

High expression of the ILT2 (LIR-1) inhibitory receptor for major histocompatibility complex class I molecules on clonal expansions of T-large granular lymphocytes in asymptomatic patients

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Background and Objectives. The lymphoproliferative disorders of large granular lymphocytes (LGLD) are divided into two groups: T-cell type and NK-cell type. These entities may be either asymptomatic or associated with autoimmune manifestations (especially cytopenias). A number of surface receptors, expressed by NK-cells and some T-lymphocyte subsets repress cytotoxicity and cytokine production upon ligation with HLA class I molecules and are clonally expressed in these lymphoproliferative disorders. These cytotoxic lymphocytes can lyse erythroid progenitors *in vitro*, and the physiologic lower levels of HLA class I antigens on the erythroid lineage may contribute to this form of autoimmunity. It is conceivable that the clinical outcome of T-LGLD might be influenced by the expression of MHC class I inhibitory receptors.

Design and Methods. We analyzed the surface expression of these molecules, lectin-like heterodimers (CD94/NKG2A) or killer immunoglobulin (Ig)-like receptors (KIR) and another Ig-like inhibitory receptor, termed ILT2 or LIR-1 in CD8⁺ cells from 12 cases of $\alpha\beta$ T-LGLD using specific monoclonal antibodies.

Results. None of the LGLD cases had anemia and 11 of 12 patients remain asymptomatic. KIR and CD94/NKG2A expression was detected on CD8⁺ populations only in some cases of T-LGLD. By contrast, our observations revealed that ILT2 expression was markedly higher in CD8⁺ cells from LGLD patients than from healthy donors.

Interpretation and Conclusions. Expression of the ILT2 inhibitory receptor for HLA class I molecules on LGLD cells might indeed contribute to preventing their autoreactivity. Further studies are required to evaluate the expression/function of the ILT2 receptor in patients who eventually become symptomatic. The development of

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cytopenias in LGLD patients must involve other self-reactive activating receptors. Analysis of the expression and function of triggering NKR in LGLD needs to be carefully addressed.

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Key words: lymphoproliferative disease of granular lymphocytes, HLA, natural killer receptors

The heterogeneous lymphoproliferative disorders of large granular lymphocytes (LGLD) have been recently included in the REAL classification of lymphoid neoplasias.¹ They are divided into two groups: CD3 positive (T-cell type) and CD3 negative (NK-cell type), the former being more frequent.² The clinical impact of these entities is variable and they may be either asymptomatic or associated with cytopenias, autoimmune manifestations such as rheumatoid arthritis, neuropathies or recurrent infections.³⁻⁵ The Yorkshire Leukaemia Study Group proposed that LGLD might be more common than previously suspected; this group showed that most patients with persistent LGLD remain asymptomatic.⁶ Minor subpopulations of CD8⁺ clonal T-cells have been identified in healthy elderly persons.⁷ Some data suggest that clonal expansions of CD3⁺ LGLD may represent activated cytotoxic T-lymphocytes (CTLs): expression of perforin, Fas ligand,⁸ tumor necrosis factor (TNF) or TNF-receptor-related molecules.⁹ Moreover, non-MHC-restricted cytotoxic activity can be triggered in T-cell LGLD upon stimulation with anti-CD3, CD16 or with superantigens.¹⁰ Both CD3 positive and negative LGLD are phenotypically het-

erogeneous, but they usually express NK-cell-associated markers (i.e. CD16, CD56 or CD57).² Recently, it has been proposed that leukemic CD3⁺T-LGL represent the normal counterpart of CD3⁺CD8⁺CD57⁺ lymphocytes.¹⁰ This subset is frequently expanded *in vivo* after chronic antigen-specific stimulation of CD8⁺ T-cells due to viral infections, namely human immunodeficiency virus (HIV) and cytomegalovirus (CMV), or after allogeneic transplantation and displays the cytologic characteristics of LGLD. These expanded cell populations are not monoclonal although a remarkable restriction of TCR V β usage is found in CD8⁺CD57⁺ cells.¹¹ Moreover, differences in the expression of adhesion molecules (CD58, CD11c, CD54) between leukemic and oligoclonal cell populations have been reported.¹⁰ Recently, Handgretinger *et al.*¹² described a patient with red cell aplasia who had an expansion of large granular $\gamma\delta$ T-lymphocytes, expressing HLA-specific inhibitory receptors. The study showed that these cytotoxic lymphocytes could lyse erythroid progenitors *in vitro*, and proposed that the physiologic lower levels of HLA class I antigens on the erythroid lineage may contribute to this form of autoimmunity. A number of surface receptors, expressed by NK-cells and some T-lymphocyte subsets repress cytotoxicity and cytokine production upon ligation with HLA class I molecules. These inhibitory molecules are either lectin-like heterodimers (CD94/ NKG2A) or killer immunoglobulin (Ig)-like receptors (KIR);¹³ remarkably, both molecular families include homologous proteins that display a triggering role. Another Ig-like

