Detection of monoclonal T populations in patients with KIR-restricted chronic lymphoproliferative disorder of NK cells

Cristina Gattazzo,[1,2] Antonella Teramo,[2] Francesca Passeri,[1,2] Elena De March,[1] Samuela Carraro,[1] Valentina Trimarco,[1,2] Federica Frezzato,[1,2] Tamara Berno,[1] Gregorio Barilà,[1] Veronica Martini,[1,2] Francesco Piazza,[1,2] Livio Trentin,[1,2] Monica Facco,[1,2] Gianpietro Semenzato,[1,2] and Renato Zambello[1,2]

[1]Department of Medicine, Hematology and Clinical Immunology Branch, Padua University School of Medicine; and [2]Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

ABSTRACT

The etiology of chronic large granular lymphocyte proliferations is largely unknown. Although these disorders are characterized by the expansion of different cell types (T and natural killer) with specific genetic features and abnormalities, several lines of evidence suggest a common pathogenetic mechanism. According to this interpretation, we speculated that in patients with natural killer-type chronic lymphoproliferative disorder, together with natural killer cells, also T lymphocytes undergo a persistent antigenic pressure, possibly resulting in an ultimate clonal T-cell selection. To strengthen this hypothesis, we evaluated whether clonal T-cell populations were detectable in 48 patients with killer immunoglobulin-like receptor-restricted natural killer-type chronic lymphoproliferative disorder. At diagnosis, in half of the patients studied, we found a clearly defined clonal T-cell population, despite the fact that all cases presented with a well-characterized natural killer disorder. Follow-up analysis confirmed that the TCR gamma rearrangements were stable over the time period evaluated; furthermore, in 7 patients we demonstrated the appearance of a clonal T subset that progressively matures, leading to a switch between killer immunoglobulin-like receptor-restricted natural killer-type disorder to a monoclonal T-cell large granular lymphocytic leukemia. Our results support the hypothesis that a common mechanism is involved in the pathogenesis of these disorders.

Introduction

Large granular lymphocyte (LGL) disorders are characterized by the chronic proliferation of cytotoxic lymphocytes. These LGL expansions can be sustained by two distinct subpopulations: $CD3^+$ cytotoxic T lymphocytes (CTLs) or CD3- natural killer (NK) cells.[1,2] According to the specific lymphoid cell involved, the 2008 World Health Organization (WHO) classification defined chronic LGL disorders as T-large granular lymphocytic leukemia (T-LGLL) and as chronic lymphoproliferative disorder of NK cells (CLPD-NK).[3,4] Both diseases are characterized by an abnormal expansion of cytotoxic clonal populations; in T-LGLL, the marker of clonality is represented by the rearrangement of TCRγ whereas a restricted pattern of killer immunoglobulin-like receptor (KIR) expression has been used as a surrogate of clonality in CLPD-NK, NK cells lacking a clonotypic structure.[2,5]

In recent years, progress in research has widened our understanding of the pathogenetic events that sustain T-LGL leukemia and CLPD-NK.[6] The identification of specific mechanisms and biological differences between neoplastic NK and T clones seem to strengthen the subclassification of these disorders made by the WHO. In CLPD-NK, the pathogenetic mechanism is intrinsically nested in a genetic background which determines a biased response of cytotoxic NK cells equipped with activating NK receptors,[7,8] this pattern being mainly related to the reduced expression of other activating receptors, such as natural cytotoxic receptors. This impaired expression of inhibitory receptors is dependent on the patient's genotype, and is characterized by the presence of multiple activating KIR genes[9] and on a discrete silencing of inhibitory KIR genes through their promoter methylation.[10] On the other hand, attempts to understand the pathogenesis of T-LGLL have suggested there is a crucial role for inflammatory cytokines. Leukemic T-LGLs fail to undergo activation-induced cell death (AICD), an event that is consequent to a critical impairment of Fas-induced apoptosis.[11,12]

The etiology of LGL expansions is largely unknown. This is due to the fact that no single, specific agent is responsible for the LGL proliferation, which is likely the expression of an abnormal processing of different foreign antigens.

Several data suggest the concept that different events induce the disease through a pathogenic mechanism that is common for both disorders. In this regard, a number of reports strongly support the role of a chronic/persistent antigenic stimulation provided by an auto-antigen or a foreign infectious antigen.[13-17] This would lead to the expansion of a fully differentiated effector cytotoxic LGL which is not eliminated as a consequence of an impairment of apoptotic pathways and persistence of chemokines triggering a chronic stimulation.[12] The phenomenon of clonal drift, i.e. a change in the dominant T-cell clone observed in nearly 50% of LGLL patients, has been interpreted in line with this hypothesis.[18] Similarly, in both disorders, *in vivo* activation of STAT3 and the presence of somatic STAT3 mutations have been observed.[19-22] The observation that STAT3 SH2 somatic mutations can be found with a similar frequency in NK-cell and in T-cell disorders further indicates that a common mechanism is responsible for their pathogenesis, possibly driven by a shared genetic lesion irrespective of the cell lineage.[22]

 doi:10.3324/haematol.2014.105726
The online version of this article has a Supplementary Appendix.
Manuscript received on February 19, 2014. Manuscript accepted on September 4, 2014.
Correspondence: g.semenzato@unipd.it

