Analysis of expression and function of the inhibitory receptor ILT2 (CD85j/LILRB1/LIR-1) in peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE)

A. Monsiváis-Urenda a, P. Niño-Moreno a, C. Abud-Mendoza a,b, L. Baranda a,b, E. Layseca-Espinosa a, M. López-Botet c, R. González-Amaro a,*

a Departamento de Inmunología, UASLP, Ave. V. Carranza 2405, 78210 San Luis Potosí, S.L.P., Mexico
b Unidad Regional de Reumatología y Osteoporosis, Hospital Central “Dr. Ignacio Morones Prieto”, San Luis Potosí, S.L.P., Mexico
c IMIM, Hospital del Mar, Barcelona, Spain

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Abstract

The aim of this work was to study the expression and function of the inhibitory receptor ILT2/CD85j in peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE). We studied 23 SLE patients as well as 17 patients with rheumatoid arthritis, 10 with fibromyalgia, and 23 healthy individuals. We found a variable level of expression of ILT2 in the PBMC from both SLE patients and controls, with no significant differences among them. However, when the expression of this receptor was assessed in cell subsets, significantly lower levels were detected in CD19+ lymphocytes from SLE patients compared with healthy controls. Functional assays performed in unfractionated PBMC, showed a significant diminished inhibitory activity of ILT2 in CD4+ and CD8+ cell subsets from SLE patients compared to either rheumatoid arthritis or fibromyalgia patients, and healthy individuals. Our results show that the PBMC from some patients with SLE show a defective expression of ILT2, and that most of them exhibit a poor function of this inhibitory receptor.

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1. Introduction

The immune response results from a balance between positive and negative signals [1,2]. Besides CTLA-4, which is expressed by all activated T cells, other molecules that counteract the activation of T lymphocytes have been described [3]. Ig-like transcripts (ILTs), also called lymphocyte inhibitory receptors or leukocyte immunoglobulin (Ig)-like receptors (LIR/LILRs) are a group of membrane receptors coded by more than 10 genes located in the 19q13.4 chromosome, which also includes the genes for killer cell immunoglobulin-like receptors (KIR), FcγR receptors, and the inhibitor receptor LAIR (leukocyte-associated immunoglobulin-like receptor-1) [4,5]. ILT2 (CD85j/LIR1/MIR7, LILRB1) is a membrane molecule of 110 kDa with an extracellular region of 442 amino acids, which includes four immunoglobulin-like domains, a 25-amino acid hydrophobic transmembrane domain and a 167-amino acid cytoplasmic domain [4,6]. In its intracytoplasmic domain, there are two pairs of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that upon phosphorylation, recruit SHP-1 and -2 phosphatases, which are involved in the inhibition of different intracellular signal pathways [7,8].

ILT receptors are expressed by all myelomonocytic cells and by some lymphocyte subsets. ILT2 is detected on monocytes, macrophages, dendritic cells (DCs) and B cells [9–11]. In addition, this receptor is expressed by a subset of T lymphocytes (4–20%), and NK cells (approximately 75%)
ILT2 binds to different class I HLA molecules, including HLA-G, and also interacts with the UL18 cytomegalovirus protein, which is a homologue of these molecules [5,12]. This receptor exerts a negative regulatory effect on the activation of T cells by inhibiting the phosphorylation of the TCR ζ-chain, the recruitment of ZAP-70, and the phosphorylation of LAT and ERK1/2 [13]. HP-F1 is an anti-ILT2, a monoclonal antibody (mAb) which mimics the ligand and inhibits the proliferation induced through CD3/TCR [10]. In addition, in B cells, ILT2 is able to down-regulate their activation and proliferation as well as the intracellular signals generated through the B cell receptor (BCR) [14]. Furthermore, ILT2 has a tolerogenic effect in DCs by inhibiting the production of IL-12 [15]. Finally, it has also been demonstrated that ILT2 inhibits the adhesion of NK lymphocytes to their target cells in the initial recognition process [16].

