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The raft marker GM1 identifies functional subsets of granular lymphocytes in patients with CD3+ lymphoproliferative disease of granular lymphocytes

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The raft marker GM1 is expressed at very low levels at the plasma membrane of resting T cells (GM1^{dull}). *In vitro* **T-cell activation induces synthesis of this lipid, which is then expressed at very high levels (GM1^{bright}) at the membrane of activated/effector cells. By flow cytometry and confocal microscopy, we analyzed the expression and organization of GM1 in a series of 15 patients with CD3$^+$ lymphoproliferative disease of granular lymphocytes (LDGL). We found that GM1^{bright} GL were detectable in fresh blood samples obtained in all LDGL patients, although the range of brightly stained cells was extremely variable. This distinctive** *in vivo* **pattern has never been shown in T lymphocytes from healthy individuals or in patients with different chronic T or B lymphoproliferative disorders or active infectious diseases. The low number of cycling cells detected in LDGL patients was always included within the GM1^{bright} GL population. Interestingly, GM1^{bright} GL were demonstrated to contain a higher amount of IFN-γ as compared to GM1^{dull} GL. These findings allow to distinguish subsets of GL at different levels of activation within the monoclonal CD3$^+$ population. The GM1^{bright} GL subset is likely to be responsible for the renewing of GL and thus for maintaining chronic proliferation.**

Introduction

The lymphoproliferative disease of granular lymphocytes (LDGL) is a disorder characterized by the chronic proliferation of granular lymphocytes (GL) belonging, in the majority of cases, to the T-cell lineage, that is, CD3$^+$CD16$^+$ T lymphocytes.[1–3] Current concepts on the pathogenesis of this disease point out that proliferating cells represent *in vivo* primed cytotoxic T lymphocytes, triggered by a still unknown (viral?) antigen[3,4] and then maintained by cytokines, notably IL-15.[5] Several lines of evidence are consistent with this interpretation, including the use of a restricted TCRVβ repertoire by patients' GL,[6] the pattern of cytotoxic molecules expressed by cells and the resistance to Fas-mediated cell lysis.[7] Several viral agents have been associated to this disorder, such as EBV- and HTLV-correlated retrovirus.[8,9]

Plasma membranes are equipped with specialized lipid microdomains, rich in cholesterol and glycosphingolipids (such as GM1), called rafts.[10–12] These structures are considered 'signaling compartments' where specific molecules are concentrated while others are excluded.[13] Indeed, a variety of signaling molecules are accumulated in raft domains, including the two Src family kinases Lck and Fyn, both implicated in T-cell activation, the T-cell linker molecule LAT, monomeric and heterotrimeric G proteins, G-coupled protein receptors and molecules involved in Ca^{2+} responses.[14–17] It has been shown that resting and *in vitro* activated human T cells differ in terms of the expression and the subcellular distribution of the raft marker GM1, with activated T cells expressing higher levels of this molecule at the plasma membrane.[18,19] However, whether the upregulation of GM1 in activated T cells occurs even *in vivo* and, more interestingly, could be used for the recognition of differently activated cells is not known.

To address this hypothesis, using flow cytometry and confocal microscopy, 15 patients with CD3$^+$ LDGL were studied for the expression and organization of lipid raft marker GM1. We showed that GM1 is upregulated at the plasma membrane of proliferating GL from LDGL patients, enabling to distinguish two cell populations with different functional properties within the monoclonal GL homogeneously expressing NK-related CD16 and/or CD56 markers. These results are consistent with an *in vivo* pattern of activation, resulting in a small subset of cells within GM1^{bright} GL likely involved in the continuous renewal of the GL proliferation.

Patients and methods

Patients

In all, 15 patients (seven male and eight female, mean age 58 ± 12 years) were studied. In all cases, a chronic lymphocytosis (lasting more than 6 months) sustained by at least 2000 GL/mm^3 was present in the peripheral blood.[20] At the time of study none of the patients had received treatment, with the only exception of patient #5 who was receiving methotrexate therapy for an associated disease (idiopathic pulmonary hypertension). Using Southern blot analysis, all CD3$^+$ LDGL patients were found to be monoclonally rearranged for TCRβ and/or γ genes. Relevant immunological data of cases under study are shown in Table 1. As controls, 22 age-matched individuals were included in the study.