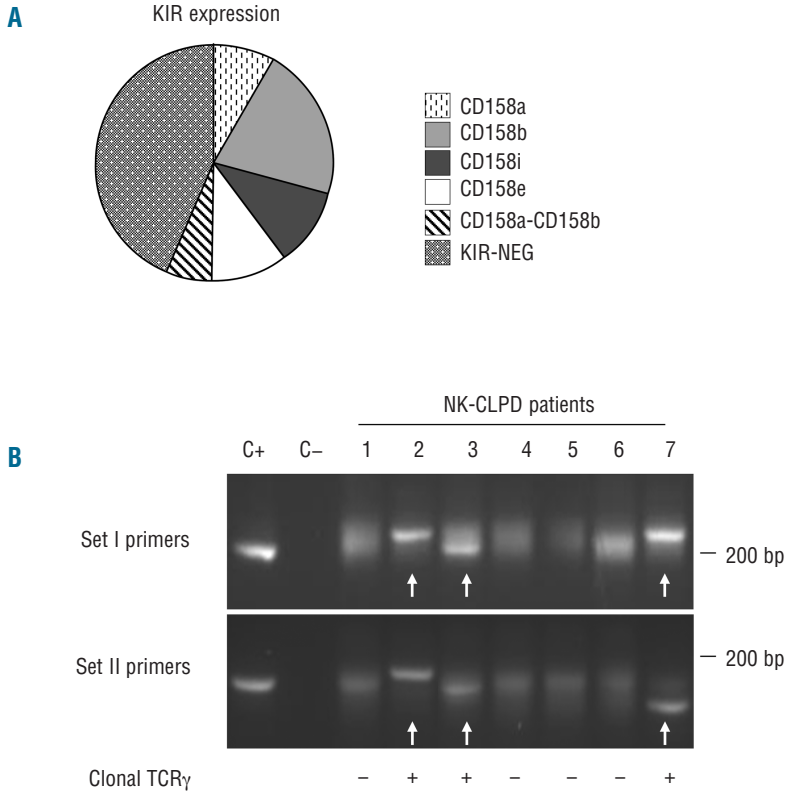

Figure 1. KIR expression on NK cells and analysis of TCRγ rearrangement on T cells from patients with CLPD-NK. (A) KIR expression on NK $CD16^+/CD3^-$ cells from patients with CLPD-NK was evaluated by flow cytometry analysis. Cells were stained with CD158a mAb against the KIR2D1, CD158b mAb against the KIR2DL2/3 and KIR2DS2, CD158i mAb against the KIR2DS4 and CD158e mAb against the KIR3D1. (B) TCRγ rearrangement was evaluated by PCR at the time of diagnosis. Specific primers for the variable region of the rearranged TCRγ chain gene were used as two mixes (Set I primers and Set II primers; see Methods section). Twenty-three patients (48%) presented monoclonal TCRγ rearrangement; figure reports 7 representative patients. The presence of clonal rearrangement is indicated by white arrows.

Given this, we hypothesized that the antigenic pressure favouring NK-cell proliferation in CLPD-NK would represent a relevant signal also for the T compartment, ultimately leading to the expansion of cytotoxic clonal T-cell populations. To investigate this hypothesis, we analyzed the rearrangement of TCRγ in the residual 'normal' T lymphocytes in 48 patients with CLPD-NK. Our data show that, in half of these patients, a clonal T-cell population was detectable at the time of diagnosis, whereas in 27% of our patient cohort it also occurred during follow up. In some cases, this phenomenon leads to a switch from a KIR-restricted CLPD-NK to a monoclonal T-LGLL, strengthening the suggestion that a common pathogenetic mechanism takes place in these chronic LGL disorders.

Methods

Patients

Forty-eight patients were enrolled in this study. All patients were affected by CLPD-NK, characterized by a chronic proliferation of $CD16^+/CD3^-$ NK cells. Diagnoses were made according to reported criteria.[5,23,24] At the time of enrollment, none of the patients had received treatment. Twenty-nine patients were available for serial measurements over an extended time period (3-14 years). Patients' samples and clinical features were collected from the Hematology Division of the Department of Medicine in accordance with a protocol approved by the Institutional Review Board for experimental studies (Protocol n. 2362P, approved on July 6, 2011). Written informed consent was obtained in accordance with the Declaration of Helsinki.

Cells purification and isolation

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Sigma Aldrich) gradient centrifugation. $CD16^+/CD3^-$ NK cells of patients were obtained using magnetic separations over columns (MACS; Miltenyi Biotec, Auburn, CA, USA) with magnetic micro-beads coated with anti-human CD16 antibodies (isotype: mouse IgM; Miltenyi Biotec). $CD3^+/CD57^+$ T cells were obtained from PBMCs by the FACSAria cell sorter (BD biosciences, San Jose, CA, USA). Sorted populations were analyzed for purity and viability (both 95%). We performed preliminary experiments to rule out the possibility that cells obtained by FACSAria sorting or by magnetic micro-beads were functionally different.

Flow cytometry analysis

Peripheral blood samples were collected from patients at clinical testing as detailed in the *Online Supplementary Appendix*.

Statistical analysis

The comparison of characteristics between patients with monoclonal and polyclonal TCRγ rearrangement was performed using Student's t-test. $P<0.05$ or $P<0.01$ were considered statistically significant.

Result

Patients' characteristics

We studied a well-reported cohort (n=48) of patients with CLPD-NK (Table 1). Mean age at diagnosis was 61 years (range 25-86 years). As assessed by flow cytometry analysis, mean percentage of NK $CD16^+/CD3^-$ cells was 63% (range 42-91%). The presence of a relevant KIR expression was detected in 27 patients (56%) (Figure 1A). More specifically, 4 patients (9%) presented a restricted expression of CD158a (KIR2D1), 10 patients (21%) pre-

sented the CD158b (KIR2DL2/3 and KIR2DS2), 5 patients (10%) presented the CD158i (KIR2DS4), and 5 patients (10%) presented the CD158e (KIR3D1). The cohort also included 3 patients (6%) with restriction of both CD158a and CD158b. Finally, 21 patients (44%) were characterized by the lack of KIR expression on the cell surface. Analysis of NK activity at the time of diagnosis showed that all cases were characterized by the ability to induce a strong cytolytic response (*data not shown*).

Table 1. Patients' clinical and laboratory characteristics.

Variable	CLPD-NK patients (n=48)
Mean age, years (range)	61 (25-86)
Male/female	34/14
Mean % lymphocytes (range)	65 (44-88)
Mean $CD16^+/CD3^-$ count in PB cells $x10^9$/L (range)	3.2 (1.1-7.4)
Mean % [1]$CD16^+/CD3^-$ on lymphocytes (range)	63 (42-91)
Mean % [1]$CD16^+/CD56^+$ on lymphocytes (range)	34 (1-87)
Mean $CD3^+/CD57^+$ count in PB cells $x10^9$/L (range)	0.5 (0.05-1.9)
Mean % [1]$CD3^+/CD57^+$ on lymphocytes (range)	12 (1-28)
Mean [1]CD4/8 ratio on lymphocytes (range)	0.84 (0.07-2.19)
% neutropenia (<1.5x10^9/L) (n. patients)	27 (13)
% anemia (<10 g/dL) (n. patients)	8 (4)
% thrombocytopenia (<100x10^9/L) (n. patients)	6 (3)
% splenomegaly (n. patients)	10 (14)
% associated diseases[‡] (n. patients)	31 (15)
% positive serology for EBV and CMV infections (n. patients)	100 (48)
% patients with somatic mutation in STAT3-SH2 domain[§] (n. patients)	8 (4)