Systemic lupus erythematous (SLE) is an autoimmune disease that mainly affects the skin, joints, kidneys, brain, blood vessels, blood cells, and serous membranes [17]. It has been widely proposed that the development of this condition depends on the complex interaction between environmental and genetic factors [18]. The main immunological alterations found in SLE are the polyclonal activation of B lymphocytes, the production of many different auto-antibodies [19,20], and the defective function of T cells. Different abnormalities in the function and activation as well as in signaling pathways of SLE T cells have been described [21–27]. The cause of B cell hyperactivity in this condition has not been fully elucidated, but it is very likely that defects in immune regulation have a very important role. In this regard, it has been described as a defective function of natural regulatory CD4+ Foxp3+ T regulatory (Treg) cells, and suppressor CD8+ lymphocytes in SLE [28–32]. In addition, it has been reported that B cells from NZB mice act as antigen presenting cells, and induce T cell hyper-responsiveness [33].

As the possible role of different molecules with regulatory function as KIR and ILT receptors have not been properly studied in SLE, we decided to explore the expression and function of ILT2 in patients with this condition, and different control individuals. We have found that the PBMC from some patients with SLE show a defective expression of ILT2, and that in most of them a poor function of this inhibitory receptor is observed.

2. Materials and methods

2.1. Patients

We studied 23 patients (all females) with SLE, according to the classification criteria of the American College of Rheumatology [34]. Mean age was 31.4 years, and mean duration of disease was 3.2 years at the time of the study. Seventeen patients had active disease (according to the MEX-SLEDAI index) [35], arithmetic mean = 5.6 points, and six had inactive disease (<2.0 points). Values of disease activity were as follows: 0, 0, 0, 0, 0, 0, 2, 2, 2, 4, 4, 4, 6, 6, 6, 6, 9, 9, 10, 11, 14, and 20. All patients included in this work were not under immunosuppressive or corticosteroid therapy at the time of study. Most patients had renal disease (mainly type II and III nephropathy), but no patients with renal failure were included. We also studied 23 healthy subjects (with similar age and sex of patients) as well as 10 patients with fibromyalgia, and 17 patients with rheumatoid arthritis (RA) (15 females, and two males). Mean age of RA patients was 53.7 years, all had active disease, and none of them was receiving disease modifying anti-rheumatic drugs at the time of the study. In all cases, an informed consent was obtained, and the local University Ethics Committee approved this study. This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Sigma Chemical Co., St. Louis, MO) centrifugation. Cells were cultured in RPMI medium (GIBCO, Grand Island, NY) supplemented with 10% bovine fetal serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at a final concentration of 2 × 10⁶ cells/ml.

2.3. Flow cytometry analysis

Surface immunostaining was performed using the anti-ILT2 HP-F1 mAb, which has been previously described [11], and a secondary rabbit anti-mouse IgG conjugated with FITC (Sigma). For double staining experiments, anti-CD3, anti-CD4, anti-CD8, anti-CD19, or anti-CD56 mAb labeled with phycoerythrin (PE) (BD Biosciences PharMingen, San Diego, CA) were simultaneously used with the HP-F1 mAb. Cells were washed with PBS and fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), using the Cellquest software (Becton).

2.4. Cell proliferation assays

Cells (4 × 10⁵) were cultured in flat bottom 96-well plates previously coated with the T3b anti-CD3 mAb (10 μg/ml, provided by Dr. F. Sánchez-Madrid, Hospital Universitario de La Princesa, Madrid, Spain), in the presence or absence of the anti-ILT2 HP-F1 mAb plus a rabbit anti-mouse IgG (Sigma) as a cross-linker reagent. Cells were cultured for 72 h, and 1.0 μCi of ³H-thymidine (³H-TdR, 6.7 Ci/mM, New England Nuclear, Boston, MA) was added to each well for the last 12 h of culture. Then, cells were harvested, and the ³H-TdR incorporated was determined with a Wallac 1209 Liquid Scintillation Counter (Pharmacy, Turku, Finland). Cell proliferation was expressed as the stimulation index (SI), according to the following formula: SI = (cpm in the stimulated culture/cpm in the control culture) × 100. In other experiments, cell proliferation was assessed by a fluorescent label partition assay and flow cytometry analysis [36]. Briefly, cells were treated as above, labeled with 5.0 mM carboxyfluorescein diacetate succinimidyl ester.
(CFDA-SE, Molecular Probes, Eugene, OR), incubated for 72 h, and analyzed by flow cytometry. In some cases, an anti-CD4-PE or anti-CD8-PE (BD Biosciences) antibodies were used to analyze the cell proliferation in these lymphocyte subsets. These results were expressed as the percent of divided cells.

