stained for the CD3 and CD19 surface antigens—and intracellular cytokines—interleukin (IL)-2, IL-4, IL-10, IL-13, interferon (IFN)−γ, tumor necrosis factor (TNF)−α, and TNF−β.18 In these studies NK cells were identified as CD19−, CD3− lymphocytes.

Data acquisition was performed in each of the two centers in FACSCalibur flow cytometers [Becton/Dickinson Biosciences (BDB), San Jose, CA], equipped with two lasers using the Cell QUEST software program (BDB). Information on a minimum of 2 × 105 events was acquired and stored as list mode data for each staining. For data analysis the Paint-A-Gate PRO software program (BDB) was used. Identification and enumeration of NK cells present in the sample was performed as follows: the percentage of NK cells from all PB nucleated cells and lymphocytes was calculated by selecting CD3− lymphoid cells that expressed CD2 and/or CD7; then, the normal and abnormal NK cell populations were identified according to the unique pattern of expression of the CD56, CD2, and CD7 antigens observed in normal NK cells15,23 and the proportion of abnormal CD56−/+/dim, normal CD56+, and normal CD56−/+/dim NK cells within total NK cells was determined. Afterward, the abnormal CD56−/+/dim NK cell population was characterized for the expression of the other cell surface antigens analyzed. For each antigen studied, the following characteristics were recorded: 1) percentage of positive cells; 2) intensity of expres-
sion—absent (−), low (−/+), moderate (+), high (++), very high (+++) evaluated as their mean fluorescence intensity (MFI) (arbitrary relative linear units scaled from 0 to 10,000 fluorescence channels) and; 3) pattern of antigen expression (homogeneous versus heterogeneous), evaluated by the coefficient of variation (CV).

Flow Cytometry DNA Cell Content Studies
DNA studies were performed in a subset of patients (n = 6). Briefly, PB cells were double stained for either surface CD16 or CD94 and nuclear DNA (DNA-Prep; Beckman-Coulter, Hialeah, FL) and acquired in an EPICS-XL-MCL flow cytometer using the XL2 software (Beckman-Coulter). Both the DNA ploidy status and the cell cycle distribution of PB NK cells were evaluated using the MultiCycle software (Phoenix Flow Systems, San Diego, CA).

Human Androgen Receptor Gene Assay (HUMARA)
To confirm the clonal origin of the aberrant NK cell population, the pattern of inactivation of chromosome X was studied in 4 of 15 female patients by assessing the methylation of human androgen receptor gene using the HUMARA polymerase chain reaction (PCR)-based assay. For that purpose, purified CD56dim NK cells (mean, 97 ± 2.6%; range, 93.6 to 99.6%) and the corresponding NK cell-depleted leukocyte fractions from the four female patients were studied in parallel. NK cells were purified by a single immunomagnetic depletion step using the Human NK Cell Isolation Kit II in the automated magnetic cell separator autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), strictly following the manufacturer’s recommendations. Digestion of genomic DNA with the methylation-sensitive restriction endonuclease HapII and subsequent PCR amplification of the methylated (inactivated) alleles was performed, as previously described.

Molecular Analysis of TCR Gene Rearrangements
Molecular analyses of TCR gene rearrangements were performed in most cases using either conventional Southern blot-based TCR-β gene (n = 2) or PCR-based TCR-γ gene (n = 12) techniques or both (n = 6). Briefly, DNA was extracted from mononuclear cells obtained by density gradient centrifugation, using the chloroform method. For Southern blot, DNA was digested with the EcoRI and HindIII restriction enzymes and DNA fragments were separated by a 0.8% agarose gel electrophoresis and transferred to nitrocellulose membranes by vacuum blotting, UV fixed, and hybridized with 32P-labeled probes for the TCR-β gene region (Cβ, TCRBC, and TCRBJ2; DAKO A/S, Glostrup, Denmark). PCR amplification of the TCR-γ gene was done using the BIOMED-2 primer sequences and protocols, with a limit of detection varying from 1 to 10%, depending on the polyclonal TCR repertoire complexity, and the type of TCR-γ gene rearrangements.