CLPD-NK: chronic lymphoproliferative disorder of NK cells; PB: peripheral blood; MGUS: monoclonal gammopathy of undetermined significance; HCV: hepatitis C virus; HBV: hepatitis B virus; EBV: Epstein-Barr virus; CMV: cytomegalovirus. [‡]Polymyalgia, polymyositis, non-Hodgkin lymphoma, MGUS, HCV infection, HBV infection, prostate cancer, sarcoidosis, thyroditis, essential thrombocythemia. [§]Somatic STAT3 mutations were found in 4 patients, Y640F in 2 cases and D661Y in 2 cases. [1]$CD16^+/CD3^-$, $CD16^+/CD56^+$, $CD3^+/CD57^+$ and CD4/8 ratio are calculated on the percentage of total lymphocytes.

Table 2. Comparison of clinical characteristics in patients with or without monoclonal TCRγ rearrangement.

Variable	CLPD-NK patients without monoclonal TCRγ rearrangement (n. 25)	CLPD-NK patients with monoclonal TCRγ rearrangement (n. 23)
Mean age, years (range)	65 (44-86)	56* (25-77)
Male/female	18/7	16/7
Mean % lymphocytes (range)	67 (45-88)	63 (44-79)
Mean $CD16^+/CD3^-$ count in PB cells $x10^9$/L (range)	3.1 (1.1-7.0)	3.1 (1.1-7.4)
Mean % [1]$CD16^+$ on lymphocytes (range)	63 (42-91)	63 (47-80)
Mean % [1]$CD16^+$ on lymphocytes (range)	32 (5-54)	36 (1-87)
Mean % [1]$CD3^+/CD57^+$ on lymphocytes (range)	261 (49-1035)	904** (284-1971)
Mean % [1]$CD3^+/CD16^+$ on lymphocytes (range)	1 (0-4)	1 (0-5)
Mean % [1]$CD3^+/CD57^+$ on lymphocytes (range)	6 (1-25)	19** (9-28)
Mean [1]CD4/8 ratio on lymphocytes (range)	0.88 (0.13-2.19)	0.79 (0.07-2.19)
% symptoms at diagnosis	20	34
% neutropenia (<1.5x10^9/L)	43	43
% anemia (<10 g/dL)	4	4
% thrombocytopenia (<100x10^9/L)	8	4
% splenomegaly	8	9
% associated diseases[‡]	28	35
% positive serology for EBV and CMV infections	100	100
% patients with somatic mutation in STAT3-SH2 domain[§]	8	9

*CLPD-NK: chronic lymphoproliferative disorder of NK cells; PB: peripheral blood; MGUS: monoclonal gammopathy of undetermined significance; HCV: hepatitis C virus; HBV: hepatitis B virus; EBV: Epstein-Barr virus; CMV: cytomegalovirus. ‡Polymyalgia, polymyositis, non-Hodgkin lymphoma, MGUS, HCV infection, HBV infection, prostate cancer, sarcoidosis, thyroditis, essential thrombocytemia. §Somatic STAT3 mutations were found in 4 patients, Y640F in 2 patients and D661Y in 2 patients. *Student's t-test, patients without TCRγ rearrangement versus patients with TCRγ rearrangement; P=0.012. **Student's t-test, patients without TCRγ rearrangement versus patients with TCRγ rearrangement, P=0.0001. [1]$CD16^+/CD3^-$, $CD16^+/CD56^+$, $CD3^+/CD57^+$ and CD4/8 ratio are calculated on the percentage of total lymphocytes.*

Rearrangement of the TCRγ gene in residual T CD3⁺ population in CLPD-NK patients

Rearrangements of the T-cell receptor γ (TCRγ) genes were identified by PCR on PBMCs of all patients with CLPD-NK enrolled in the study. At the time of CLPD-NK diagnosis, we found that 23 patients (48%) presented one or two discrete bands of PCR product, indicative of a monoclonal rearrangement of TCRγ (Figure 1B). The product of PCR ranged from a fairly detectable band to a strong signal, suggesting a different percentage of abnormal T cells in the patients. Criteria for diagnosis of T-LGLL consider the proliferation of a clonal T-cell population that expresses the membrane surface marker CD3, CD8, CD57, often associated to CD16 and characterized by TCR rearrangement. In fact, in our patients, CD4/CD8 ratio was significantly reduced with respect to the normal value (0.84±0.07 *vs.* 1.80±0.70; P=0.0011) and a subset of T CD3⁺/CD57⁺ cells was clearly detectable. This CD3⁺/CD57⁺ cell expansion was correlated to the presence of TCR clonality. In fact, in patients with monoclonal rearranged TCR, percentage of CD3⁺/CD57⁺ cells was 19% *versus* 6% of patients with polyclonal T-cell population (**P=0.0001) (Table 2). Mean CD3⁺/CD57⁺ count was higher in patients with monoclonal rearranged TCR (0.9 x 10^9 cells/L; range 0.3-2.0 x 10^9 cells/L) *versus* patients with polyclonal TCR (0.3 x 10^9 cells/L; range 0.05-1.0 x 10^9 cells/L; P=0.001). In the first group, 18 of 23 patients met the diagnostic criteria for T-LGL, since CD3⁺/CD57⁺count was over 0.5 x 10^9 cells/L together with the presence of TCR monoclonal rearrangement. Monoclonal T expansion did not correlate with other parameters, such as the patient's clinical characteristics (Table 2) or immunophenotype of NK cells (*Online Supplementary Table S1*). Finally, since clonal T-cell populations with a characteristic CD8⁺, CD28⁻, CD57⁻ phenotype have been reported to be detectable in normal individuals over 65 years of age,[29] in 6 patients the expanded CD3⁺/CD57⁺ population was sorted and TCRγ rearrangement was analyzed in these cells, confirming the monoclonal nature of this abnormal cell subset (*data not shown*).

