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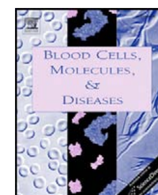

Chemokine receptor repertoire reflects mature T-cell lymphoproliferative disorder clinical presentation

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ABSTRACT

The World Health Organization classification of mature T-cell lymphoproliferative disorders, combines clinical, morphological and immunophenotypic data. The latter is a major contributor to the classification, as well as to the understanding of the malignant T-cell behavior.
The fact that T-cell migration is regulated by chemokines should, in theory, enable us to identify tissue tropism and organ involvement by neoplastic T-cells by monitoring chemokine receptor surface expression. To address this issue we compared the expression of several early and late inflammatory, homeostatic, and organ specific chemokine receptors on blood T-cells from normal individuals and patients with T-cell large granular lymphocytic leukemia and peripheral T-cell lymphoma.
T-cell large granular lymphocytic leukemia cells mainly express late inflammatory chemokine receptors (CXCR1 and CXCR2), whereas peripheral T-cell lymphoma cells usually express one or more organ homing receptors (CCR4, CCR6 and CCR7). Nevertheless, no clear correlation was found between CCR4 and CCR7 expression and skin and lymph node involvement, respectively. Compared to their normal counterparts, lymphoma T-cells displayed an exaggerated CCR4 expression, whereas leukemic T-cells had abnormally high CXCR1 and CXCR2 expression.
Further analysis revealed that, in leukemia patients, the percentage of neoplastic cells expressing CCR5 correlates directly with lymphocytosis. In addition, in the case of CD8 T-cell leukemia patients, an inverse correlation with neutropenia was found. In lymphoma patients, higher CCR4 and CCR7 expression is accompanied by lower to absent CCR5 expression.

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Introduction

T-cell lymphoproliferative disorders (LPD) are uncommon diseases, arising from an abnormal proliferation or accumulation of neoplastic mature T-cells. They are broadly divided into leukemia or lymphoma depending on their presentation at the time of diagnosis. The World Health Organization (WHO) classifies mature T-cell neoplasms according to clinical, morphological and phenotypic characteristics. Some of the most representative groups of T-cell LPD are the large granular lymphocytic leukemia (LGLL) and the peripheral T-cell lymphoma (PTCL).

PTCLs are usually sub-classified into nodal and extra-nodal lymphomas [1]. While the former affect the lymph nodes at diagnosis, the latter is evident in other organs and tissues, such as the skin, the gastrointestinal and the respiratory tract. Most nodal and extra-nodal cutaneous T-cell lymphoma cases arise on T-cell receptor α/β^+ $CD4^+$ T-cells, and cutaneous T-cell lymphomas account for the majority of extra-nodal PTCLs [2].

T-cell LGLL, one of the most common T-cell leukemias, is characterized by the proliferation of monoclonal large granular lymphocytes (LGL) in the blood, usually of the CD8 subset, and is diagnosed mainly based on the morphologic and phenotypic characteristics of the monoclonal T-cell population, including the absence of the CD28 co-stimulatory receptor, the expression of NK-cell associated molecules and the presence of cytotoxic granules containing perforin and granzymes [3,4]. $CD8^+$ T-cell LGLL usually is accompanied by neutropenia and other cytopenias, whereas $CD4^+$

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T-cell LGLL frequently is associated with other hematological or non-hematological neoplasms [3]. Except for splenomegaly, which is relatively frequent in patients with CD8$^+$ T-cell LGLL, other organ enlargement is rarely observed and, the marrow and spleen are the principal sites of accumulation of LGLL T-cells [4]. Unlike T-cell prolymphocytic leukemia [5], LGLL doesn't display any recurrent cytogenetic abnormality, nor does it usually result in high blood lymphocyte counts [6].

These diverse characteristics of T-cell diseases make the differential diagnosis difficult and demand for a different and better approach to classification.

Over the years, the classification systems have evolved from purely morphologic systems combined with prognostic categories (Working Formulation) to classification systems which integrate clinical, biological and laboratorial data (REAL, WHO and EORTC classifications) [7,8].