Serological and Molecular Studies for Viral Infection
Sera from nine patients were tested for the presence of IgM and IgG antibodies against cytomegalovirus (CMV), EBV viral capsid (EBV-VCA), and nuclear (EBNA) antigens, parvovirus, herpes simplex (HSV) type 1 and 2, varicella zoster (VZV), rubella, and hepatitis A, B, and C viruses. In addition, 13 patients were tested for the presence of antibodies against human immunodeficiency (HIV) and human T-cell leukemiy/lymphoma (HTLV) type I and type II viruses.

The presence of HTLV-1, CMV, EBV, HSV, VZV, and human herpesvirus type 6 (HHV-6) DNA sequences was also evaluated in nine patients by real-time PCR assays using DNA extracted from PB mononuclear cells with the MagNA Pure machine and the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Mannheim, Germany). Amplification of CMV, HSV, and HHV-6 DNA was done using an in-house developed real-time PCR assay with synthetic genetic primers and probes (Epoch, USA) in the SmartCycler system (Cepheid, USA) whereas EBV and VZV DNA were detected using commercially available kits (Artus, Hamburg, Germany) and the LightCycler System (Roche Diagnostics). HTLV-1 DNA was detected using an in-house developed real-time PCR assay with previously described primers and probes or the LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics).

Other Laboratory Parameters
Other laboratory parameters included a full hematological blood cell count, a morphological evaluation of blood smears, and a routine biochemical survey including serum levels of liver enzymes, creatinine, lactate dehydrogenase, β2-microglobulin, immunoglobulins (Igs), antinuclear antibodies, and rheumatoid factor.

Statistical Analyses
For all variables under study, median, mean, SD, and range values were calculated. To explore for the statistical significance of the differences observed between groups the Mann-Whitney U-test was used (SPSS software, version 11; SPSS, Chicago, IL). P values <0.05 were considered to be associated with statistical significance.

Results
Clinical and Laboratory Findings
Table 1 summarizes the clinical and hematological findings at diagnosis for the 26 patients studied. As may be seen in Table 1, all patients were adults more than 35 years of age and there was a slight predominance in females (male/female ratio of 0.73). In the great majority of the cases (18 of 26) lymphocytosis was an occasional finding in either a routine blood analysis (n = 9) or in
with HZV being confirmed with serological analysis in one of them. Thrombocytopenia was obtained in blood analyses performed because of another pathological condition (n = 2) or because of trauma (n = 1). At diagnosis, all except three patients were asymptomatic. In these three cases symptoms were related to cytopenias and/or an associated-disease. Physical examination (Table 1) showed the presence of splenomegaly, adenomegaly, and hepatomegaly in only a minor proportion of cases (15%). Only one patient suffered from arthritis, which was diagnosed as rheumatoid arthritis. Skin lesions were not found neither were fever or vasculitis.

Increased lactate dehydrogenase (mean ± SD, 484 ± 185 U/L; range, 208 to 856 U/L) and β₂-microglobulin (mean ± SD, 3.24 ± 1.61; range, 1.30 to 7.10) serum levels were observed in 50% and 80% of the cases, respectively. Ig serum levels were abnormal in 15 of 21 patients and consisted of increased IgM (n = 3), IgG (n = 7), IgA (n = 5), and/or IgE (n = 2); in addition, 5 patients had moderate IgA (n = 4) or IgG (n = 1) hypogammaglobulinemia. Serum rheumatoid factor was positive and serum levels of anti-nuclear antibodies were increased in 4 of 9 and 3 of 11 patients tested, respectively.

Studies aimed at detecting underlying viral infections confirmed the presence of HIV infection in three patients, being associated with HTLV1 infection in one of them. Hepatitis C virus infection was detected in three additional patients. Moreover, serological studies suggested past infection with CMV, EBV, and HSV-1 each in nine of nine cases tested, HSV-2 in three of seven cases, VZV in seven of eight patients studied, parvovirus in five of nine, and hepatitis B virus in two of nine cases tested. Serological data from a control group of 5853 outcare adult patients showed a high incidence of previous infection with the viruses analyzed here (CMV, 80%; EBV/anti-VCA, 88%; EBV/anti-EBNA, 80%; HSV, 88%; VZV, 85%; and parvovirus, 77%).