Monoclonal antibodies

The commercially available FITC-, phycoerythrin (PE)- and PeCy5-conjugated monoclonal antibodies (mAbs) used were from Becton Dickinson (Sunnyvale, CA, USA) and Immunotech (Marseille, France), and included anti-CD3 (Leu4), anti-CD4 (Leu3), anti-CD8 (Leu2a), anti-CD16 (Leu11c), anti-CD56 (Leu19) and anti-CD57 (Leu7). The TCRVβ repertoire was investigated in all patients using the TCRVβ repertoire kit (Immunotech, Marseille France). The raft marker GM1 was analyzed using FITC-labeled cholera toxin B (CTB) subunit

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Table 1 Phenotypic analysis of LDGL patients

Case #	GL × 10³/mm³	CD3%	CD16%	CD56%	CD57%	CD94%	NKG2A%	NKG2D%	RelevantTCRVβ[a]	GM1bright%[b]
1	6.4	93	42	63	57	4	4	55	13.1	51
2	8.7	95	16	75	65	3	3	7	1	29
3	4.7	97	13	83	79	2	2	59	2	10
4	6.1	94	44	2	39	3	2	5	8	4
5	4.2	87	5	52	53	2	2	71	13.1	11
7	7.6	94	69	1	45	41[c]	2	75	14	4
8	16.6	96	58	2	52	2	2	65	20	12
9	2.1	89	56	1	3	1	1	61	14	7
10	4.7	88	41	50	55	3	3	70	20	5
11	4.6	89	56	19	38	65[c]	1	42	ND	20
12	3.5	87	47	13	33	2	2	67	8	6
14	5.7	89	56	19	38	1	1	67	13.1	5
15	2.0	73	16	27	32	1	1	51	21.3	4
CTR (n:22)	0.4±0.2	75±6	13±3	14±4	17±6	12±5	12±4	35±3	ND	0

[a]Expression of relevant TCRVβ receptor in >60% of GL.
[b]Percentage of GM1bright expression is referred to the percentage of pathological GL.
[c]GL expressed the heterodimer CD94/NKG2C as detected with the mAb P25.
ND = not determined.

(Sigma), as described.[21] Anti-CD94 (Xa185 mAb), anti-NKG2A (Z270 mAb) and NKG2D (BAT221 mAb) were kindly provided by A Moretta (Genova, Italy).

Flow cytometry analysis

The expression of the above-mentioned antigens on GL was assessed by flow cytometry analysis using direct or indirect immunofluorescence assay combining two or three fluorescences, as described previously.[5] A gate on pathological GL, identified according to the abnormal phenotypic profile (CD3$^+$CD16$^+$TCRVβ$^+$CD57$^+$ or CD3$^+$CD56$^+$TCRVβ$^+$CD57$^+$), was defined for each case and the expression of antigens of interest was analyzed only on this cell subset. NK cells were excluded from the gate of analysis, taking advantage of the lack of CD3 antigen, which is on the other hand expressed on pathological GL. Cells were scored using a FACScan® analyzer (Becton Dickinson), and data were processed using CELLQuest (Becton Dickinson).

Intracellular detection of IFN-γ

Patients' cells were sorted according to the CTB pattern of using FACSCalibur Cell Sorter (Becton Dickinson). The resulting cell populations were pure at 99% for each sorted subset (ie CTB^{high} and CTB^{dull}). The decision regarding the cutoff point for separating the two subsets of GM1 cells was obviously crucial. Taking advantage of the finding that GM1^{dull} lymphocytes are present in all control subjects in a stable manner, we calculated the mean channel of expression of GM1^{dull}-positive cells in normal individuals using flow cytometry. This value was highly stable, not only for control subjects but also for patients studied, as demonstrated by overlying the histograms of patients and controls (not shown). Accordingly, the cutoff for the expression of GM1^{dull} was defined considering the highest channel of expression detected in normal and pathological lymphocytes as upper limits.