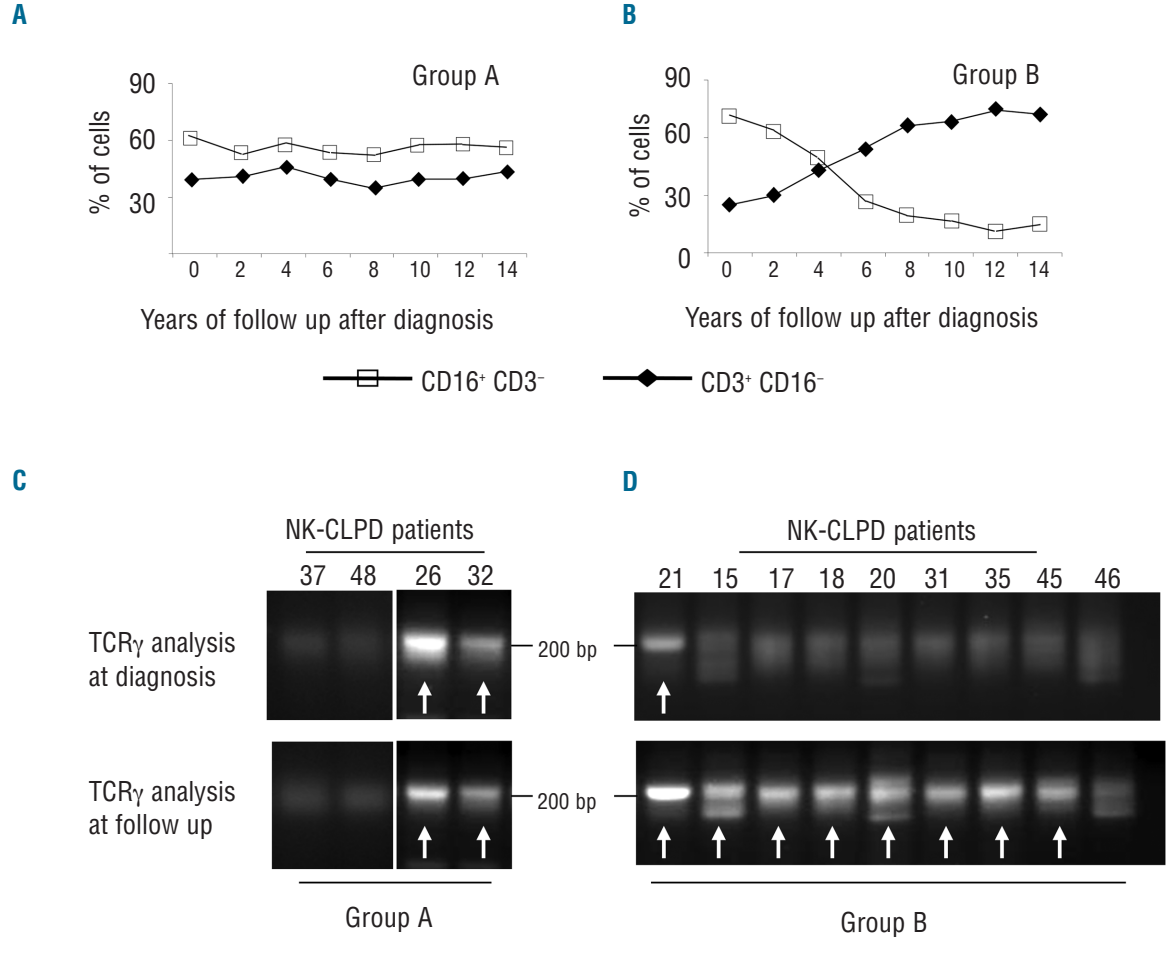

Figure 2. Long-term evaluation of T CD3⁺ and NK CD16⁺/CD3⁻ percentage and TCRγ rearrangement. According to the behavior of NK- and T-cell patterns, we divided patients into 2 groups: Group A and Group B. (A and B) show one representative patient for each group. (A) Group A (n=20, 69%) patients with a percentage of NK cells stable over time. (B) Group B (n=9, 31%) patients in whom NK cells, that represented the predominant population at the diagnosis, progressively decreased to values comparable to controls, with a concomitant increase of T-cell population (range 3-14 years). (C) Analysis of TCRγ rearrangement on T cells at diagnosis and at follow up (range 3-14) in Group A patients. Figure reports 4 representative patients. (D) Analysis of TCRγ rearrangement on T cells at diagnosis and at follow up (range 3-14 years) in Group B patients. Figure reports all 9 patients of Group B. The presence of clonal rearrangement is indicated by white arrows.

STAT3 mutations in NK cells

Somatic STAT3 mutations at diagnosis were demonstrated in 4 patients of the 48 studied: Y640F in 2 cases and D661Y in 2 cases. In all these cases, the data were also confirmed when purified NK cells were used for the analysis. In 5 patients, the presence of STAT3 mutations at different times along the progression of disorder (range 3-14 years). In Patient 21, rearranged TCRγ was detected already at the time of diagnosis, whereas in Patients 15, 18, 31 and 35, rearrangement was observed during follow up. In all these patients, STAT3 mutations were not found either at diagnosis during follow up.

Clonotypic dynamics and disease progression

In 29 patients, the presence of a clonal T-cell population was investigated during an extended period of follow up ranging from 3 to 14 years. Analysis of TCR gene rearrangement at different yearly intervals allowed the identification of different patterns of T-cell clonal population behaviour during the course of the disease. Among these 29 patients, in 9 patients (31%) a T-cell clonal population was never observed, even several years (range 3-14 years) after diagnosis. In 13 patients (45%), the TCRγ rearrangement, already detectable at diagnosis, was persistent during years of follow up. In 7 patients (24%), the TCRγ rearrangement appeared a few years after diagnosis. Interestingly, whereas in some patients a shift from a polyclonal to a monoclonal T-cell population was documented, none of the patients with an initial clonal TCR regained a normal polyclonal TCR repertoire.

Table 3. Long-term evaluation of patients in group B.