T lymphoblastic lymphoma/leukemia originates from the stages of T-cell differentiation in the thymus and peripheral T-cell neoplasms originate from post-thymic mature T-cells. T-cell prolymphocytic leukemia is thought to originate from naive T-cells whereas PTCL and LGLL originate from various stages of antigen-dependent T-cell activation, with LGLL resembling the phenotype of terminally activated cytotoxic T-cells [7]. The fact that the normal blood T-cell population is a mixture of cells in different activation stages, from naïve to effector, in conjunction with their resemblance to pathological T-cells, makes the identification of neoplastic T-cells difficult [9]. The same line of thought applies to migration in that lymphoid neoplasms should follow the homing patterns of their normal counterparts, despite the fact that no clear correlation has yet been made between the CR repertoire and cell activation on normal T-cells.

To test this hypothesis we looked at chemokine receptor (CR) expression, since T-cell traffic can be indirectly assessed by the expression of these CRs [10,11].

For this, we compared the expression of organ specific (CCR4, CCR6 and CCR7) [12–14], early inflammatory, Th1 related (CCR5 and CXCR3) [15,16] late inflammatory (CXCR1 and CXCR2) [17], and homeostatic (CXCR4) [18] CRs between the abnormal peripheral blood (PB) T-cells from patients with CD4$^+$ PTCL and both CD4$^+$ and CD8$^+$ LGLL patients. We also compared the CR repertoire from the abnormal T-cells of these LPD with normal PB T-cells with an equivalent phenotype.

Materials and methods

Patients and controls

The CR repertoire was studied on the abnormal PB T-cells from 18 CD4$^+$ PTCL, 10 CD4$^+$ LGLL and 18 CD8$^+$ LGLL patients and twelve normal age-matched adult individuals (blood donors) were used as controls.

All patients had normal hemoglobin values (11–13 g/dL), as well as normal platelet counts (150–300 × 10^3/μL). White cell counts were usually high (5–25 × 10^3/μL) due to lymphocytosis caused by T-cell proliferation. The majority of PTCL patients had either lymph node or skin infiltration by the neoplastic T-cells. The percentage of abnormal T-cells per total lymphocytes was always above 15%.

T-cell LPD was diagnosed and classified based on clinical and laboratorial data according to the WHO schema [7]. Abnormal T-cell identification was accessed by aberrant expression of cell-surface markers by flow cytometry [3]. T-cell clonality was accessed by at least one of the two following methods: a) expression of a single family of the TCR beta chain accessed by flow cytometry [3] and, or b) monoclonal TCR gamma and/or beta chain genes rearrangement accessed by molecular PCR-based studies [19].

Samples

PB samples were collected on EDTA-K3 containing tubes.

Four-color flow cytometry

Immunophenotypic analysis of surface antigen expression on PB T-cells was performed in all cases using a stain-and-then-lyse four-color direct immunofluorescence technique, as previously described in detail [3]. Briefly, 100 μl of whole blood containing 1–2 × 10^6 cells was incubated for 15 min at room temperature with antigen-specific fluorochrome-conjugated monoclonal antibodies (mAb), followed by erythrocyte lysis and cell fixation using FACS lysing solution (Becton-Dickinson BioSciences, BD, San José, CA USA), according to manufacturer instructions. The mAb indicated in Table 1 were used in different combinations in order to ensure the best selection of the abnormal T-cell population. In accordance, allophycocyanin conjugated anti-CD4 or anti CD8 mAb (for CD4$^+$ and CD8$^+$ T-cell LPD, respectively) were combined with Phycoerythrin (PE) conjugated anti-CR mAb in all cases. The specificity of the other mAb used was variable, depending on the best combination to identify the phenotypically abnormal T-cells in blood. In accordance, Cy5-Phycoerythrin (PC5) conjugated anti-CD28 and fluorescein (FITC)-conjugated anti-CD27 mAb were used in all LGLL cases, whereas Peridin–chlorophyll–protein complex. (PerCP) conjugated anti-CD3 and FITC conjugated anti-CD7 mAb was the preferred combination to identify PTCL cells. CD8$^+$ LGLL cells were further subdivided into three groups according to their CD11c and CD57 expression pattern (CD11c$^+$CD57$^-$, CD11c$^+$CD57$^+$ and CD11c$^-$CD57$^+$).

CD4$^+$ and CD8$^+$ LGLL cells were selected based on the absence of CD27 and CD28 expression whereas CD4$^+$ PTCL cells were identified based on their abnormal light scatter characteristics and abnormal levels of CD3 and/or CD7 expression.

All experiments were done on a FACSCalibur cytometer (BD Biosciences), using CellQuest software version 3.1 (BD Biosciences) for sample acquisition and Paint-a-gate Pro software (BD Biosciences) for data analysis.