For the intracellular expression of IFN-γ, cells were fixed using Fix and Perm kit (Caltag) according to the manufacturer's instructions. Briefly, cells were fixed using reagent A for 15 min

at room temperature, washed in PBS and then permeabilized with reagent B and stained with PE-conjugated anti-IFN-γ (Beckton Dickinson) for 30 min at room temperature. Data were processed using the CellQuest software program (Becton Dickinson Immunocytometry Systems). The amount of cytokine expression was quantified by calculating the increase of the mean fluorescence intensity (MFI) value of the positively stained sample as compared to the MFI value of isotype control, indicated as MFI ratio.

Cell cycle analysis

The percentage of cells in replication phases was evaluated in sorted GM1^{high} and GM1^{dull} cell populations by staining with propidium iodide (PI), in 70% ethanol fixed cells. Cells were washed with PBS, fixed with 70% cold ethanol for 30 min at 4°C, and incubated with a solution containing 50 μg/ml PI and 1 mg/ml Rnase (Sigma Chemical Co, St Louis, MO, USA). The tubes were placed at 4°C in the dark for 30 min before analysis using flow cytometry to discriminate cells in each phase of cell cycle.

Confocal microscopy

Cells were immobilized on poly-L-lysine-coated slides, fixed in 2% paraformaldehyde and permeabilized in 0.1% Triton X-100 before staining with FITC-labeled CTB or with a mouse mAb specific for Lck (MOL171, Pharmingen) followed by an FITC-labeled goat anti-mouse IgG1 (Southern Biotechnology Associates), as described.[21,22] The nuclei were stained with PI.

Results

Patients' GL express GM1bright. Table 1 reports the phenotypic analysis of the patients examined in this study. In all patients, a variable but well-defined fraction of GL expressed the raft marker GM1 at an intensity higher than normal resting T cells. A comparative GM1 expression in two representative LDGL patients and a normal control is shown in Figure 1. To
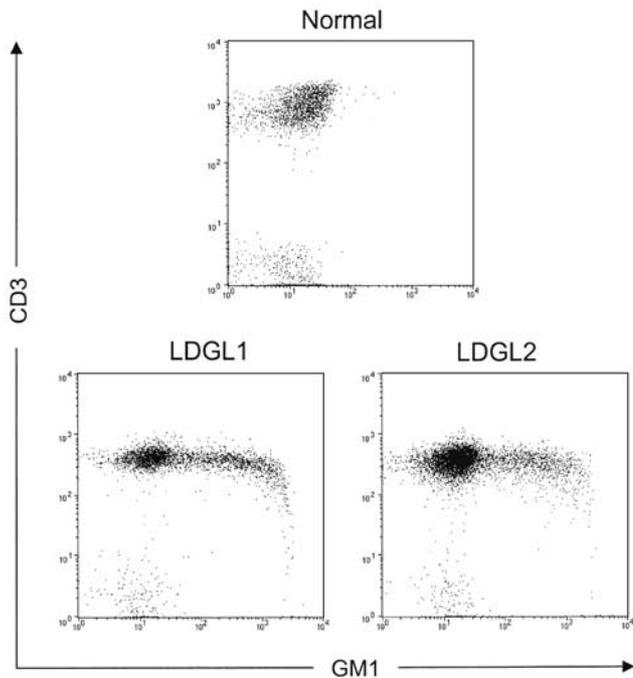
Figure 1 High expression of GM1 at the plasma membrane of proliferating GL from LDGL patients. FACS analysis of PB lymphocytes from a healthy donor (normal) and two LDGL patients, showing the presence of CD3$^+$GM1^{bright} cells in the peripheral blood of LDGL patients. Cells were stained with PE-labeled anti-CD3 and FITC-labeled CTB.

Figure 2 Normal CD3 + CD8^{bright} + CD16 + lymphocytes were characterized by the expression of GM1^{dull}. A gate was designed around CD3 + CD8^{bright} + CD16 + lymphocytes, which can be detected in a discrete number of normal individuals, usually comprising 2–3% of the total lymphoid population. Results from two representative normal controls are shown. In both cases, within the gated areas (gate 1 for control #1 and gate 2 for control #2), the selected cell population showed virtually no GM1bright expression (<0.01).