Patient #	Group	Clonal TCR γ rearrangement	Years follow up	% ^{1}CD16^{+}	% ^{1}CD3^{+} CD16^{+}	% ^{1}CD3^{+} CD57^{+}	% ^{1}CD3^{+}	% ^{1}CD3^{+} CD56^{+}	% ^{1}CD8^{+}	^{1}CD4/8 ratio	% ^{1}CD56^{+} CD57^{+}	KIR restriction
15	B	–	0	80	0	14	3	2	22	0.3	1	CD158a-b
		+	6	54	0	40	23	3	35	0.5	1	CD158b
		+	13	33	0	64	22	3	52	0.4	3	CD158b
17	B	–	0	47	0	44	9	1	34	1.0	7	CD158i
		–	3	26	0	56	15	2	31	1.0	21	CD158i
		+	5	20	0	60	17	1	31	1.0	20	CD158i
18	B	–	0	59	0	35	4	3	27	1.5	13	KIR NEG
		+	5	22	0	71	11	3	31	1.5	10	KIR NEG
		+	9	24	2	67	10	3	32	1.3	16	KIR NEG
20	B	–	0	49	0	44	4	2	28	1.1	26	CD158e
		–	3	38	0	45	4	4	20	1.8	27	CD158e
		+	5	26	0	58	16	3	45	0.6	18	CD158e
31	B	–	0	89	0	9	2	5	13	0.6	16	CD158i
		+	4	33	1	45	19	7	41	0.4	11	CD158i
		+	12	7	1	43	22	2	52	0.5	7	NORMAL
		+	14	9	5	46	22	5	47	0.6	6	NORMAL
35	B	–	0	88	5	10	1	2	15	1.3	62	KIR NEG
		+	8	16	1	68	24	3	37	1.1	10	NORMAL
		+	12	15	1	72	23	5	37	1.1	10	NORMAL
45	B	–	0	74	0	12	6	2	64	0.1	0	KIR NEG
		–	3	36	0	53	17	1	42	1.0	2	KIR NEG
		+	5	38	0	51	16	3	46	0.9	1	KIR NEG
21	B	+	0	48	0	51	22	2	24	1.4	5	CD158b
		+	3	27	0	61	18	0	35	0.7	10	NORMAL
		+	6	22	0	62	26	1	31	1.8	15	NORMAL
46	B	–	0	45	0	34	6	6	27	1.0	24	KIR NEG
		–	10	25	4	64	1	3	31	1.4	19	NORMAL
		–	14	19	3	69	1	0	29	1.5	6	NORMAL

^{1}CD16^{+}, CD3^{+}/16^{+}, CD3^{+}, CD3^{+}/CD357^{+}, CD3^{+}/56^{+}, CD8^{+} and CD4/8 ratio, CD56^{+}/CD57^{+} are calculated on the percentage of total lymphocytes.

Table 4. Analysis of Vβ expansion over time (range 3-14 years).

Patient #	Clonal TCRγ at diagnosis	Vβ at diagnosis	Clonal TCRγ at follow up	Vβ at follow up
20	–	normal Vβ repertoire	+	Vβ 7, 9
31	–	normal Vβ repertoire	+	Vβ 7, 9, 20
46	–	normal Vβ repertoire	–	normal Vβ repertoire
11	+	Vβ 1, 7, 8, 9, 17	+	Vβ 1
14	+	Vβ 7	+	Vβ 7
41	+	normal Vβ repertoire	+	Vβ 7, 9, 17

Analysis of NK CD16⁺/CD3⁻ and T CD3⁺ population in CLPD-NK patients during the course of disease

The observation that T-cell clones were detected in nearly half of CLPD-NK cases, and that some patients developed monoclonal TCRγ rearrangement during the natural history of disease, prompted us to investigate the kinetics of the T-cell population in our cohort of patients. In 29 patients, it was possible to evaluate the percentage of CD3+ T lymphocytes and CD16⁺/CD3⁻ NK cells by flow cytometry analysis at the time of diagnosis and within a follow up ranging from three to 14 years. According to the behaviour of the NK- and T-cell patterns, we divided the patients under study into 2 groups: 20 patients (69%) with a percentage of NK and T cells stable over the time period evaluated (Group A) (Figure 2A), and 9 patients (31%) in whom the percentage of KIR-restricted NK cells, representing the dominant population at the diagnosis, progressively decreased to within the normal range, with a concomitant increase in T-cell population (Group B) (Figure 2B). By matching these data with the presence of detectable T clones, we found that in Group A, made up of patients with stable disease, the presence or absence of T-cell clone was constant. In fact, if the clone was not presented at diagnosis (n=7), it was also never detected after years of follow up (range 3-14 years) (Patients 37 and 48; Figure 2C). Otherwise, if the T clone was present at the time of diagnosis (n=13), it was constantly detected during the course of the disease (Patients 26 and 32; Figure 2C). In contrast, in Group B, 7 patients (Patients 15, 17, 18, 20, 31, 35 and 45) showed the appearance of a clonal T-cell population during follow up (Figure 2D). Interestingly, all these cases were characterized by a shift from a KIR-restricted NK expansion to a T CD3⁺/CD57⁺ abnormal population, with monoclonal TCR rearrangement (Table 3). Moreover, at the last follow up, the initial KIR-restricted population was still documented in 5 of these cases (Patients 15, 17, 18, 20, 45), whereas in 2 patients the KIR-restricted population was observed to disappear, with normalization of NK-cell subsets (Patients 31 and 35) (Table 3). In contrast, Patient 21 presented a monoclonal T CD3⁺/CD57⁺ cell population already at diagnosis (Patient 21) (Figure 2D) that persisted during follow up, with a concomitant decrease in NK-cell population characterized by a shift from a KIR-restricted NK expansion to a normal KIR repertoire. Finally, the only patient in Group B in whom a monoclonal TCRγ rearrangement was never found (Patient 46) (Figure 2D) achieved hematologic remission paralleled by a gradual disappearance of the aberrant NK population, an increase to normal value of a T CD3⁺/CD57⁻/CD16⁻ cell population with polyclonal TCR, and normal CD4/CD8 ratio, suggesting a spontaneous remission of CLPD-NK (Table 3).