2.5. Cell cycle analysis

Cell cycle analysis was performed by a DNA content assay by using propidium iodide staining and flow cytometry. Cells (1 × 10^6) were poured in 48-well flat bottom plates, stimulated with 10 μg/ml anti-CD3 mAb, and cultured in the presence or absence of the anti-ILT2, HP-F1 mAb plus a rabbit anti-mouse IgG. After 72 h of culture, cells were harvested and stained with 30 μg/ml propidium iodide (Sigma) and 0.5 mg/ml RNase (Sigma), incubated for 30 min at 4°C and analyzed in a FACSCalibur flow cytometer.

2.6. Statistical analysis

Data were entered into GraphPad InStat, 3.06 version program, and analyzed using non-parametric tests. Flow cytometry data for surface expression of ILT2 and percentage of cell proliferation were evaluated using the Kruskal–Wallis test (non-parametric ANOVA). For multiple comparisons, the Dunnet test was used. Flow cytometry data for surface expression in cell subsets were analyzed by the Mann–Whitney U test. The analysis of correlations between variables was based on Spearman’s rank test. p < 0.05 was considered statistically significant.

3. Results

3.1. Expression of ILT2 by PBMC from SLE patients

Flow cytometry analysis showed that there were individuals with high (40–80%), intermediate (10–30%) and low (less than 10%) levels of ILT2-positive cells (Fig. 1). Thus, a heterogeneous level of expression of ILT2 in peripheral blood lymphocytes (defined by their side and forward scatter characteristics) from SLE patients, and control individuals were detected (Fig. 1B). Accordingly, no significant differences were found when the level of expression of this receptor by lymphocytes in SLE patients was compared to healthy controls or patients with RA or fibromyalgia (Fig. 1B). However, an additional analysis showed a significantly diminished expression of ILT2 in CD19+ B cells from SLE patients compared to controls (p < 0.05, Fig. 2B). In contrast, no significant differences in the expression of ILT2 by CD4+, CD8+ and CD56+ cells were detected (Fig. 2B). Finally, no significant correlation was observed between the percent of ILT2+ cells and disease activity in SLE patients (r = −0.16, p > 0.05, data not shown).

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Fig. 1. Expression of ILT2 in lymphocytes from patients with SLE and control individuals. Peripheral blood mononuclear cells from SLE patients, healthy donors as well as patients with rheumatoid arthritis and fibromyalgia were stained with the anti-ILT2 HP-F1 mAb and analyzed by flow cytometry, as stated in Section 2. (A) Expression of ILT2 by lymphocytes from two SLE patients, with intermediate (upper panel) and high (lower panel) levels of ILT2+ cells. (B) Levels of ILT2+ lymphocytes in peripheral blood from 23 patients with SLE, 17 with rheumatoid arthritis (RA), 13 with fibromyalgia (FM), and 23 healthy controls. Median is indicated with a horizontal line. No significant differences were detected in any of the cases. Numbers in (A) correspond to the percent of positive cells.
3.2. Inhibition of PBMC proliferation by ILT2

To explore whether a deficient function of ILT2 may contribute to the pathogenesis of SLE, we cultured PBMC from patients with this condition as well as with fibromyalgia, and RA and healthy controls, in the presence or absence of the anti-ILT2 HP-F1 mAb. Then, the cell proliferation response was analyzed when T lymphocytes were stimulated through CD3. We found a similar level of cell proliferation induced through CD3 in lymphocytes from patients with SLE and healthy controls (data not shown). When cells were cultured in the presence of an ILT2 mAb plus a cross-linker secondary
antibody, a significant reduction in the cell proliferation induced through CD3 was observed in the three control groups but not in SLE patients (Fig. 3). Accordingly, ILT2 engagement induced a significant reduction in the cell proliferation of CD4+ and CD8+ lymphocytes in response to stimulation through CD3 in healthy controls, but not in SLE patients (Fig. 4). As expected, when the PBMC were cultured with the cross-linker antibody alone or with the HP-F1 mAb with no stimulation through CD3, the cell proliferation was similar to that observed in non-stimulated cells (data not shown). In addition, when in these assays the cross-linker secondary antibody was not added, the inhibition of cell proliferation induced by ILT2 was not observed (data not shown). As in the case of ILT2 expression, we did not detect a significant correlation between disease activity and the suppressive function of this inhibitory receptor ($p > 0.05$, data not shown). Moreover, no significant correlation between the inhibitory function of ILT2 and its level of expression was found both in healthy controls and in SLE patients.