Comparison between neoplastic T-cells and their normal counterparts

Normal terminally activated CD4$^+$CD27$^-$CD28$^-$ and CD8$^+$CD27$^-$CD28$^-$ T-cells were considered as the normal counterparts for CD4$^+$ and CD8$^+$ LGLL T-cells.

CD4$^+$ PTCL cells have no clear homology to any specific subset of normal PB CD4$^+$ T-cells except for the fact that they are always CD28$^+$ and usually express activation-related molecules [20]. Considering these two aspects normal PB CD4$^+$CD28$^+$CD45RO$^+$ T-cells were used as normal counterparts for CD4$^+$ PTCL cells in this study.

Table 1
Specificities, clones and sources of the monoclonal antibodies used

Specificity	Clone	Conjugate[a]	Source[b]
CD3	SK7	PerCP	BD
CD4	SK3	APC	BD
CD7	CLB-3A1/1,7F3	FITC	CLB
CD8	SK1	APC	BD
CD11c	SHCL-3	PE	BD
CD27	9F4	FITC	CLB
CD28	CD28.2	PC5	IOT
CD57	HNK-1	FITC	BD
CCR4	1G1	PE	PH
CCR5	3A9	PE	PH
CCR6	11A9	PE	PH
CCR7	3D12	PE	PH
CXCR1	5A12	PE	PH
CXCR2	6C6	PE	PH
CXCR3	1C6	PE	PH
CXCR4	12G5	PE	PH

[a] APC — Allophycocyanin; FITC — fluorescein isothiocyanate; PC5 — Cy5-Phycoerythrin; PerCP — Peridin–chlorophyll–protein complex.
[b] IOT — Immunotech, Marseille, France; BD — Becton-Dickinson, San José, CA, USA; CLB — Sanquin, Amsterdam, The Netherlands; PH — Pharmingen, San Diego, CA, USA.

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Statistical analysis

All statistical analysis was done with Mann–Whitney *U* test. Differences were considered significant for $p<0.05$.

Ethical implications

All samples were collected after informed approval, and the work was approved by the local Ethical Committee.

Results

Comparison between PTCL and LGLL CR repertoires

Our results showed that phenotypically abnormal T-cells from patients with PTCL and LGLL had quite different CR repertoires (Fig. 1A), which were consistent with their different clinical presentation (Fig. 1B). When lining up the CR repertoire on these two groups of T-cell LPD, it was clear that there was a large