TCR Vβ usage in CLPD-NK patients

TCR Vβ clonotyping was available in 6 patients with a mean 6-year follow up (range 3-14 years). In Patients 20, 31 and 46 who did not present clonal TCRγ rearrangement at diagnosis, we found a normal TCR Vβ repertoire that, in Patients 20 and 31 became restricted at follow up with the appearance of TCR clonality (Table 4). Patients 11 and 14 are of particular interest; they presented monoclonal TCRγ rearrangement already at diagnosis. Patient 11 had a preferential usage of Vβ regions 1, 7, 8, 9 and 17 that became further restricted to Vβ region 1. Only Patient 14 displayed a dominant clone from the outset of clinical presentation and the clone remained stable over prolonged periods of time. In Patient 41, even if TCRγ monoclonal rearrangement was detected at diagnosis, we found a normal Vβ repertoire. In fact, the pathological T cells were so few that the analysis of TCR Vβ gene expression did not highlight any dominant T-cell population. During the 5-year follow up, Patient 41 developed a skewed TCR repertoire restricted to the Vβ 7, 9 and 17 regions. The change in the dominant TCR Vβ clone or the progressive appearance of a dominant clone from an oligoclonal background, referred to as clonal drift, is a typical feature of T-LGL leukemia. Clemente *et al.* suggested that the clonal drift is a subtle characteristic of the disease in its early manifestation.[18]

Clinical behavior

The primary hematologic manifestation was neutropenia (<1.5 x 10^9 /L; 27% of patients), although never reaching the level of severe neutropenia, followed by anemia (<10 gr/dL; 8%), and thrombocytopenia (<100,000 x 10^9/L; 6% of patients). Associated diseases were reported in 15 cases (polymyalgia n=2, polymyositis n=3, non-Hodgkin lymphoma n=1, monoclonal gammopathy of undetermined significance (MGUS) n=2, hepatitis C virus (HCV) infection n=1, hepatitis B virus (HBV) infection n=1, prostate cancer n=1, sarcoidosis n=1, thyroiditis n=1, essential thrombocythemia n=2). All patients were found to be positive for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) IgG antibodies. One patient had undergone splenectomy for non-defined reasons several years before the diagnosis of CLPD-NK. Nine patients needed treatment due to associated diseases other than CLPD-NK. In particular, 2 patients for each group were receiving low-dose methotrexate for autoimmune disorders. Concerning patients with and without a monoclonal T population, no differences were demonstrated between the groups, except that patients with TCRγ clonal rearrangement had a median age of 56 years (range 25-77 years) compared to the other group of patients who had a median age of 65 years (range 44-86 years; Student's t-test, $^*P$=0.012) (Table 2). No clinical differences were demonstrated in patients shifting from NK to T LGL proliferation.

Discussion

Our results demonstrate the presence of monoclonal T-cell populations in 48% of cases in this large cohort of patients with CLPD-NK. Even more intriguing is the finding that these monoclonal populations can be detected not only at the time of diagnosis, but in some cases they can occur during the natural history of disease, indicating that the association of T and NK proliferations is much more frequent that initially thought. The T-cell clone can eventually became so relevant as to be dominant, leading to the shift from CLPD-NK to T-LGLL. This concept supports the hypothesis that the immunological reaction ongoing in patients with CLPD-NK is able to sustain the clonal expansion of both NK and T cells. In this contest, it is likely that CLPD-NK and T-LGLL should not be viewed as separated entities, but should be considered to be the different sides of the same disorder, with a common pathogenic mechanism. This hypothesis is apparently in contrast with the WHO 2008 classification that classifies CLPD-NK and T-LGLL as two different diseases.[3,4] However, the WHO classification relies on different lineage features of T and NK cells, not taking into account putative common pathogenic

mechanisms and clinical features. In fact, chronic NK- and T-cell expansions display a similar morphology and, on clinical grounds, in both types of patients cytopenia and other co-morbidities are often associated, including autoimmune conditions and/or malignancies.[30,31] Furthermore, the treatment is very similar in CLPD-NK and T-LGLL patients, entailing an immunosuppressive regimen or careful observation of chronic lymphocytosis.[1] The identification of patients with a NK population bearing a restricted KIR expression and an accompanying $CD3^+/CD57^+$ clonal T-cell population(s) demonstrated in the present study questions the accuracy of this subdivision, also considering that there are no clinical differences between those patients with or those without clones.

The etiopathogenesis of these disorders is not completely understood, and this is particularly the case for CLPD-NK. In CLPD-NK cases, several experimental data support the involvement of a genetic background. In fact, NK cells equipped with an activating KIR gene aplotype are thought to be more prone to clonal expansion, with a concomitant marked silencing of inhibitory KIRs.[9,10,32] The selection of T cells during the T-LGLL pathogenesis has been more precisely investigated. T-LGL cells exhibit the activation of multiple survival signaling pathways,[33] are characterized by the failure to undergo AICD,[11] and are not sensitive to Fas-induced apoptosis.[12] Although CLPD-NK and T-LGLL seem different, the steps that take place during their etiopathogenesis are similar. In fact, a number of reports strongly support the role of a chronic/persistent antigenic stimulation by an auto-antigen or a foreign infectious antigen as the initial step.[13-17] Since disorders of large granular lymphocytes are often associated to an inflammatory background, it is likely that the chronic stimulation that triggers the clonal expansion of NK cells during the pathogenesis and progression of CLPD-NK is also able to sustain the expansion of the T population characterized by a TCRγ monoclonal rearrangement. Antigens and cytokines involved in this process have already been the subject of investigation, given that IL-2 and IL-15 are well-known cytokines that regulate the biology of NK and T cells, and both have been claimed to be involved in LGL clonal expansion.[34-36] A pivotal role is also played by the JAK/STAT pathway, an overexpression and overactivation of STAT3 by IL-6 being responsible for the survival of neoplastic T clone.[20] Furthermore, somatic mutations of STAT3 are crucial in both T-LGLL and CLPD-NK, as recently reported.[21,22] The relevant role of STAT family genes in the pathogenesis of LGL has been recently reinforced by the demonstration that STAT5b mutations can be detected, although at a very low frequency (4 of 211 cases), in patients with LGL proliferations.[37] The frequency of mutated STAT3 in our cohort of patients is lower than that reported by other.[21,22,38,39] Differences between the study populations could explain the different results. In particular, the more indolent clinical features of our patients, as compared with those reported by Koskela *et al*.,[21] represent a plausible explanation. As a matter of fact, a recent paper by Clemente *et al*.[40] shows a lower incidence of STAT3 mutation than that reported by the same group in a previous cohort.[22] Finally, the suggestion that STAT3 mutation might occur in a common T/NK cell precursor[41] is unlikely, the most relevant reason being represented by the fact that the number of STAT3 mutated cells might account for only a fraction of the whole proliferating clone, eventually becoming dominant along the natural history of disease. The identification of mutations both into NK and T clones raises the question whether NK and T expansions present in the same patient might share the same mutation. Studies are in progress to test this possibility in clonal purified T or NK populations.