inhibitory receptor, termed ILT2 or LIR-1, which interacts specifically with different HLA class I molecules and with a human cytomegalovirus protein (UL18) has been identified.^{13,14} It is conceivable that the clinical outcome of T-LGLD might be influenced by the expression of MHC class I inhibitory receptors. We analyzed the surface expression of these molecules in CD8⁺ cells from 12 cases of $\alpha\beta$ T-LGLD using specific monoclonal antibodies (MAbs). KIR and CD94/NKG2A expression was detected on CD8⁺ populations only in some cases of T-LGLD. By contrast, our observations revealed that ILT2 expression was markedly higher in CD8⁺ cells from LGLD patients than from healthy donors. Further studies are required to evaluate the expression/function of the ILT2 receptor in patients who eventually become symptomatic.

Design and Methods

Patients

Twelve patients with T-type LGLD were identified during standard screening of lymphocytosis according to the recently redefined diagnostic criteria¹⁵ with lymphocyte counts from 2,000 to 14,800/ μ L. All cases had granular lymphocytes in peripheral blood smears, a clonal T-cell population defined by T-cell receptor (TCR) rearrangement, and the presence of NK-associated markers such as CD16, CD56 or CD57 cells. Clinical, hematologic and immunophenotypic features are detailed in Tables 1 and 2. Two patients with NK-type LGL, one patient with chronic polym-

Table 1.

NID	Age (yr)	Sex (M/F)	Past medical history	Leukocyte count (x 10 ⁹ /L.)	Lymphocytes (%)	Follow-up (months)	CD3 %	CD8 %	HP-3B1 *	Z199 *	HP-F1 *	5.133 *	GL183 *	HP-3E4 *	DX9 *
3	82	M		13,4	48	23	83	68	42	0,4	87	5	4	5	
6	22	M		5,2	38	114	62	54	35	1	35	9	18	23	4
9	58	M		13,4	70	11	89	83 ^{dim}	3,2	0,4	67	2	3	0,5	0,3
11	76	M		15,1	38	9	88	62	38	4	74	7	5	5	
12	68	F		8,1	54	9	64	47	8	0,3	90	1	2	0,3	
13	75	M		12,1	47	10	90	59	79	11	78	19	23	13	
14	37	M		10,3	50	8	63	44	74	15	60	24	23	35	2
15	52	M		8,3	52	28	92	36	47	22	59	18	12	7	3
16	38	M	Liver transplantation 8 months previously	19,2	77	24	65	60	5	0,3	84	0,7	1	0,2	0,3
18	45	F	BMT because of MDS 4 months previously No GVHD.	6,6	46	16	81	62	16	3	96	5	4	7	5
19	72	F		9,5	41	27	88	40	65	4	81	22	15	15	
20	78	M		4,6	25	3	77	44	46	20	69	25	17	13	2
Mean (S.D.)									38 (26)	7 (8)	74 (17)	12 (9)	11 (8)	11 (10)	6 (7)
Controls (n=23) Mean (S.D.)									6 (10)	5 (5)	26 (21)	5 (7)	9 (5)	7 (11)	3 (3)

*Numbers correspond to the percentage of CD8^{right} cells stained with each monoclonal antibody. In case #9 the percentage is referred to the CD3 cells, as the immunophenotype was CD3⁺ CD8^{dim}. All cases were CD3⁺, TCR $\alpha\beta$ ⁺, CD2⁺, CD5⁺, CD7⁺ CD8⁺ and CD57⁺ (data not shown) except in case #9 that display a CD3⁺ CD7⁺ CD8^{dim} phenotype. Monoclonal antibodies tested were: HP-3B1 (antiCD94 IgG 2a), Z199 (anti CD94-NKG2A), HP-F1 (anti ILT2), GL183 (anti p58.2 and p50.2 KIR), HP-3E4 (anti p58.1 and p50.1 KIR), DX9 (anti p70 KIR), and 5.133 (anti p70/p140 KIR) (Table 1). It is of note that some KIR-specific monoclonal antibodies may cross-react with homologous triggering and inhibitory receptors.