investigate whether this phenotype was restricted to patients with LDGL, we analyzed peripheral blood lymphocytes obtained from healthy donors (22 cases), patients with infectious disease (eight active infectious mononucleosis and two acute cytomegalovirus infections), patients with lymphoproliferative disorders of B lymphocytes (29 chronic lymphocytic leukemia, three hairy cell leukemia and 21 leukemic phase of non-Hodgkin's lymphomas, namely mantle cell lymphomas, marginal zone lymphomas and follicular lymphomas) and with different lymphoproliferative disorders of T lymphocytes (seven cases CD4$^+$ CLL). In all the cases tested, the percentage of GM1^{bright} peripheral blood T cells was undetectable, thus pointing out that the presence of GM1^{bright} lymphocytes in the blood is a highly uncommon event. We would like to emphasize the evidence that, even if the number of GM1^{bright} T cells in some LDGL patients is low (5% of GL), a similar percentage has never been detected on T lymphocytes in the peripheral blood in any other condition (normal, reactive or neoplastic) explored, pointing to the high peculiarity of this finding for LDGL. As further proof, we analyzed the expression of GM1 on the small subset of CD3 + CD8 + CD16 + cells, which could be detected in a discrete number of normal individuals. As reported in Figure 2, virtually no GM1^{bright} expression can be reported within this cell subset.

Using a combination of mAbs, we were able to define that the GM1^{bright} phenotype was restricted to the proliferating GL identified on the basis of the aberrant phenotypic expression (Figure 3). Apart from the different expression of GM1, the population of CD3 + TCRβV + expressing NK-related markers was homogeneous. No expression of activation antigens, as CD69, CD27 or CD25 could be documented (not shown).
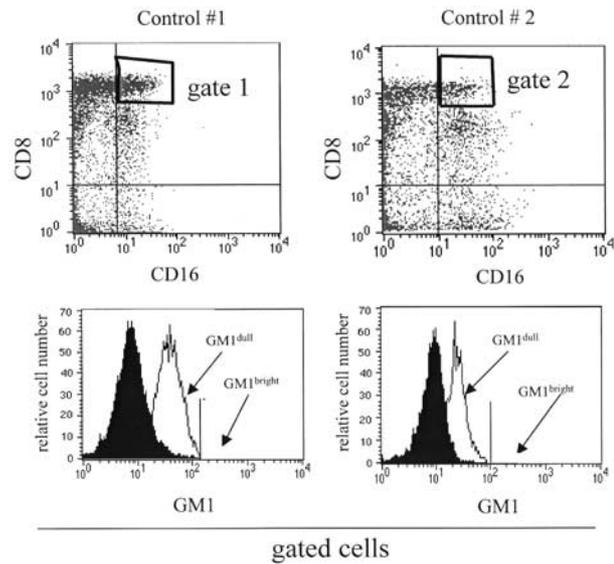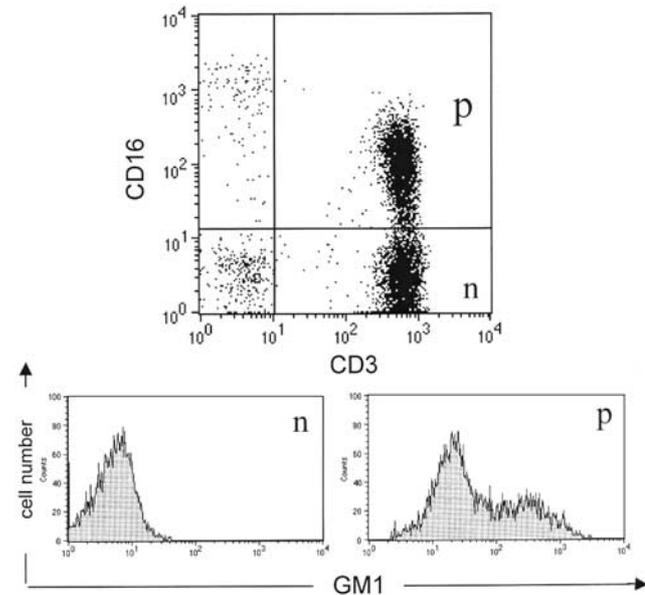

Figure 3 CD3$^+$GM1^{bright} cells are the proliferating (p) GL (CD3$^+$CD16$^+$). No CD3$^+$GM1^{bright} cells were found in the normal counterpart (n) from the same patient. Data consistent with these results were obtained in all 15 LDGL patients studied.