### Table 1. Clinical and Laboratory Characteristics of Individuals with Chronic CD56−/+dim NK Cell LGD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68 ± 17 (37–94)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>11/15 (42%/58%)</td>
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<tr>
<td>Reason for consulting</td>
<td></td>
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<tr>
<td>Routine blood analysis</td>
<td>(65%)</td>
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<tr>
<td>Adenomegaly</td>
<td>(4%)</td>
</tr>
<tr>
<td>Viral infection</td>
<td>(15%)</td>
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<tr>
<td>Concomitant malignant neoplasias</td>
<td>(12%)</td>
</tr>
<tr>
<td>Other (ITP)</td>
<td>(4%)</td>
</tr>
<tr>
<td>Physical examination</td>
<td></td>
</tr>
<tr>
<td>Adenophathies</td>
<td>(13%)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>(15%)</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>(4%)</td>
</tr>
<tr>
<td>Skin lesion</td>
<td>(0%)</td>
</tr>
<tr>
<td>Arthritis</td>
<td>(4%)</td>
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<tr>
<td>Peripheral blood cell counts</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>123 ± 27 (66–163)</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>186 ± 83 (26–417)</td>
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<tr>
<td>WBC (×10⁹/L)</td>
<td>128 ± 96 (2–43)</td>
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<tr>
<td>Neutrophils (×10⁹/L)</td>
<td>2.3 ± 2.4 (0.1–11.4)</td>
</tr>
<tr>
<td>Lymphocytes (×10⁹/L)</td>
<td>9.0 ± 8.9 (1.2–40.8)</td>
</tr>
<tr>
<td>NK cells (×10⁹/L)</td>
<td>6.7 ± 8.3 (0.5–38.7)</td>
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</tbody>
</table>

* Four cases had splenectomy before diagnosis of CNKL for an associated condition corresponding to an idiopathic thrombocytopenia purpura (n = 1), autoimmune hemolytic anemia (n = 1), trauma (n = 1), or splenic B-cell lymphoma (n = 1).

† Two of the patients had past history of herpetic skin lesions, infection with HZV being confirmed with serological analysis in one of them.

blood analysis performed during investigation or monitoring of another pathological condition (n = 9), consisting of diabetes mellitus (n = 1), arthritis (n = 1), adenomegaly (n = 3), idiopathic thrombocytopenia purpura (n = 1), autoimmune hemolytic anemia (n = 1), and B-cell lymphoma (n = 2). In the remaining eight cases, diagnosis was obtained in blood analyses performed because of co-existing active pathological conditions: HIV infection (n = 3), prostatic carcinoma (n = 1), colon carcinoma, refractory anemia with excess of blasts, autoimmune hemolytic anemia (n = 1), hepatitis C virus infection (n = 1), idiopathic thrombocytopenia purpura (n = 1), and breast carcinoma (n = 1).

At the moment of diagnosis 96% of the patients had lymphocytosis (>5.0 × 10⁹/L). Hemoglobin, neutrophil, and platelet counts were decreased in 46%, 44%, and 28% of the cases, respectively. Symptomatic anemia (<75 g/L) requiring red blood cell transfusion was observed in only three patients who suffered from severe Coomb’s-negative (n = 2) or Coomb’s-positive (n = 1) hemolytic anemia; in addition, one patient had a past history of autoimmune hemolytic anemia that responded completely to corticosteroids plus splenectomy and another patient had a past episode of hemolytic anemia of unknown origin that solved spontaneously. Neutropenia was severe (<500 × 10⁹/L) in six cases, although only one patient had a past history of repeated or severe infections. Clinically relevant thrombocytopenia (<50 × 10⁹/L) was observed at diagnosis of CNKL in only two cases, one of them being diagnosed has idiopathic thrombocytopenic purpura; in addition, three other patients had a past history of idiopathic thrombocytopenic purpura. One of these cases had a partial response to corticosteroids and a complete response to splenectomy; the other two cases had been successfully treated with corticosteroids. Four patients underwent splenectomy 2 to 12 years before the diagnosis of CNKL, either for an associated condition (n = 3) or because of trauma (n = 1).