Table 2. Clinical and hematologic features of LGLD.

Feature	Number of affected patients (N=12)
B-symptoms	0/12
Enlarged liver or spleen	0/12
Adenopathies	0/12
Abnormal karyotype	0/2
High $\beta 2$ microglobulin	4/9
Anemia	1/12
Thrombocytopenia ($<10^5/\mu\text{L}$)	1/12
Neutropenia ($\text{ANC} < 1.5 \times 10^9/\text{L}$)	1/12
Lymphoid bone marrow infiltration	8/8
Polyclonal hypergammaglobulinemia	5/12
Positive Coombs' test	0/7
Rheumatoid factor	2/12

phocytic T-cell leukemia (T-PLL/T-PL) and 23 healthy donors were studied as a control group.

Immunofluorescence analysis and clonality assays

Heparinized peripheral blood samples were drawn. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation, washed in PBS and frozen in a medium containing 40% fetal calf serum and 10% dimethylsulfoxide. After thawing, cells were stained with HP-3B1 (antiCD94 IgG 2a),¹⁶ Z199 (antiNKG2A), HP-F1 (antiILT-2/LIR 1),¹⁴ GL183 (which recognised both p58.2 and p50.2, gene KIR2DL2/KIR2S2),¹⁷⁻¹⁹ HP-3E4 (which recognised p58.1, p50.1, p50.3, p58.2, p50.2 gene KIR2DL1/KIR2DS1/KIR2DS4/KIR2DL2/KIR2S2),²⁰ DX9 (which recognised p70/NKB1 gene KIR3DL1),²¹ and 5.133 (which recognised p70, p50.3, p140 gene KIR3DL1/KIR3DL2/KIR2DS4)²² followed by FITC conjugated goat anti mouse IgG1 and counter-stained with CD8 PE, CD4 PE, CD56 PE or CD3 PerCP (Becton Dickinson) depending on the immunophenotype profile observed in preliminary studies (Table 1). Stained cells were analyzed by flow cytometry on a FacSort (Beckton Dickinson) using LYSIS II or Cell Quest software. Percentages of NKR positive cells within the CD3 and CD8 subsets from T-LGLD cases and healthy subjects were shown. T-cell receptor (TCR) rearrangements were assessed by means of polymerase chain reaction using TCR γ primers as previously described²³ in all fifteen cases of T-type LGLD, NK-type LGLD and T-CLL/T-PLL.

Results

Patients

Twelve T-type LGLD patients (9 males and 3 females) and two NK-type LGLD patients (1 male, 1 female) were studied. Their median age was 68 years (range: 22-82) at the time of diagnosis. The median

follow-up period was 15 months (range: 3-113). The disorder arose without any significant prior medical history except for two cases. The first patient (NID 18) presented this disorder after an allogeneic bone marrow transplantation while being in complete remission of the hematologic malignancy and with complete donor chimerism; the other patient (NID 16) developed T-type LGLD during immunosuppressive therapy for liver transplantation. All but two patients have remained asymptomatic. Case number 6 had mild neutropenia (10^9 neutrophils/L), mild thrombocytopenia ($50 \times 10^9/\text{L}$) and recurrent infections; patient number 19 had concomitant iron-deficiency anemia. They had neither B-symptoms, lymph nodes, hepatomegaly nor splenomegaly. Bone marrow specimens were obtained in eight cases and all of them showed interstitial T-lymphocytosis. No progression to aggressive lymphomas has been observed during the follow-up.

Immunologic and molecular features

Clonality was established by analyzing the TCR rearrangement in all CD3 LGL patients and in the patient with T-CLL/T-PL. All cases expressed an $\alpha\beta$ TCR and exhibited the typical CD4⁻ CD8^{+bright} CD57⁺ profile except for one case (patient #9), who had a CD4⁻ CD8^{+dim} phenotype.