Expression of GM1^{bright} on GL surface is stable during follow-up of patients

We investigated whether the presence of GM1^{bright} GL was stable during the course of disease, thus representing a specific

marker for detecting a persisting functional state of GL in these patients. Two cases were studied, who were characterized by high percentages of GM1^{bright} GL. This feature allowed the detection of even small differences during time. As reported in Figure 4, the expression of GM1^{bright} in GL was consistent during time along a period of up to 7 years. Since the presence of GM1^{bright} on T lymphocytes reflects a dynamic condition of these cells,[19] it is conceivable that this finding could be related to a continuous renewal of circulating cells sustained by GM1^{bright} GL.

Cycling cells are included in the GM1^{bright} GL population

Since GM1 upregulation is related to the events leading to T-cell activation and proliferation,[18] we evaluated whether cycling cells could be demonstrated within the GL population, and whether they are restricted to a specific population (ie GM1^{bright} GL or GM1^{dull} GL). As indicated in Figure 5, in a representative patient characterized by the high percentage of GM1^{bright}-positive cells, a very small amount of proliferating cells can be detected in the GM1^{bright} GL subset, whereas no cycling cells were detected in the GM1^{dull} GL. It should be pointed out that, due to the very low amount of cells proliferating *in vivo*, cycling GL are usually not detected in patients with LDGL. Indeed, we were not able to detect cycling cells in patients characterized by a low percentage of circulating GM1^{bright} GL.

GM1^{bright} GLs include IFN-γ higher producer cells

In order to investigate whether the different expression of GM1 could be related to a different functional status of cells, we analyzed GM1^{bright} and GM1^{dull} GL for the intracellular content of IFN-γ, a cytokine produced by GL of LDGL patients.[23] To this aim, we sorted GM1^{bright} and GM1^{dull} cells and evaluated the different intracellular content of IFN-γ by flow cytometry. As shown in Figure 5 in a representative case, GM1^{bright} GL demonstrated a higher content of IFN-γ (MFI ratio: 14.98) when compared with GM1^{dull} cells (MFI ratio: 8.89), indicating that these two cell populations are functionally distinct.

GM1^{bright} GL do not present intracellular raft-like structures

Normal resting T cells express intracellular raft-like structures, which are stored inside the cell and targeted to the plasma membrane upon T-cell activation.[18,19,21–26] In contrast, *in vitro* activated human T cells, as well as *ex vivo* effector and memory murine T cells, express raft markers (ie GM1 or Lck) only at the plasma membrane.[18,19,21–26] To study the localization of rafts in GL, we performed Lck and GM1 intracellular staining of sorted GM1^{bright}CD3+ GL obtained from LDGL patients. In contrast to normal peripheral blood T cells, GM1^{bright} GL do not contain intracellular raft-like structures, as shown by the fact that Lck as well as GM1 staining was restricted to the plasma membrane (Figure 6).