### Immunophenotypic Features of CD56−/+dim NK Cells

NK cells accounted for 68 ± 18% of all PB lymphocytes (range, 19 to 95%) increased PB NK cell counts being observed in all cases. The vast majority (97.6 ± 4.1%) of all PB NK cells had an abnormal immunophenotype as illustrated in Figure 1 and the main phenotypic differences and similarities between CD56−/+dim NK cells, normal, and activated CD56+ NK cells are detailed in Figure 2 and illustrated in Figure 3. As shown there, the most relevant immunophenotypic feature consisted on an abnormally low (P < 0.0001) CD56 expression on the majority of PB NK cells (CD56−/+dim NK cells); most of the CD56−/+dim NK cells (78 ± 21%) were CD56− and a minority of them (22 ± 21%) showed dim and heterogeneous CD56 expression, as compared to normal NK cells. Residual CD56+ and CD56−+ NK cells were detected in all but one patient, accounting for only 2.3 ± 4.1% and 0.3 ± 0.3% of total PB NK cells, respectively.
As normal CD56$^+$ cells, CD56$^{-/-dim}$ NK cells were granzyme B$^+$, CD3/TCR$^+$, and CD5$^-$ and displayed a variable and heterogeneous reactivity for CD8 except in three patients who had abnormally high and homogeneous CD8 expression. Besides showing defective CD56 expression, the expanded NK cells also had abnormally low expression of CD7 ($P < 0.0001$), CD38 ($P < 0.0002$), and CD16 ($P < 0.05$) (Figure 2). In contrast, abnormal CD56$^{-/-dim}$ NK cells had a stronger and more homogeneous (ho) CD2 ($P < 0.0001$) and CD11c expression ($P < 0.0005$) (Figure 2).

In that concerning the expression of killer receptors, most CD56$^{-/-dim}$ NK cells were CD94$^+$ with a higher ($P = 0.03$) and more homogeneous ($P = 0.02$) expression than that observed in normal CD56$^+$ NK cells. CD161 and CD158a were dimly and heterogeneously expressed in a variable fraction of CD56$^{-/-dim}$ NK cells whereas NKB1 expression was found in a variable proportion (49 ± 34%) of CD56$^{-/-dim}$ NK cells in only 29% of the patients.

As normal PB NK cells, CD56$^{-/-dim}$ NK cells were CD25$^+$, CD5$^+$, CD11a$^{bright, ho}$, CD45RA$^{bright, ho}$, and CD122$^-$. Nevertheless, CD56$^{-/-dim}$ NK cells expressed much higher levels of HLA-DR ($P < 0.0001$) than normal CD56$^+$ NK cells, the majority of CD56$^{-/-dim}$ NK cells being HLA-DR$^+$ (mean of 62 ± 23%, versus 16 ± 14% observed on normal CD56$^+$ NK cells; $P < 0.0001$). In addition, CD122 and CD45RA expression were weaker ($P < 0.0001$ and $P = 0.0009$, respectively) and more heterogeneous ($P = 0.01$ and $P = 0.03$, respectively) whereas reactivity for CD45RO was slightly higher ($P = 0.03$) in the aberrant CD56$^{-/-dim}$ NK cells as compared to normal PB NK cells.

Some of the immunophenotypic differences found between normal and CD56$^{-/-dim}$ NK cells—ie, overexpression of CD11c and HLA-DR and down-regulation of CD45RA accompanied in some cases by co-expression of CD45RO in a variable fraction of NK cells—do in fact reproduce the immunophenotypic changes that typically occur on recently activated CD56$^+$ NK cells, whereas other—ie, increased CD2 expression and decreased and heterogeneous expression of CD7, CD11b, and CD38—were similar to those typically observed in conditions of chronic NK cell stimulation.15 Despite these similarities between CD56$^{-/-dim}$ NK cells and either recently or chronically activated CD56$^+$ NK cells, major differences were observed in the pattern of expression of the above-mentioned markers. Accordingly, CD56$^{-/-dim}$ NK cells...
had higher and more homogenous \((P = 0.002)\) expression of CD11c, greater percentages of CD57^+ cells \((P = 0.007)\), and lower levels of HLA-DR \((P = 0.0006)\) as compared to recently activated NK cells. Additionally, CD2 expression was more homogeneous \((P = 0.004)\), down-regulation of CD7 and CD11b were more pronounced \((P < 0.0001)\), and CD7 expression was even more heterogeneous \((P = 0.02)\) on CD56^{+/dim} NK cells than on chronically activated CD56^{+} NK cells. Moreover, disagreement between down-regulation of CD11b and CD38 expression, consisting of a CD11b^+/CD38^- phenotype, was frequently found in CD56^{+/dim} NK cells (10
CD2 (CV): 0.004; CD7 (MFI): 0.0001; CD11c (CV): 0.002; CD161 (CV): 0.0002; HLA-DR (MFI): 0.0006. 2) CD56^dim NK cells showed a diploid DNA content and a very low fraction of cells in the S phase (4.0 ± 0.5%) and G2/M (0.3 ± 0.4%) cell cycle phases.