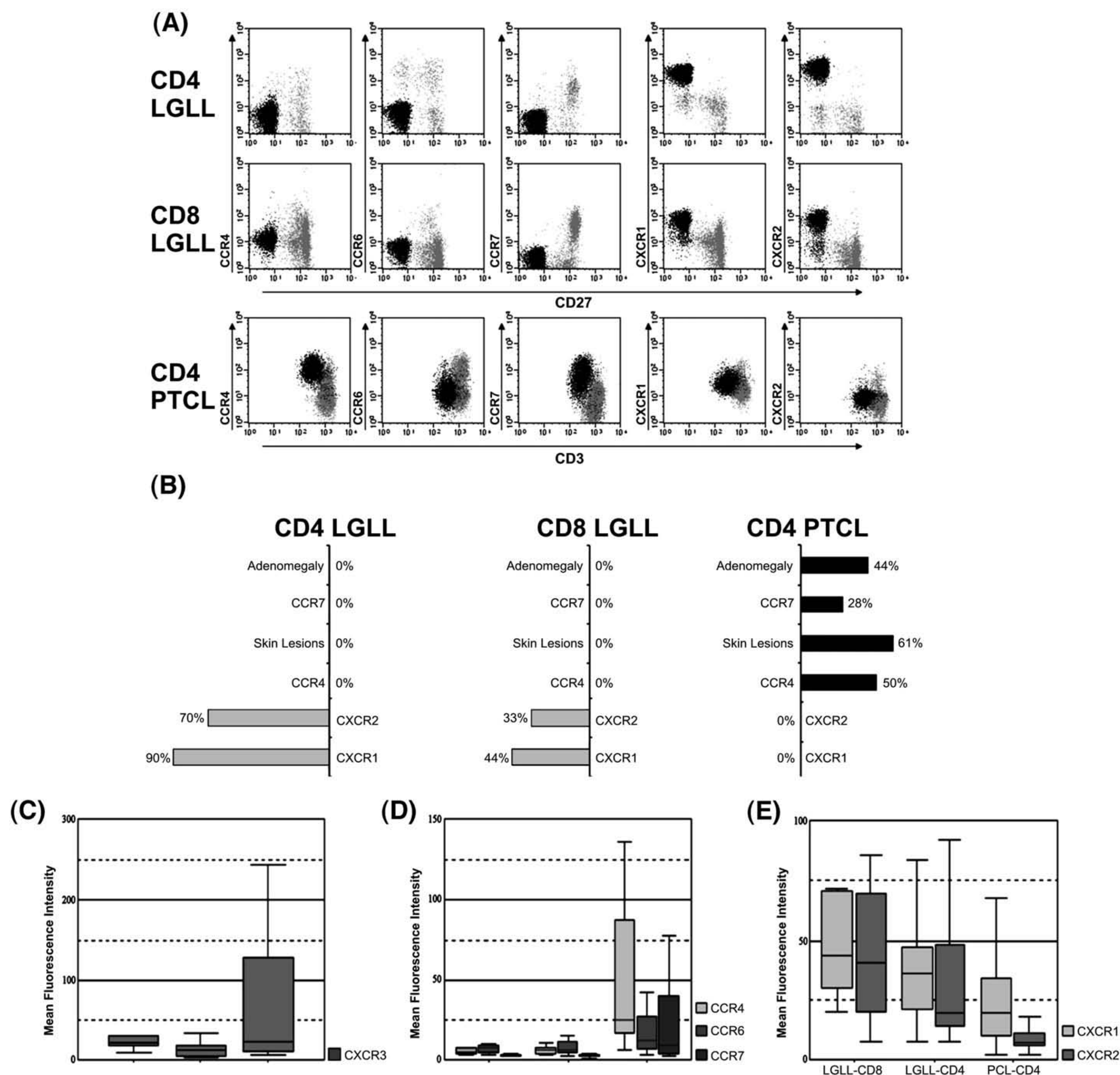

Fig. 1. (A) Dot plots showing typical patterns of expression of organ specific (CCR4, CCR6 and CCR7) and late inflammatory (CXCR1, CXCR2) CRs on abnormal PB T-cells (black dots) in representative cases of CD8⁺ LGLL, CD4⁺ LGLL and CD4⁺ PTCL and normal T-cells (grey dots). (B) Percentage of cases with CR membrane expression measured by flow cytometry in PB neoplastic cells from 18 CD4⁺ PTCL, 10 CD4⁺ LGLL and 18 CD8⁺ LGLL patients and associated tissue involvement from these pathologies. Panels C, D and E represent the mean fluorescence intensity of cell-surface expression of (C) early inflammatory (CXCR3), (D) organ specific (CCR4, CCR6 and CCR7) and (E) late inflammatory (CXCR1 and CXCR2), on abnormal PB T-cells from 10 patients with CD4⁺ LGLL, 18 patients with CD8⁺ leukemia and 18 patients with CD4⁺ PTCL.

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difference between PTCL and LGLL cells, concerning both the percentage of cases expressing each of the CRs (Fig. 1B) and their expression intensity (Figs. 1C, D and E). PTCL cells usually expressed a variety of organ specific CRs, including CCR4, CCR6 and CCR7, whereas LGLL cells expressed the two late inflammatory IL-8 receptors (CXCR1 and CXCR2).

Abnormal T-cells from the majority (78%) of patients with PTCL expressed one or more organ specific CRs, with half expressing CCR4 and nearly 1/3 expressing CCR6 or CCR7, which correlates with the high incidence of skin lesions and lymph node enlargement observed in those patients (Fig. 1B). Nevertheless, no clear correlation was found between CCR7 expression and lymph node involvement, either between CCR4 expression and skin lesions. Actually, the neoplastic T-cells from 2 out of 8 PTCL patients with lymphadenopathy had no CCR7 expression and T-cells from 3 out of 12 patients with skin lesions had no CCR4 expression. PTCL cells have no late inflammatory CR expression.

On the other hand, CXCR1 and CXCR2 were expressed in the majority of $CD4^+$ (90% and 70% respectively) and in a large fraction of $CD8^+$ (44% and 33%, respectively) LGLL cases. In accordance to the absence of skin, lymph node or other organ infiltration observed in LGLL patients (Fig. 1B), LGLL cells never expressed organ specific CRs.