Discussion

Our data indicate that in LDGL patients a variable but well-defined fraction of GL expresses the raft marker GM1 at levels higher (GM1^{bright}) than normal resting T cells, with a pattern that is consistent with recently activated T lymphocytes.[18] This result is in line with the suggestion that GL from LDGL patients are stimulated *in vivo* by an unknown antigen.[27] Interestingly, since
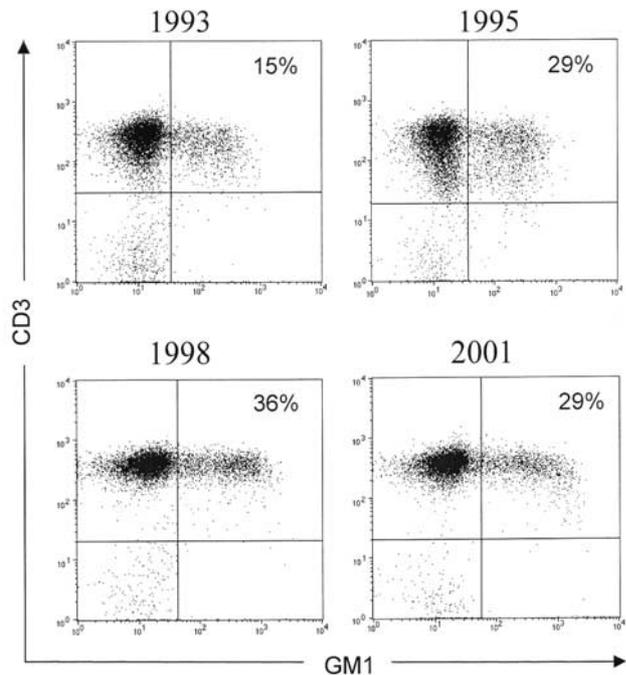
Figure 4 GM1^{bright} GL are stably detected in the peripheral blood of LDGL patients. Longitudinal analysis of GM1^{bright} expression in CD3+ GL from a single LDGL patient (case #1). This analysis was performed in two patients, providing consistent results.
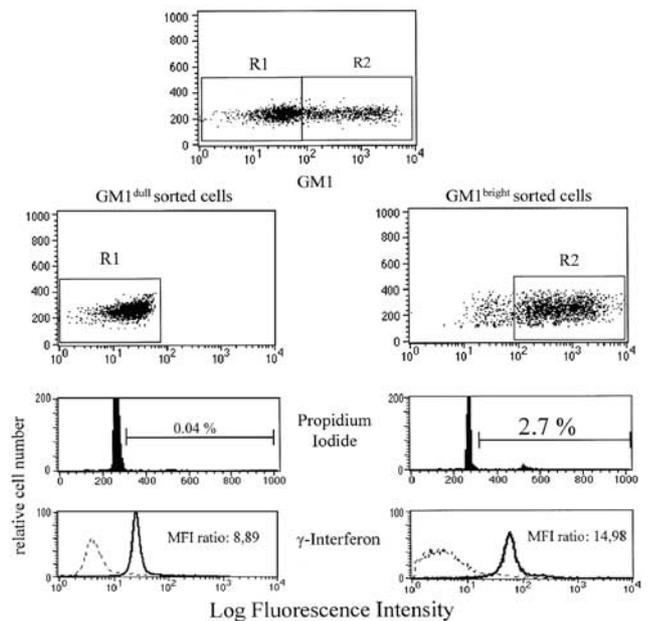
Figure 5 GM1^{bright} GL include cycling cells and lymphocytes with a high expression of IFN-γ. GM1-positive cells of patient #2 were sorted according to the expression of GM1 (GM1^{dull} and GM1^{bright}) and analyzed for the presence of cycling cells and for the expression of cytoplasmic IFN-γ. Consistent results were obtained in two additional experiments (cases #1 and 8).
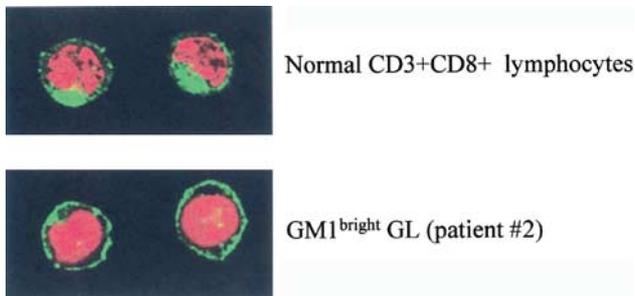
Figure 6 Absence of cytosolic rafts in GL of LDGL patients. Confocal microscopy of permeabilized normal T cells (obtained from healthy donors) and GM1^{bright} GL stained with anti-Lck. Nuclei were stained with PI (red). The results are representative of three different experiments. Consistent results were obtained with anti-GM1 cholera toxin.

no upregulation of markers indicating recent activation was observed in patients' cells (<1% of GL population was positive for CD25, CD27, CD69 antigens), it is likely that GM1^{bright} expression in our patients identifies a previously unrecognized subset of activated CD3$^+$ GL. Accordingly, the fact that within GM1^{bright} GL both replicating cells and cells with the highest content of IFN-γ are included suggests that GM1^{bright} and GM1^{dull} GL are two distinct populations in terms of their functional properties.