Figure 2. Immunophenotypic characteristics of PB NK cells from patients with CD56^dim CNKCL (red boxes, n = 26) as compared to healthy individuals (gray boxes, n = 12), patients with acute viral infection (green boxes, n = 15), and patients with CD56^+ CNKCL associated with chronic viral infection or neoplasias (blue boxes, n = 15). The notched box and whiskers show nonparametric statistics, including the median, the lower and upper quartiles, and the 95% confidence interval around the median (notched box); the dotted lines connect the nearest observations within 1.5 interquartile ranges of the lower and upper quartiles, red crosses or circles indicate outliers. Differences between the groups were explored using the Mann-Whitney U-test. Statistically significant P values (\(\leq 0.05\)) 1) CD56^dim CNKCL versus healthy individuals: CD2 (CV): < 0.0001; CD7 (MFI): < 0.0001; CD7 (CV): < 0.0001; CD38 (MFI): 0.04; CD11a (CV): 0.006; CD11b (MFI): < 0.0001; CD11c (MFI): 0.002; CD11c(CV): 0.005; CD16 (MFI): 0.04; CD18 (MFI): 0.0002; CD38(CV): 0.003; CD45RA (MFI): 0.0009; CD45RA(CV): 0.05; CD45RO (MFI): 0.03; CD56 (MFI): < 0.0001; CD56 (CV): < 0.0001; CD57 (MFI): 0.0008; CD57 (CV): 0.0002; CD11b (MFI): 0.0005; CD11c (MFI): 0.002; CD11c(CV): 0.002; CD16 (MFI): 0.05; CD38 (MFI): < 0.0001; CD38 (CV): < 0.0001; CD45RA (MFI): 0.003; CD45RO (MFI): 0.0007; CD56 (MFI): < 0.0001; CD56 (CV): < 0.0001; CD57 (MFI): 0.0003; CD57(CV): 0.003; CD94(CV): 0.01; CD11c(CV): 0.0002; HLA-DR (MFI): < 0.0001.

Figure 3. Representative bivariate dot plots illustrating the most relevant phenotypic differences between PB NK cells (red dots) from a healthy individual (column A), and patients with acute (column B) and chronic (column C) viral infections as well as a case of CD56^-dim CNKCL (column D).

of 26 cases) but not on chronically activated CD56^+ NK cells.

Cytokine Production by CD56^--/+/dim NK Cells

As normal PB NK cells, CD56^-/-dim NK cells showed a typical Th1 pattern of cytokine production after stimulation with phorbol-12 myristate 13-acetate plus ionomycin (Figure 4). Accordingly, these cells produced IFN-\(\gamma\) and TNF-\(\alpha\), at the same time they failed to secrete IL-2, IL-4, IL-10, IL-13, and TNF-\(\beta\) under these stimulatory conditions. A more detail comparison with normal PB NK cells showed that the proportion of CD56^-/-dim NK cells producing both IFN-\(\gamma\) and TNF-\(\alpha\) and the amount of these cytokines produced per cell was similar to that of normal CD56^+ PB NK cells (75 ± 31% versus 75 ± 21%, \(P = 0.7\), and 52 ± 36% versus 36 ± 12%, \(P = 0.5\), respectively).