Our results support the concept that the mechanism accounting for the proliferation of pathological NK and T clones could be common for CLPD-NK and T-LGLL. This hypothesis is supported by the presence of both NK and T clones at the time of the diagnosis in a large number of cases, and by the evidence that clonal T rearrangements can occur years after diagnosis, both clones persisting in some cases during the natural history of the disease. By flow cytometry, in nearly 50% of the patients with T-LGLL studied, Clemente *et al*. reported the phenomenon recognized as clonal drift in the Vβ usage.[18] These authors observed that the clone size was smaller in the clonal drift group with respect to stable patients, supporting the concept that we are dealing with an early manifestation of the disease. CDR3 AA sequence was also shown to remain unaltered in stable clones, whereas expansions with different Vβ restriction presented markedly different CDR3 sequences, suggesting that clonal drift was not associated with a conserved TCRγ homology. We extended this observation by describing patients in whom a clonal switch takes place, from a KIR-restricted NK population to a dominant T monoclonal population, with no further demonstration in some cases of the initial NK clonal population. We also presented data on a patient who completely recovered from the disorder, with normal NK- and T-cell values, a non-restricted KIR expression cellular pattern, and polyclonal TCRγ rearrangement. The possibility of a complete recovery of CLPD-NK or T-LGLL had already been previously reported.[42,43] Taken together, these data are consistent with the suggestion that the putative inciting factors accounting for these disorders still persist for many years, and possibly for the patient's entire life-span. This implies that: i) long-term follow up of these patients is required; and ii) the possibility that clinical features will worsen, even in indolent cases, can never be ruled out, as reported in the literature.[44,45]

Our data contribute significantly to the field of knowledge on chronic T- and NK-lymphoproliferative diseases. Since a common event is likely to trigger clonal expansion in both disorders, further studies are needed to investigate the factors driving both NK- and T-clonal expansion.

Acknowledgments

The authors would like to thank Professors A. and L. Moretta and Dr. M. Vitale for providing mAbs for KIRs flow cytometry analysis.

Funding

This study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC), Cariparo, Cariverona, Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica (MIUR) and Lions Club International. Fr.Pa. and S.C. received a fellowship from AIRC. A.T. and V.T. were recipients of a fellowship from Associazione Italiana contro le Leucemie, Linfomi e Mieloma (AIL) and Lions Club, Padua.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at REFERENCES.