Early inflammatory, Th1-related CRs (CCR5 and CXCR3) were expressed in a fraction of both PTCL (28% and 50% respectively) and LGLL cells (10% and 26% respectively), with no statistically significant differences, except for a higher CXCR3 mean fluorescence intensity in PTCL (Fig. 1C), as compared to LGLL ($p<0.05$). No significant difference in CXCR4 expression was found.

Similarities and differences between the CR expression on neoplastic T-cells and their normal counterparts

When comparing the pattern of CR expression from $CD4^+$ (Fig. 2A) and $CD8^+$ (Fig. 2B) LGLL T-cells to normal terminally differentiated ($CD3^+CD27^-CD28^-$) $CD4^+$ and $CD8^+$ LGL, respectively, we observed that LGLL cells had a very significant decrease on the expression of CCR5 and CXCR3 ($p<0.005$). At the same time, LGLL cells had an increased expression of CXCR1 and CXCR2, although differences were statistically significant only in the case of $CD4^+$ LGLL ($p<0.05$).

Phenotypically abnormal $CD4^+$ PTCL cells showed, although not homogenously, a significantly higher expression of the skin homing receptor CCR4 ($p<0.05$) and a significant decrease in the expression of CCR6, CCR7 and CXCR4 ($p<0.05$) in comparison with normal blood $CD4^+CD28^+CD45RO^+$ T-cells (Fig. 2C).

The relation between CCR5 expression, lymphocytosis and neutropenia

We then compared the percentage of $CCR5^+$ cells in the blood of LGLL patients with both the lymphocyte and neutrophil PB counts.

In the case of $CD4^+$ LGLL patients, the percentage of $CCR5^+$ cells, within the abnormal population, linearly correlated with lymphocytosis ($R^2=0.91$) (Fig. 3A), while in the case of $CD8^+$ LGLL patients this correlation, although existent, was less strong ($R^2=0.32$) (Fig. 3B). The five $CD8^+$ LGLL cases with higher lymphocyte counts corresponded to those with no CCR5 expression.

In the case of PTCL patients, there was no direct correlation between CCR5 expression and lymphocytosis, although, PTCL patients that did not express CCR5 had significantly higher lymphocyte counts ($p<0.05$).

When comparing non-neutropenic and neutropenic $CD8^+$ LGLL cases, we observed that the percentage of $CCR5^+$ cells was significantly lower than normal in the case of non-neutropenic LGLL patients and no expression of CCR5 was observed in neutropenic LGLL cases (Fig. 3C).

Inflammatory CR dependence on CD11c and CD57 expression in $CD8^+$ LGLL

We further considered three groups of $CD8^+$ LGLL patients based on the patterns of CD11c and CD57 expression ($CD11c^+CD57^-$, $CD11c^+CD57^+$, $CD11c^-CD57^+$) on the abnormal T-cell population. We observed that $CD11c^+CD57^-$ cases showed the highest levels of late inflammatory CR (CXCR1 and CXCR2) expression and the lowest levels of early inflammatory CR (CCR5 and CXCR3) expression. In contrast the highest levels of CCR5 and CXCR3 expression occurred in $CD11c^-CD57^+$ LGLL cells, whereas the highest levels of CXCR1 and CXCR2 expression were observed on $CD11c^+CD57^-$ LGLL cells (Fig. 4A).

Lymphocyte counts directly correlated with the difference between late and early inflammatory CR expression, with the highest lymphocyte counts being related to $CD11c^+CD57^-$ LGLL cells. Severe neutropenia occurred in $CD11c^+CD57^+$ LGLL cases, where intermediate percentages of cells expressing each of the previously referred CRs were observed (Fig. 4B).

Discussion

The relation between CR expression on neoplastic T-cells and their migration pattern has not been satisfactorily explained, despite the thorough characterization of the CCR4-mediated T-cell migration to the skin in cutaneous PTCL, such as Sézary syndrome and mycosis fungoides [21,22]. Moreover, no clear correlation has yet been