### 3.3. Cell cycle analysis

To further explore the functional role of ILT2 in lymphocytes from patients with SLE, PBMC were cultured as indicated above, and analyzed for DNA content by flow cytometry. We found similar levels of resting cells (G0/G1 phase of cell cycle) in SLE patients and control individuals in cell cultures stimulated through CD3 (Fig. 5). When the functional role of ILT2 was assessed in these assays, we found that in healthy controls, the engagement of this receptor tended to diminish the percent of cells in synthesis (S) and G2/mitosis (G2/M) phase of cell cycle. As in the case of 3H-TdR incorporation assays, this effect of ILT2 engagement was less apparent in cells from SLE patients (Fig. 5).

As expected, a higher percent of hypodiploid (apoptotic) cells were detected in SLE patients (Fig. 5D). Interestingly, the addition of the anti-ILT2 HP-F1 mAb, tended to increase the fraction of hypodiploid (apoptotic) cells in healthy controls but not in SLE patients (Table 1).

### 4. Discussion

The activation of lymphocytes and the immune response are under the control of different inhibitory signals, generated through several regulatory receptors. In this regard, it has been described that, in different animal models, the defective function of receptors expressed by immune cells and those with inhibitory motifs (ITIMs), including the ILT molecules, can lead to serious autoimmune diseases [37–39]. However, with the presence of anti-CD3 and HP-F1 mAb. (B) Data from all patients studied are shown (10 RA, 10 FM, 15 SLE patients, and 23 healthy controls). Cell proliferation of lymphocytes stimulated with anti-CD3 was considered as 100%, and that of cells cultured in medium alone was considered as 0%. Data correspond to the median and 25–75 percentiles. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with autologous cells cultured with the anti-CD3 mAb alone.

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Fig. 3. Effect of ILT2 engagement on the proliferation of lymphocytes from patients with SLE and control individuals. PBMC from patients with SLE, rheumatoid arthritis (RA), fibromyalgia (FM), and healthy controls (H) were cultured in the presence or absence of anti-CD3 and anti-ILT2 mAb for 72 h in complete medium in 96 well plates. Then, cell proliferation was determined by both 3H-TdR incorporation (B), and by a fluorescent label partition assay and flow cytometry analysis (A), as described in Section 2. (A) Representative data from a healthy control (upper panel), and an SLE patient (lower panel) are shown. The lack of inhibitory effect of ILT2 engagement is evident in the cells from the SLE patient. Numbers correspond to the percent of divided cells. Dotted line histogram corresponds to medium alone, filled histogram to cells cultured with anti-CD3 mAb, and thick line histogram to cells cultured in the presence of anti-CD3 and HP-F1 mAb. (B) Data from all patients studied are shown (10 RA, 10 FM, 15 SLE patients, and 23 healthy controls). Cell proliferation of lymphocytes stimulated with anti-CD3 was considered as 100%, and that of cells cultured in medium alone was considered as 0%. Data correspond to the median and 25–75 percentiles. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with autologous cells cultured with the anti-CD3 mAb alone.
Fig. 4. Effect of ILT2 engagement on the proliferation of CD4+ and CD8+ cells from patients with SLE and control individuals.Peripheral blood mononuclear cells from patients with SLE, rheumatoid arthritis (RA), fibromyalgia (FM), and healthy controls (H) were cultured in the presence or absence of anti-CD3 and anti-ILT2 mAb for 72 h in complete medium in 96 well plates. Then, cell proliferation was determined by a fluorescent label partition assay. In addition, these cells were stained with an anti-CD4 or anti-CD8 labeled with PE and analyzed by flow cytometry, as described in Section 2. (A) Representative data from a healthy control (right panel), and an SLE patient (left panel) are shown. The lack of inhibitory effect of ILT2 engagement is evident in the cells from the SLE patient. Dot plots correspond to cells cultured in the presence of anti-CD3 and HP-F1 mAb, filled histogram to cells cultured with anti-CD3 mAb, and thick line histogram to cells cultured in the presence of anti-CD3 and HP-F1 mAb. Numbers correspond to the percent of divided cells. (B) Data from all patients studied are shown. Cell proliferation of lymphocytes stimulated with anti-CD3 was considered as 100%, and that of cells cultured in medium alone was considered as 0%. Data correspond to the median and 25–75 percentiles. *p < 0.05 and ***p < 0.001 compared with autologous cells cultured with the anti-CD3 mAb alone.
exception of a study on the detection of several ILT receptors in the synovial membrane of RA patients [40], the possible role of these regulatory molecules has not been explored in human autoimmune diseases. Therefore, we decided to study the expression and function of ILT2 in the PBMC from patients with SLE.