The ganglioside GM1 is a commonly used raft marker and its association with membrane lipid microdomains has been proved by microscopy and biochemical experiments.[25,26] Even if the upregulation of GM1 on cell membrane does not necessarily imply that more raft domains are expressed at the cell surface, the finding that in GL the raft tyrosine kinase Lck is fully targeted to the plasma membrane strongly suggests that GM1 upregulation might be interpreted as upregulation of raft-like domains at the cell surface.

The interpretation of the two different GL populations in the light of pathogenetic features in LDGL patients is a matter of debate. Indeed, in every patient, both GM1^{bright} and GM1^{dull} GL are characterized be the same monoclonal rearrangement of TCR. As indicated previously, the expression of GM1^{bright} on T-cell surface represents a highly unusual *in vivo* feature, even in conditions characterized by a high number of activated cells, as in the case of infectious mononucleosis or acute cytomegalovirus infection. Similarly, in disorders mediated by the chronic proliferation of B cells, as chronic lymphoproliferative disorders, the expression of GM1^{bright} lymphocytes is undetectable. The only other situation in which we could observe upregulation of GM1 is represented by tumor-infiltrating lymphocytes (Viola, ms in preparation). These data point out that the chronic persistent antigenic stimulation might represent the most crucial factor conditioning the upregulation of GM1.

As compared to normal T cells, GL of patients showed a low expression of CD28, which indicates that a major pathway of T-cell activation (ie the engagement of TCR and CD28) has nocentral role in this disease. It is not clear whether the lack of CD28, together with the presence of activating NK receptors (namely CD94/NKG2C and NKG2D), which are commonly demonstrated in GL of CD3+ LDGL patients, could indeed be related to the peculiar pattern of rafts' expression.[28] It is interesting to note that CD94 has been reported to bind HLA-E antigens[29,30] and that this interaction is particularly efficient in the recognition of some viral antigens, namely CMV, EBV and influenza viruses.[31] It can be speculated that the multiple activating signals released by different activating receptors (CD94/NKG2C, NKG2D)[28,32] might be involved in the expression of GM1^{bright} on the GL surface, although it should be noted that the percentage of cases characterized by the expression of CD94 includes only a fraction of patients under study and NKG2D was not expressed in two cases.

Our data are consistent with the suggestion that the putative and still unknown inciting event leading to maintenance of GL lymphocytosis involves the upregulation of rafts, this process being central for the stabilization of LDGL. Unfortunately, no information is available on the factors responsible for maintaining GM1^{bright} cells, although cytokines are highly suspected to play a role. However, we could not demonstrate upregulation or even modification of GM1 expression after short-time culture with IL-15 (data not shown). Unfortunately, the derangement of rafts structure by using drugs (ie methyl-*b*-cyclodextrin) definitively damages cells, thus preventing their functional analysis. Up to now, even using cell-sorting procedures, we were unable to obtain high enough GM1^{bright} cells for additional functional studies.

It has been suggested that clonal GL populations, with features identical to those responsible for LDGL, can be commonly recovered in the peripheral blood of normal individual, particularly in aged persons, representing clonal cytotoxic effector T cells.[28] According to this hypothesis, in patients with LDGL the accumulating lymphocytes should be considered as a clonal population likely recognizing viral antigens, which persists *in vivo* after the clearance of antigens. As a consequence, a small amount of activated cells should be detected among the clonal population they found, as the expression of the persisting stimulation.[28] Our results seems to validate this hypothesis, by demonstrating the expression of the raft marker GM1^{bright}, identifying activated cells, expressed in a small but well-defined percentage of GL in all patients with LDGL studied. In addition, since the percentage of GM1^{bright} GL and the lymphocytosis are extremely stable during time in all the patients analyzed (see Figure 3), our results point to a role of these activated cells in the biology of GL proliferation, possibly involved in the renewal of circulating GL.

In conclusion, the presence in the peripheral blood of GM1^{bright} T lymphocytes is a relevant feature of CD3$^+$ LDGL patients and might represent a useful criterion to distinguish true LDGL from other similar conditions. In addition, we believe that the analysis of these different populations (ie GM1^{bright} and GM1^{dull} GL) might help to further investigate the mechanisms leading to the cell expansion in LDGL and might represent a useful model for studying the role of lipid domains in the biology of lymphocytes in human disease states.

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