Flow Cytometry DNA Cell Contents of CD56^-/-dim NK Cells

In all cases analyzed, CD56^-/-dim NK cells had a diploid DNA content and a very low fraction of cells in the S phase (0.4 ± 0.5%) and G2/M (0.3 ± 0.4%) cell cycle phases.
Clonality of CD56<sup>−/−dim</sup> NK Cells

Phenotypically aberrant CD56<sup>−/−dim</sup> NK cell from four female patients studied showed a monoclonal pattern of chromosome X inactivation with a single HUMARA gene allele; in contrast, the NK-depleted leukocyte fractions from the same female patients, used as polyclonal controls, showed an heterozygous genotype for the HUMARA gene, with a random pattern of chromosome X inactivation.

TCR Gene Rearrangement Molecular Studies

Presence of (mono)clonal TCR-β and/or gamma gene rearrangements was found in three cases (none of them corresponding to patients whose CD56<sup>−/−dim</sup> NK cells were proven to be monoclonal by HUMARA PCR-based assays). A detailed analysis of PB T cells, including the study of the repertoire of the TCR-β chain variable regions (TCR-V<sub>β</sub>),<sup>25</sup> revealed T-cell abnormalities compatible with a clonal T-cell proliferation in one of these three CD56<sup>−/−dim</sup> CNKCL cases. This patient had a phenotypically abnormal CD8<sup>+</sup>/CD56<sup>+</sup> TCR-β-restricted T-cell population, representing 36% of CD8<sup>+</sup>/TCRαβ<sup>+</sup> PB T cells (2% of lymphocytes). No phenotypically aberrant T-cell populations showing a TCR-β pattern suggestive of T-cell (mono)clonality were detected in the other two cases.

Follow-Up and Clinical Outcome

At the moment of closing the study, median follow-up was 12 months (range, 1 to 63 months) and all patients remained alive except four patients who died because of disease progression (n = 1) or an associated malignant neoplasia (n = 3).

Lymphocyte counts remained stable during follow-up in all but three cases: one patient showed a progressive increase in PB lymphocyte counts from 4.1 × 10<sup>9</sup>/L up to 80.0 × 10<sup>9</sup>/L throughout a 58-month follow-up period, associated with lung infiltration and death; in another patient lymphocyte counts decreased from 5.4 × 10<sup>9</sup>/L toward normal values after an episode of septicemia, although the abnormal NK cell population still persisted in blood, increasing again afterward up to 20.0 × 10<sup>9</sup>/L; and a third patient experienced spontaneous complete remission. At the moment of closing this study none but one of the patients required specific cytotoxic treatment because of CNKCL.

During follow-up, recurrent/severe infections occurred in six cases, four of whom corresponded to patients with severe neutropenia. Overall, seven patients had a past or present history of hematological (n = 3) or nonhematological (n = 3) tumors, or developed nonhematological tumors during follow-up (n = 2). Hematological malignancies corresponded to B-cell lymphomas (n = 2) and to a myelodysplastic syndrome (n = 1). Nonhematological tumors corresponded to prostatic, bladder, colon, breast, and skin carcinomas (one case each). Three of these seven cases died because of the evolution of the neoplastic disease.

Discussion

LGL leukemia has been recognized as a distinct entity in the Revised European American<sup>29</sup> and World Health Organization<sup>30</sup> classifications and a number of NK cell-derived cases have been included in LGL-leukemia series.<sup>16</sup> Nevertheless, assessment of NK cell clonality still remains a challenge and no definitive consensus exists on the criteria to establish the diagnosis of NK cell leukemia. Indeed, although in T-cell disorders clonality can be detected through molecular analyses of TCR genes, molecular markers for the assessment of the clonal nature of NK cells such as X-linked DNA analysis and clonal integration of viral DNA in the genome of the infected cell<sup>17</sup>,<sup>34</sup> are frequently not applicable or available in most laboratories. Moreover, cytogenetic studies are of limited utility because recurrent chromosomal abnormalities have not been described in patients with CNKCL.