The proportion of cells bearing inhibitory receptors was referred to the CD8 subset in all cases except in patient #9 in whom it was referred to the CD3 subset. The ILT-2 antigen expression was detected on a high fraction of CD3⁺ CD8⁺ cells in all cases, with a mean of 74% (range 35-96%). CD94 was detected in samples from 8 out of 12 patients, whereas KIR were either not detected or their expression levels were similar to those observed in the control group. Both CD94 and ILT-2 were expressed at higher levels than in controls (Figures 1 and 2). ILT-2 expression was similar in both patients with past medical history of allogeneic transplantation and immunosuppressive therapy (84 and 96.% in CD8⁺ cells). It is of note that the only symptomatic patient (case #6) showed the lowest level of ILT2 expression. T-PLL cells (CD4⁺ cells) were negative for all NKR. In contrast, NK-LGL cells strongly displayed different NKR, especially CD94/NKG2A, which were detectable in >90% of the cells (Table 3).

Discussion

The actual prevalence of clonal LGLD is unknown. We have studied twelve cases of T-type LGLD who had mild or no symptoms, moderate increments of lymphocyte count and no progression to aggressive disease during a median follow-up period of 15 months. This clinical setting argues in favor of a dis-

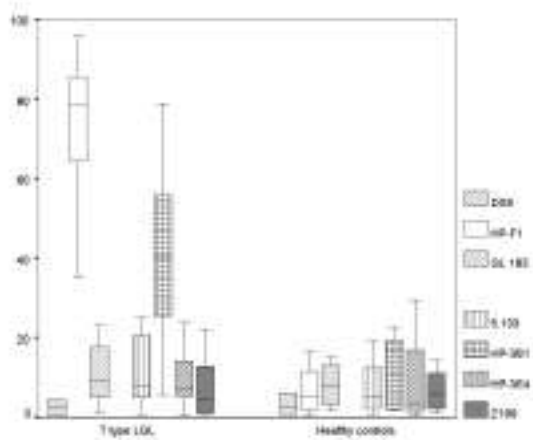


Figure 1. Expression of HLA-specific NKR: a comparison between T-LGLD patients and healthy controls. Data are displayed as the mean % positive cells, standard deviation and range. Monoclonal antibodies tested were HP-3B1 (antiCD94 IgG 2a),¹⁶ Z199 (anti NKG2A), HP-F1 (anti ILT-2 / LIR 1),¹⁴ GL183 (which recognizes both p58.2 and p50.2, gene KIR2DL2/KIR2S2)¹⁷⁻¹⁹ HP-3E4 (which recognizes p58.1,p50.1,p50.3, p58.2, p50.2 gene KIR2DL1/KIR2DS1/KIR2DS4/KIR2DL2/KIR2S2),²⁰ DX9 (which recognizes p70/NKB1 gene KIR3DL1),²¹ and 5.133 (which recognizes p70, p50.3, p140 gene KIR3DL1/KIR3DL2/KIR2DS4).²²

order that could be more frequent than previously suspected. Indeed, the Yorkshire Leukaemia Study Group performed a large survey involving almost 900 healthy adult blood samples, showing that LGL populations with NK-cell-associated markers are quite frequent²⁴ and that there is an apparently increasing incidence with age. These populations would be, as our cases, phenotypically heterogeneous, although there is a predominance of the CD4⁻ CD8^{+bright} phenotype.

As clonality does not necessarily imply malignancy, the usage of the term *leukemia* in these LGL disorders remains controversial. Furthermore, while the presence of more than 2,000 granular lymphocytes / μ l for at least 6 months was initially considered as a required diagnostic criterion,²⁵ more recent reports have outlined the fact that there are patients with the same clinical, immunophenotypic and molecular features but with normal or low granular lymphocyte counts.¹⁵ The boundaries between a normal aging event and pathology need to be defined.