We have found a variable level of expression of ILT2 by lymphocytes from both SLE patients and healthy controls. In this regard, it has been reported the presence of several single nucleotide polymorphisms in the ILT2 gene, which can be associated with a low level of expression of this receptor [41]. Therefore, our results suggest that it would be of interest to study the frequency of these genetic polymorphisms in both healthy individuals and patients with different autoimmune diseases in the Mexican mestizo population. It would be also important to explore the possible role of these polymorphisms on the function of ILT2.

We have also assessed the expression of ILT2 on different lymphocyte subsets, and our results are in agreement with a previous report on the detection of this receptor in bone marrow derived cells [11]. Interestingly, our results indicate that CD19+ B lymphocytes from SLE patients have a significantly low levels of ILT2 expression. These results indicate that it would be interesting to assess the function of ILT2 in isolated B cells.

In addition to the diminished expression of ILT2 found in CD19+ cells from SLE patients, we have detected a clear-cut defective inhibitory function of this receptor in the PBMC from these patients. These data were corroborated through two different types of cell proliferation assays. In
contrast, patients with RA or fibromyalgia showed an apparent normal function of this receptor. These results suggest that the defective function of this receptor is a characteristic feature of SLE patients. It is worth mentioning that although our cell proliferation assays were performed in unfractinated PBMC, our data indicate that the defective function of this receptor is not confined to one specific subset of immune cells, and that, therefore, this phenomenon may have different consequences. Thus, the diminished expression of ILT2 by B cells may contribute to the enhanced synthesis of auto-antibodies, and in the case of CD4+ lymphocytes, the defective function of this receptor may favor the enhanced activation of Th2 cells. It would be of interest to further study the function of this molecule in SLE as well as to explore it in additional autoimmune and inflammatory conditions, including type I diabetes mellitus. As stated above, we also consider that it would be important to assess the function of ILT2 as well as other regulatory receptors of this family in isolated lymphocyte subsets.

It is worth mentioning that different abnormalities in intracellular signaling pathways have been described in lymphocytes from SLE patients, including a defective function of CD3/TCR, a diminished expression of the ζ-chain [25,26], a reduced expression but increased activity of the lck kinase [42], and an impaired activation of PKA and MAPK [43,44]. It is feasible that these abnormalities could contribute to the defective function of ILT2 in the SLE lymphocytes. In this regard, it has been described that the activation of ILT2 depends on its phosphorylation by src kinases, mainly lck [8].

It has been described that the inhibitory effect of ILT2 in T lymphocytes could be related to an arrest in the G1 phase of cell cycle [45]. However, our data on DNA content analysis in HP-F1 treated cells did not show a significant accumulation of lymphocytes in this phase. Since the previous work was performed with T cell lines [45], this apparent discrepancy could be due to the different experimental conditions employed.

As it has been widely described [46,47], we have found that the baseline level of apoptosis of lymphoid cells is higher in SLE patients compared to healthy controls. This phenomenon seems to have a key role in the loss of tolerance to self-antigens, and in the synthesis of auto-antibodies. Interestingly, we found that the apoptosis of PBMC from healthy individuals, but not from SLE patients, tended to augment when ILT2 was engaged with the HP-F1 mAb. These results further support the defective function of this receptor in SLE, and suggest that ILT2 may have a role in the regulation of programmed cell death of lymphocytes, an issue that deserves future studies.

In summary, our results show that the PBMC from SLE patients exhibit a poor function of ILT2, and that their B lymphocytes show a diminished expression of this receptor. We think that it is very likely that these phenomena may significantly contribute to the complex pathogenesis of this autoimmune condition.

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References


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Table 1

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<td>6.8 (0.4–23.4)</td>
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* Median and 95% confidence limits of the percent of hypodiploid cells.