References

1. Lamy T, Loughran TP, Jr. How I treat LGL leukemia. Blood 2011;117(10):2764-74.
2. Semenzato G, Marino F, Zambello R. State of the art in natural killer cell malignancies. Int J Lab Hematol 2012;34(2):117-28.
3. Chan WC, Foucar KM, Morice WG, Catovsky D, in WHO "Classification of Tumours of Haematopoietic and Lymphoid Tissues, T-cell large granular lymphocytic leukaemia", eds. Swerdlow SH, Campo E, Harris NL, Jaffe EJ, Pileri SA, Stein H et al. (IARC Press, Lyon) 2008; pp 272-3.
4. Villamor N, Morice WG, Chan WC, Foucar K, in WHO "Classification of Tumours of Haematopoietic and Lymphoid Tissues, Chronic lymphoproliferative disorders of NK cells", eds. Swerdlow SH, Campo E, Harris NL, Jaffe EJ, Pileri SA, Stein H et al. (IARC Press, Lyon) 2008; pp 274-5.
5. Semenzato G, Zambello R, Starkebaum G, Oshimi K, Loughran TP Jr. The lymphoproliferative disease of granular lymphocytes: updated criteria for diagnosis. Blood 1997; 89(1):256-60.
6. Zambello R, Semenzato G. Large granular lymphocyte disorders: new etiopathogenetic clues as a rationale for innovative therapeutic approaches. Haematologica 2009;94(10):1341-5.
7. Zambello R, Falco M, Della Chiesa M, Trentin L, Carollo D, Castriconi R et al. Expression and function of KIR and natural cytotoxicity receptors in NK-type lymphoproliferative disease of granular lymphocytes. Blood 2003;102(5):1797-805.
8. Epling-Burnette PK, Painter JS, Chaurasia P, Bai F, Wei S, Djeu JY et al. Dysregulation of NK receptor expression in patients with lymphoproliferative disease of granular lymphocytes. Blood 2004;103(9):3431-9.
9. Scquizzato E, Teramo A, Miorin M, Facco M, Piazza F, Noventa F et al. Genotypic evaluation of killer immunoglobulin-like receptors in NK-type lymphoproliferative disease of granular lymphocytes. Leukemia 2007;21(5):1060-9.
10. Gattazzo C, Teramo A, Miorin M, Scquizzato E, Cabrelle A, Balsamo M et al. Lack of expression of inhibitory KIR3DL1 receptor in patients with natural killer cell-type lymphoproliferative disease of granular lymphocytes. Haematologica 2010; 95(10): 1722-9.
11. Krueger A, Fas SC, Baumann S, Krammer PH. The role of CD95 in the regulation of peripheral T-cell apoptosis. Immunol Re 2003;193:58-69.
12. Zhang R, Shah MV, Yang J, Nyland SB, Liu X, Yun JK et al. Network model of survival signaling in large granular lymphocyte leukemia. Proc Natl Acad Sci USA 2008; 105:(42)16308-13.
13. Loughran TP Jr, Hadlock KG, Yang Q, Perzova R, Zambello R, Semenzato G et al. Seroreactivity to an envelope protein of human T-cell leukemia/lymphoma virus in patients with CD3- (natural killer) lymphoproliferative disease of granular lymphocytes. Blood 1997;90(5):1977-81.
14. Lamy T, and Loughran TP, Jr. Current concepts: large granular lymphocyte leukemia. Blood Rev 1999;13(4):230-40.
15. Lamy T, and Loughran TP. Large Granular Lymphocyte Leukemia. Cancer Control 1998;5(1):25-33.
16. Sokol L, Agrawal D, and Loughran TP, Jr. Characterization of HTLV envelope seroreactivity in large granular lymphocyte leukemia. Leuk Res 2005(4);29:381-7.
17. Zambello R, Loughran TP Jr, Trentin L, Pontisso P, Battistella L, Raimondi R et al. Serologic and molecular evidence for a possible pathogenetic role of viral infection in CD3-negative natural killer-type lymphoproliferative disease of granular lymphocytes. Leukemia 1995;9(7):1207-11.
18. Clemente MJ, Wlodarski MW, Makishima H, Viny AD, Bretschneider I, Shaik M et al. Clonal drift demonstrates unexpected dynamics of the T-cell repertoire in T-large granular lymphocyte leukemia. Blood 2011; 118(16):4384-93.
19. Epling-Burnette PK, Liu JH, Catlett-Falcone R, Turkson J, Oshiro M, Kothapalli R et al. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. J Clin Invest 2001;107(3):351-62.
20. Teramo A, Gattazzo C, Passeri F, Lico A, Tasca G, Cabrelle A et al. Intrinsic and extrinsic mechanisms contribute to maintain the JAK/STAT pathway aberrantly activated in T-type large granular lymphocyte leukemia. Blood 2013;121(19):3843-54.
21. Koskela HL, Eldfors S, Ellonen P, van Adrichem AJ, Kuusanmäki H, Andersson EI et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. N Engl J Med 2012;366(20):1905-13.
22. Jerez A, Clemente MJ, Makishima H, Koskela H, Leblanc F, Peng Ng K et al. STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T cell large granular lymphocyte leukemia. Blood 2012;120(15): 3048-57.
23. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R et al. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 1995; 182(3):875-84.
24. Kang YH, Park CJ, Seo EJ, Huh J, Kim SB, Kang YK et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer 2002;94(12):3073-82.
25. Williams WV, Fang Q, Demarco D, VonFeldt J, Zurier RB, Weiner DB. Restricted heterogeneity of T cell receptor transcripts in rheumatoid synovium. J Clin Invest 1992;90(2):326-33.
26. Johansson C, Jeddi-Tehrani M, Grunewald J, Tengvall Linder M, Bengtsson A, Halldén G et al. Peripheral blood T-cell receptor beta-chain V-repertoire in atopic dermatitis patients after in vitro exposure to Pityrosporum orbiculare extract. Scand J Immunol 1999;49(3):293-301.
27. Chen Y, Chew CT, and Chan SH. T-cell receptor gene expression in tumour-infiltrating lymphocytes and peripheral blood lymphocytes of patients with nasopharyngeal carcinoma. Br J Cancer 1995;72(1): 117-22.
28. Choi YW, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. Proc Natl Acad Sci USA 1989;86(22):8941-5.
29. Posnett DN, Sinha R, Kabak S, and Russo C. Clonal populations of T cells in normal elderly humans: The T cell equivalent to "Benign Monoclonal Gammapathy". J Exp Med 1994;179(2):609-18.
30. Semenzato G, Pandolfi F, Chisesi T, De Rossi G, Pizzolo G, Zambello R et al. The lymphoproliferative disease of granular lymphocytes. A heterogeneous disorder ranging from indolent to aggressive conditions. Cancer 1987;60(12):2971-8.
31. Pandolfi F, Loughran TP Jr, Starkebaum G, Chisesi T, Barbui T, Chan WC et al. Clinical course and prognosis of the lymphoproliferative disease of granular lymphocytes. A multicenter study. Cancer 1990;65(2):341-8.
32. Scquizzato E, Zambello R, Teramo A, Baesso I, Varotto S, Albergoni MP et al. KIR/HLA-I mismatching and risk of relapse in paediatric patients undergoing non-haploidentical allogeneic haematopoietic stem cell transplantation. Pediatr Transplant 2011;15(2):198-204.
33. Epling-Burnette PK, and Loughran TP, Jr. Survival signals in leukemic large granular lymphocytes. Semin Hematol 2003;40(3): 213-20.
34. Zambello R, Facco M, Trentin L, Sancetta R, Tassinari C, Perin A et al. Interleukin-15 triggers the proliferation and cytotoxicity of granular lymphocytes in patients with lymphoproliferative disease of granular lymphocytes. Blood 1997;89(1):201-11.
35. Hodge DL, Yang J, Buschman MD, Schaughency PM, Dang H, Bere W et al. Interleukin-15 enhances proteasomal degradation of bid in normal lymphocytes: implications for large granular lymphocyte leukemias. Cancer Res 2009;6(9):3986-94.
36. Zambello R, Trentin L, Pizzolo G, Bulian P, Masciarelli M, Feruglio C et al. Cell membrane expression and functional role of the p75 subunit of interleukin-2 receptor in lymphoproliferative disease of granular lymphocytes. Blood 1990;76(10):2080-5.
37. Rajala HL, Eldfors S, Kuusanmäki H, van Adrichem AJ, Olson T, Lagström S et al. Discovery of somatic STAT5b mutations in large granular lymphocytic leukemia. Blood 2013;121(22):4541-50.
38. Sekiguchi N, Matsuda K, Momose K, Makishima H, Ito T, and Ishida F. STAT3 Gene Mutations and the Association with Pure Red cell Aplasia in Large Granular Lymphocyte Leukemia. 54th ASH Annual Meeting and Exposition, Atlanta, GA, December 8-11, 2012. Abs#2668.
39. Fasan A, Kern W, Grossmann V, Haferlach C, Haferlach T, Schnittger S. STAT3 mutations are highly specific for large granular lymphocytic leukemia. Leukemia 2013; 27(7):1598-600.
40. Clemente MJ, Przychodzen B, Jerez A, Dienes BE, Afable MG, Husseinzadeh H et al. Deep sequencing of the T cell receptor repertoire in CD8+ T-large granular lymphocyte leukemia identifies signature landscapes. Blood 2013;122(25):4077-85.
41. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. J Exp Med 2006; 203(4):1033-43.
42. Zambello R, Loughran TP Jr, Trentin L, Rassu M, Facco M, Bortolin M et al. Spontaneous resolution of p58/EB6 antigen restricted NK-type lymphoproliferative disease of granular lymphocytes: role of Epstein Barr virus infection. Br J Haematol 1997;99(1):215-21.
43. Takeuchi M, Tamaoki A, Soda R, Takahashi K. Spontaneous remission of large granular lymphocyte T cell leukemia. Leukemia 1999;13(2):313-4.
44. Huang Q, Chang KL, Gaal KK, and Weiss LM. An aggressive extranodal NK-cell lymphoma arising from indolent NK-cell lymphoproliferative disorder. Am J Surg Pathol 2005;29(11):1540-43.
45. Roullet MR, and Cornfield DB. Large natural killer cell lymphoma arising from an indolent natural killer cell large granular lymphocyte proliferation. Arch Pathol Lab Med 2006;130(11):1712-4.