If it is difficult to ascertain the real incidence of

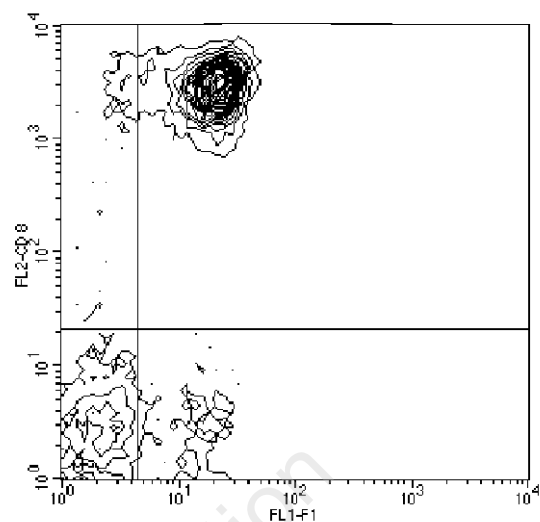


Figure 2. Two-color immunofluorescence analysis of HP-3F1 anti ILT2 MoAb reactivity in peripheral blood lymphocytes of a LGLD patient (NI 12). It is evident that the majority of circulating CD8⁺ cells co-expressed ILT2.

these disorders, it is even more complicated to determine the incidence of the clinical impact of these entities as most reports are biased, to at least some extent, by including those cases that present with symptoms or higher lymphocyte counts. Thus, the clinical spectrum has ranged from asymptomatic subjects with an uneventful course to patients with peripheral cytopenias (pure red cell aplasia, hemolysis or myelodysplastic features) and a variety of associated conditions including rheumatoid arthritis, neuropathies, solid and hematologic tumors, endocrinopathies and solid organ or hematopoietic precursors transplantation.²⁻⁶ Our twelve cases fulfilled the recently updated criteria for LGL disorders, i.e. they arose in a compatible clinical setting, and had an increased proportion of granular lymphocytes in peripheral blood smear with NK-associated markers, mainly CD57 and clonal TCR rearrangements. Bone marrow was affected in eight of the studied cases. Eleven subjects were asymptomatic, clinical features did not change from the time of diagnosis to the time of last contact in any patient and only one patient

Table 3.

NID	Age (yr)	Sex (M/F)	Disease	Leukocyte Count ($\times 10^9/L$)	% Lymphocyte	% CD56	HP-3B1 *	Z199 *	HP-F1 *	5.133 *	GL183 *	HP-3E4 *
1	58	F	NK-LGL	18	60	96	94	94	36	89	4	58
2	26	M	NK-LGL	5,6	50	26	49	41	26	26	20	12
4	55	F	T-PLL	100	95		0,5	0,25	1,3	4,5	0,45	2,07

*Numbers correspond to the percentage of CD56 cells stained with each monoclonal antibody. In case no. 4 the percentage is referred to the CD4 cells, as the immunophenotype was CD3⁺ CD4⁺. Monoclonal antibodies tested were HP-3B1 (antiCD94 IgG 2a) Z199 (antiCD94-NKG2A), HP-F1 (antiILT2), GL183 (anti p58.2 and p50.2 KIR), HP-3E4 (anti p58.1 and p50.1 KIR), DX9 (anti p70 KIR), and 5.133 (anti p70/ p140 KIR) (Table I). It is of note that some specific monoclonal antibodies may cross-react with homologous triggering and inhibitory receptors.

(NID 6) required treatment because of associated neutropenia and soft tissue infections. The expression of CD56, which some authors have related to an adverse outcome,²⁶ was found in 11 out of 12 cases, who have an uneventful course. Recently Handgretinger *et al.*¹² suggested the possibility that a clone of cytotoxic large granular lymphocytes expressing killer-cell inhibitory receptors can destroy erythroid progenitors *in vivo* due to the physiologic down-regulation of HLA class I antigens in the erythroid lineage. Natural killer and some T-lymphocyte subsets regulate cytotoxic activity by sensing the expression of HLA class I molecules on target cells by means of different cell-surface receptors.^{27,28} Two receptor families have been identified: the C-type lectin molecules (which include CD94 and NKG2)^{29,30} and molecules belonging to the Ig superfamily known as killer-cell inhibitory receptors (KIR). The KIR family includes about 12 genes and is subdivided depending on the structure (two or three Ig domains) and the length of the cytoplasmic tail. Typically, KIR with a long cytoplasmic tail deliver an inhibitory signal, while KIR with a short cytoplasmic tail may activate NK- and T-cell responses. It is of note that an important limitation to studying NKR by serologic methods is that the same antibodies (i.e. GL183, EB6, CH-L) may cross-react with functionally different homologous molecules (KIR2DL/p58 and KIR2DS/p50).¹⁹ Another protein family homologous to KIR has been termed Ig-like-transcript (ILT) or leukocyte Ig like receptor (LIR). The ILT2 / LIR 1 molecule functions as an inhibitory receptor specific for HLA class I molecules, and is widely distributed on subsets of NK-cells, T-cells, B-cells and myelomonocytic cells.¹⁴ Hitherto, just KIR2DL/p58 and KIR2DS/p50 molecules have been described on the surface of cells from patients with T- and NK-type LGL disorders.^{15,31,32} As NK-type LGL do not rearrange TCR, the presence of discrete NK subsets defined by the homogeneous expression of GL183 and EB6 molecules,¹⁵ has been proposed to constitute an indication of the clonal nature of these