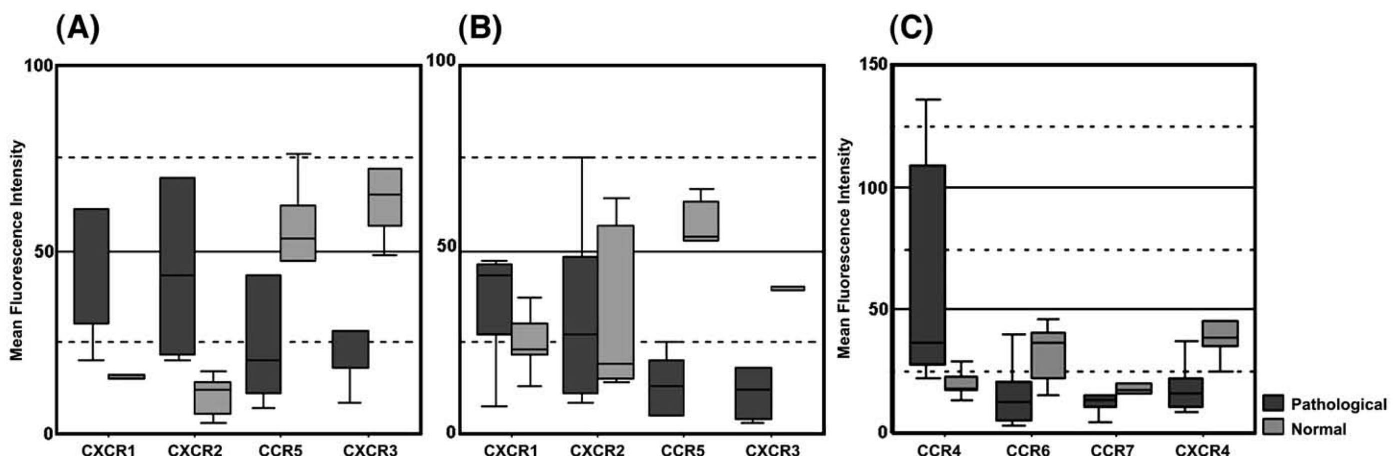

Fig. 2. Comparison of the CR repertoire between normal and pathological T-cells accessed by flow cytometry. Mean fluorescence intensity of CCR5, CXCR1, CXCR2 and CXCR3 expression was compared between PB $CD27^-CD28^-$ T-cells from 10 normal individuals and neoplastic T-cells from (A) 10 $CD4^+$ LGLL patients and (B) 18 $CD8^+$ LGLL patients. (C) Mean fluorescence intensity of CCR4, CCR6, CCR7, and CXCR4 expression on PB $CD4^+CD28^+CD45RO^+$ T-cells from 10 normal individuals and PB neoplastic T-cells from 18 $CD4^+$ PTCL patients.

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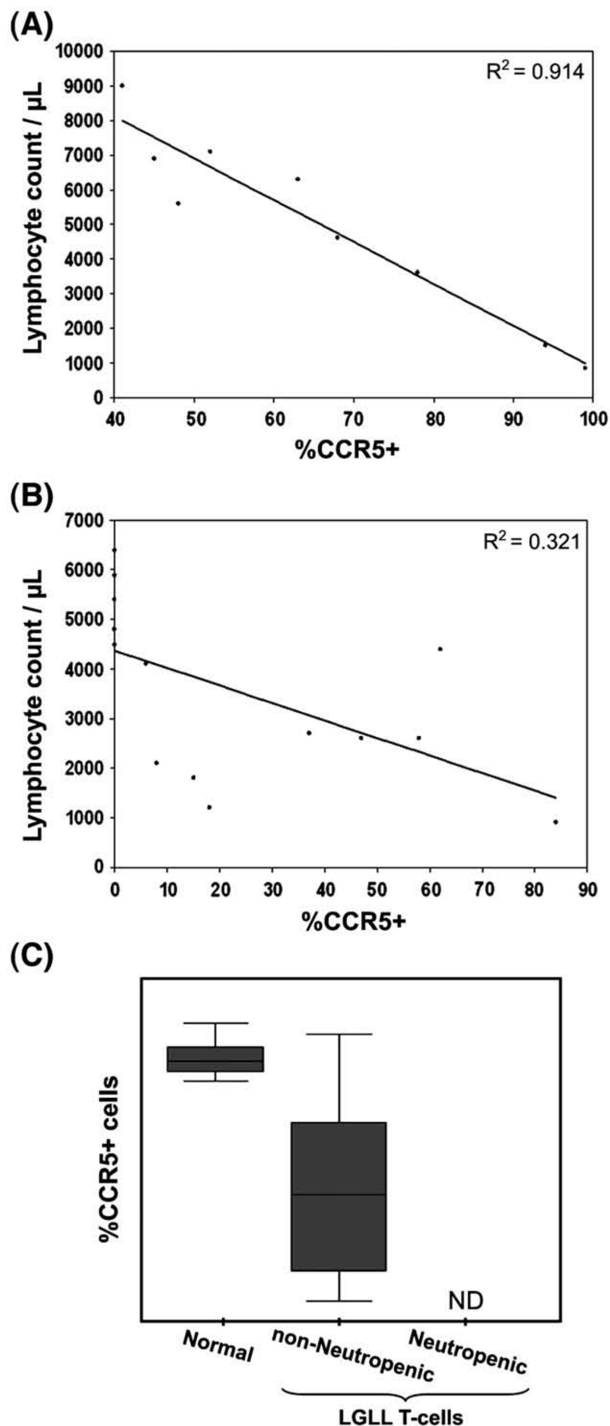