Recent studies show that neoplastic cells from almost every chronic and acute leukemia display phenotypic aberrations that can be used for the reliable identification of neoplastic cells.<sup>33</sup> Although no similar phenotypic study has been performed in CNKCL and NK cell leukemia/lymphoma, we have recently established the bases to define aberrant immunophenotypes in CNKCL through a detailed analysis of the immunophenotypes of CD56<sup>+</sup> and CD56<sup>++</sup> PB NK cells in normal individuals<sup>32</sup> and the phenotypic changes that occur in vivo in early and late phases of NK cell activation.<sup>15</sup> This strategy allowed us to identify a particular type of CNKCL, whose most striking abnormal immunophenotypic feature consisted on lack or dim CD56 expression. Our results indicate that CD56<sup>−/−dim</sup> CNKCL accounts for an important proportion of all CNKCL cases; despite this, cases of CD56<sup>−/−dim</sup> CNKCL have been only sporadically described in the literature.<sup>10</sup>–<sup>14</sup>

Besides displaying abnormally low or even no CD56 expression, CD56<sup>−/−dim</sup> NK cells showed other immunophenotypic differences with respect to normal blood CD56<sup>+</sup> NK cells: they had a more homogeneous CD2 expression, an increased and more homogenous expression of CD11c and CD94, and an increased percentage of HLA-DR<sup>+</sup> and CD45RA<sup>+</sup>/CD45RO<sup>+</sup> cells, together with a lower reactivity for CD7, CD11b, CD38, and CD45RA<sup>+</sup>. Interestingly, once individually considered, most of these features are also observed after NK cell activation. However, once the whole phenotypic pattern of CD56<sup>−/−dim</sup> NK cell is taken into account (CD2<sup>+</sup>/CD7<sup>−/−dim</sup>/CD11c<sup>+</sup>/CD94<sup>+</sup>/HLA-DR<sup>+</sup>) it could never be detected in normal or activated/reactive NK cells. Overall, these findings would indicate that the expanded NK cell population is under the effect of some kind of activation stimulus in vivo but it does not mature normally. Such aberrant activation-related immunophenotype was, in fact, the very first argument supporting the (mono)clonal nature of these neoplastic NK cells, which was subsequently confirmed in a subset of female patients through the HUMARA assay.

Although it is currently accepted that NK cells do not rearrange the TCR genes, some cases of CNKCL with clonal rearrangement of TCR-γ and/or TCR-β genes have been sporadically reported<sup>15</sup>,<sup>34</sup> as also found here. The
fact that concomitant clonal CD8^+ T-LGL and CNKCL have also been described in the literature^35 and that a detailed molecular and phenotypic analysis of blood T cells revealed T-cell abnormalities compatible with a clonal T-cell proliferation in one of the three CD56^+/-dim CNKCL cases here described, indicates that the possibility of co-existence of a monoclonal T-cell LGL proliferation and a CNKCL in a single patient should always be considered. Except for reasons related to a common origin of T and NK cells, no other reasonable explanation can be found for the other two cases who revealed monoclonal TCR-γ and/or TCR-β gene rearrangements; in fact, the monoclonal rearrangement pattern was confirmed in distinct assays although none of these patients showed immunophenotypic evidence of a concomitant clonal T-cell expansion.

The identification of those stimuli potentially responsible for the activation of CD56^+/-dim NK cells remains a challenge. Although we found evidence for a past infection with herpes viruses in most patients included in this study, a high prevalence of past herpes virus infections was also observed in controls. In agreement with previous studies,^36,37 we did not find serological or molecular evidence for EBV infection. The possibility of infection with HHV-8, has also been ruled out in patients with T-cell and NK cell LGL LPD.^38

The fact that transgenic mice for the HTLV-I tax gene develop NK cell LGL leukemia^39,40 and that sera from patients with both T-cell and NK cell LGL leukemia frequently react with HTLV-1/II p21 envelope proteins^41 had lead to the hypothesis of a direct relationship between CNKCL and infection by retroviruses. As in this series, cases of HTLV^42,43 or HIV-infected individuals^44 associated with CNKCL have been previously described. However, efforts to document association of CNKCL and infection with these^45,46 or other retroviruses^47 have been also unsuccessful.