disorders.³² A minority of T-type LGLD (4 out of 44) were found to be positive when studied with GL183 (p58.2 and p50.2 KIR), EB6 (p58.1 and p50.1 KIR) and CH-L (p58.2 and p50.2 KIR) antibodies,³¹ but clonal and uniform expression have been reported in NK-LGLD patients using selective immunoanalysis.³² In functional assays KIR appeared either to inhibit or to activate.³¹ In the present study, we searched for the presence of known NKR. First, our data revealed a clear dissociation between the expression of CD94 and the NKG2A inhibitory subunit on LGLD T-cells. CD94 may be coupled to triggering members of the NKG2 family (NKG2C, NKG2E/H). Specific reagents to identify these receptors are required to address the question. Alternatively, it is conceivable that CD94 may be expressed on the surface as a non-functional homodimer, as was originally shown in transfected cells.³⁰ Indeed, expression of non-functional CD94 in the absence of NKG2-A or NKG2-C has been detected in CD8⁺ T-cells from myeloma patients by Besostri *et al.*³³ More importantly, expression of ILT2 was systematically detected on the surface of T-type LGL. ILT2 is known to be expressed not only by NK- and T-cells, but also by B- and myelomonocytic cells. ILT2 binds class I molecules and delivers a negative signal that inhibits killing by NK- and T-cells and Ca²⁺ mobilization in B- and myelomonocytic cells triggered via the B-cell receptor and HLA-DR. The clinical outcome of T-LGLD might indeed be influenced by the expression of MHC class I inhibitory receptors. Functional analysis of CD8⁺CD57⁺ cells from LGLD indicates that these lymphocytes have cytotoxic potential.¹⁰ Though KIR are detectable on a T-cell subset, Cambiaggi *et al.*³¹ reported that only a minority of cases (4 out of 44) of CD3⁺LGLD^{31,32} expressed these molecules and, yet, all of them were asymptomatic. Expression of the ILT2 inhibitory receptor for HLA class I molecules on LGLD cells might contribute to preventing their putative autoreactivity; thus, further studies are required to evaluate the expression/function of the ILT2 receptor in patients who eventually

become symptomatic. As a preliminary approach, we performed a rADCC assay in LGL cells from two asymptomatic cases in order to determine whether signaling via ILT2 prevents CD3-induced cytotoxicity against the FcγR-bearing p815 cell line. No inhibition was detected; yet, the implication of this finding is uncertain as it has been reported that some normal T-cells may be also unresponsive to ILT2 signaling in this assay.¹⁴

On the other hand, inhibitory receptors are considered to establish a threshold for cellular activation that can be overcome by stimulatory signals. The fact that none of the LGLD cases had anemia, despite the fact that their erythroid progenitors had predictably undergone the physiologic downregulation of HLA class I antigens,¹² supports the hypothesis that development of cytopenias involves other self-reactive activating receptors. Analysis of the expression and function of triggering NKR34 in LGLD needs to be carefully addressed.

Contributions and Acknowledgments

LFC and EG: design, flow cytometry studies, clinical data and writing the paper. PA: performed the molecular analysis experiments. FN: flow cytometry and cytotoxicity studies. MCMF: responsible for the management of the patients. MLB: analysis of data, review and final approval.

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Disclosures

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Manuscript processing

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Potential implications for clinical practice

- Expression of the ILT2 inhibitory receptor for HLA class I molecules on LGLD cells might indeed contribute to preventing the autoreactivity.
- The development of cytopenias in LGLD patients must involve activating and inhibiting receptors, and their function and expression needs to be carefully addressed.

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