Fig. 3. Relation between the percentage of CCR5 positive neoplastic T-cells and the lymphocyte count per microliter of PB in (A) 9 patients with $CD4^+$ LGLL and (B) 14 patients with $CD8^+$ LGLL. Patients with other pathologies that could account for an increase in lymphocyte count were excluded from this experiment. (C) The percentage of CCR5 positive cells was accessed on PB $CD8^+CD27^-CD28^-$ T-cells from 10 normal individuals, 13 non-neutropenic $CD8^+$ LGLL patients (>1500 neutrophils) and 5 neutropenic $CD8^+$ LGLL patients (<1500 neutrophils). None of the neutropenic $CD8^+$ LGLL patients had evidence of anti-neutrophil antibodies in the blood. In all cases lymphocyte and neutrophil count was performed on fresh blood using a hematological counter and CCR5 positive cells were accessed by flow cytometry.

established between normal and neoplastic T-cell CR repertoire, and a comparison has not been made between T-cell CR repertoires expressed in cases of leukemia and lymphoma.

In the present study, the results obtained indicate that CR expression is a tool for aiding in differential diagnosis between PTCL and LGLL. In every patient studied, a high expression of CCR4 or CCR7 and absence of CXCR1 and CXCR2 expression indicates PTCL, whereas the opposite indicates LGLL. These findings are consistent with the absence of lymphadenopathy and skin lesions in T-cell LGLL patients, and the high prevalence of these clinical manifestations in PTCL patients. Despite these relationships, neither the percentage of $CCR4^+$ cells, nor the intensity of CCR4 expression correlates with skin involvement, and the same is true for CCR7 and lymph node involvement. This observation supports the idea that CCR7 is not solely responsible for T-cell migration into the lymph nodes [23].

Changes in CR expression occur during activation as judged by *in-vitro* observations that CCR1, and CCR2 are up-regulated by IL-2 and down-regulated by anti-CD3 and, or anti-CD28 [24]. CCR7 and CCR5 were the first CRs associated with naïve [25] and activated [26] T-cell populations, respectively, but since then, other CRs showed preferential expression and others will follow due to chemokine receptor redundancy. Our results reflect this relation between activation and CR expression and lead us to the conclusion that only by fully understanding the normal T-cell activation sequence and the physiological lymphoid traffic will we be able to determine the origin of T-cell LPD and pathological T-cell migration, essential aspects for a better understanding of T-cell diseases and their evolution.

Both normal and pathological LGL express a predominantly inflammatory CR repertoire, despite the fact that they differ in the expression of early and late inflammatory CRs, with normal LGL having a much higher expression of CCR5 and CXCR3 than CXCR1 and CXCR2, whereas LGLL T-cells show the opposite pattern. This observation indicates that LGLL T-cells have an exaggerated terminal inflammatory phenotype as compared to normal cytotoxic T lymphocytes, and is in accordance with the previously observed constitutive production of the pro-inflammatory cytokines RANTES, MIP-1beta and IL-18 by mononuclear PB cells in LGLL patients [27].