An alternative possibility to explain the failure in associating CNKCL with any particular type of virus is that infection by different viruses may play a role in the pathogenesis of the disease by inducing a persistent NK cell stimulation but that it may no longer be necessary in sustaining the LGL proliferation. In line with this hypothesis, and the results reported by Zambello and colleagues^48 our findings provided serological/molecular evidence for a viral infection in the large majority of patients with CD56^+/-dim CNKCL.

Data presented here clearly shows that CD56^+/-dim CNKCL has an indolent clinical course and that most of the clinical problems in patients with CD56^+/-dim CNKCL are related to the associated cytopenias, infections, and neoplasias that are typically found in a great proportion of cases. In contrast, organomegalies were rarely observed, as did symptoms directly attributed to the LPD itself. Despite this and the low tumor mass found in most cases increased lactate dehydrogenase and β₂-microglobulin levels were observed in most patients.

As in CD8^+ T-LGL, the exact mechanism by which cytopenias associate to CD56^+/-dim CNKCL is not known. The possibility that a direct effect of BM infiltration by leukemia cells is not likely, as cytopenias were usually selective and their severity did not correlate with the magnitude of the BM infiltration, which was mild in most cases and only detected when careful cytological and immunophenotypic studies were performed (data not shown). The fact that Coomb's direct-positive anemia has been previously described in association with CNKCL^49,50 and that increased levels of neutrophil and platelet-associated Igs were detected in some of our cases presenting with neutropenia and thrombocytopenia, (data not shown) may suggest an antibody-mediated immune mechanism. Nevertheless, the true significance of these findings is not known because only patients with cytopenias were tested for the presence of these antibodies and they may be only a consequence of the polyclonal hypergammaglobulinemia associated with CNKCL.^51 this was also probably the case of the increased levels of other autoantibodies, observed in some patients, without any apparent clinical significance. In fact, clinical features that were previously reported in association to CD8^+ T-cell LGL leukemia, ie, arthritis, and CNKCL, ie, fever of unknown origin and vasculitis, were sporadically found in patients with CD56^+/-dim CNKCL.

The possibility of a direct cytotoxic effect of the expanded CD56^+/-dim NK cell population against normal blood cells^52 or a Fas/Fas-ligand mediated disturbance of normal blood cells' apoptosis^53,54 should be further investigated. In line with this hypothesis, one patient with CNKCL and hemolytic anemia, whose NK cells displayed cytotoxic activity against autologous erythrocytes has been previously reported^41 and anemia associated with CNKCL usually responds to cyclosporine A. In^55 irrespective of the mechanism involved, it seems obvious that cytopenias occur as a consequence of peripheral destruction because they usually responded to corticosteroids and splenectomy and laboratorial evidence of hemolysis was a frequent finding.

The mechanism that explains the high incidence of neoplasias associated to CD56^+/-dim NK cell is also unclear. It could be speculated that the expanded CD56^+/-dim NK cell population are functionally abnormal and unable to perform an adequate immune surveillance against tumors. The marked deficiency found in the expanded NK cells from our patients on the expression of CD56 and CD11b, both of which are involved in adhesion of NK cells to their targets, and target cell killing^56–60 supports this hypothesis. Also in agreement with this hypothesis is the fact that low levels of CD16 expression were also observed in some cases. Nevertheless, the overall amounts of TNF-α and IFN-γ produced by NK cells from normal PB and by CD56^+/-dim NK cells in patients with CNKCL were similar, although the latter displayed a more heterogeneous response.

A major clinical concern in patients with CNKCL relates to its potential transformation into a more aggressive LPD. As previously described for CD8^+ and CD4^+ T-cell LGL-leukemia, transformation of CNKCL into aggressive NK cell malignancies may occur suggesting that both T-cell and NK cell LGL-leukemia could be premalignant conditions. In such cases, new chromosomal abnormalities are frequently detected suggesting that the malignant clinical behavior of the disease could probably depend on additional oncogenic events. In line with
these findings, one of our patients with longer follow-up had evidence for disease progression with lung infiltration.

In summary our results would support the neoplastic nature of expanded CD56−/dim NK cells. Despite showing an indolent clinical course CD56−/dim CNKCL is frequently associated with infections, cytopenias, and neoplasias. Although the former may be involved in the pathogenesis of the disease, the latter may translate the existence of an altered immunosurveillance.

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