Also, neoplastic T-cells from PTCL patients (with or without skin involvement) have significantly higher expression of the skin homing

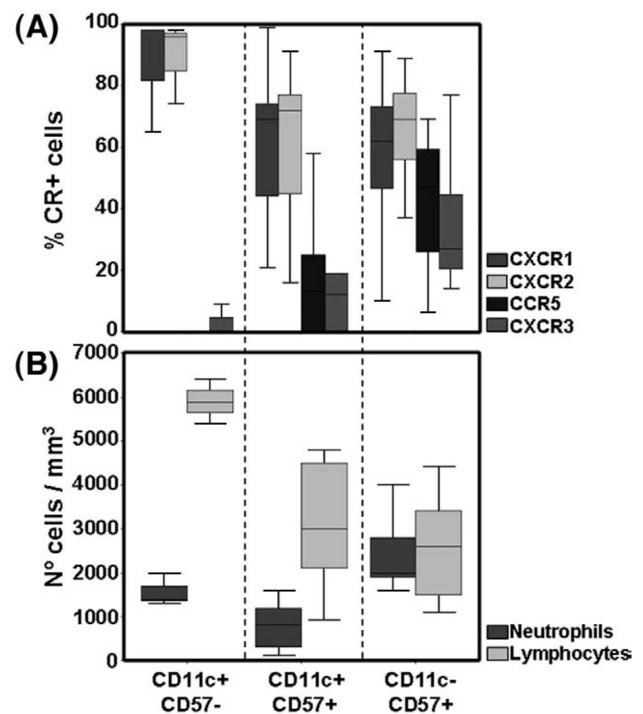

Fig. 4. Relation between the expression of the activation-related molecules CD11c and CD57 and (A) the percentage of relevant inflammatory CRs positive neoplastic T-cells and (B) the lymphocyte and neutrophil count per microliter of PB, in patients with $CD8^+$ LGLL. In all cases lymphocyte and neutrophil count was performed on fresh blood using a hematological counter and CR positive cells were accessed by flow cytometry.

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receptor CCR4 when compared to normal blood T-cells with the same phenotype (CD4$^+$CD28$^+$CD45RO$^+$).

Increased CCR4 expression is in accordance with other studies that indicate CCR4 as being responsible for the skin homing potential of Sézary syndrome [28,29] and other cutaneous lymphoma cells [30], and that this CR is also usually expressed in other PTCLs with skin involvement [21,31]. Moreover, as confirmed in this paper, lymphoma cells from mycosis fungoides patients [32,33] and other PTCL [34] express CCR4 with early inflammatory, Th1-related CR expression, namely CCR5 and CXCR3, supporting the idea that PTCL are derived from cells with an activated phenotype. In addition, when compared to the equivalent normal T lymphocytes, PTCL cells have infrequent and usually lower expression of CCR6 and CCR7, which cannot solely be explained by the predominance of cutaneous T-cell lymphomas over nodal PTCL patients in this study (61% of patients with skin lesions versus 44% of patients with lymph node involvement) because even patients with lymphadenopathy caused by PTCL cells display less CCR7 on their surface than normal counterparts.

The clear inverse correlation between the percentage of neoplastic cells expressing CCR5 and lymphocyte counts in both LGLL and PTCL patients is in accordance with a possible physiological role of CCR5 in the regulation of T-cell proliferation and/or death, as well as T-cell adhesion to endothelial cells and consequent migration to the inflamed tissue [35–37]. In contrast, the correlation between CCR5 expression and neutrophil counts in CD8$^+$ LGLL patients, which express preferentially the late rather than the early inflammatory CRs, might be explained by a cytolytic action of terminally activated CD8$^+$ cytotoxic lymphocytes on neutrophils.

Taken together these two observations suggest that, in the case of CD8$^+$ LGLL patients, the decrease in the percentage of CCR5$^+$ neoplastic cells is implicated in the development of neutropenia and lymphocytosis, which are two useful parameters for monitoring LGLL. This observation is important since it demonstrates a relation between the activation status of LGLL T-cells and both neutropenia and lymphocytosis, which in turn can be monitored by CCR5 expression.

CR expression is a determinant for the clinical presentation of T-cell LPD thus it is reasonable to assume that by monitoring the expression of these and other organ specific CRs not addressed in this study, such as CCR10 [38,39], we will be able to follow disease manifestations and predict organ involvement during the course of the disease. In fact, preliminary data suggest that it is possible to identify a group of patients whose neoplastic T-cells only express CCR4, which have the most aggressive clinical behavior (data not shown).

This makes the evaluation of the CR repertoire by flow cytometry useful for the classification of T-cell LPD, and especially important for establishing a solid premise for the distinction between leukemia and lymphoma. Moreover, the knowledge of the specificities of the CR repertoire on the different T-cell LPD may play an important role on the future anti-chemokine receptor treatment of these neoplastic disorders.

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