Altered recruitment of Lyn, Syk and ZAP-70 into lipid rafts of activated B cells in *Systemic Lupus Erythematosus*

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ABSTRACT

There is evidence that B cells from patients with Systemic Lupus Erythematosus (SLE) could be hyperactivated due to changes in their lipid rafts (LR) composition, leading to altered BCR-dependent signals. This study aimed to characterize possible alterations in the recruitment of protein tyrosine kinases (PTK) into B cells LR from SLE patients. Fifteen patients with SLE and ten healthy controls were included. Circulating B cells were isolated by negative selection and stimulated with goat Fab´2 anti-human IgM/IgG. LR were isolated with a non-ionic detergent and ultracentrifuged on 5–45% discontinuous sucrose gradients. Proteins from each fraction were analyzed by Western Blot. Total levels of Lyn, Syk, and ZAP-70 in resting B cells were similar in SLE patients and healthy controls. Upon BCR activation, Lyn, Syk and ZAP-70 recruitment into LR increased significantly in B cells of healthy controls and patients with inactive SLE. In contrast, in active SLE patients there was a great heterogeneity in the recruitment of signaling molecules and the recruitment of ZAP-70 was mainly observed in patients with decreased Syk recruitment into LR of activated B cells. The reduction in Flotilin-1 and Lyn recruitment in SLE patients seem to be associated with disease activity. These findings suggest that in SLE patients the PTK recruitment into B cell LR is dysregulated and that B cells are under constant activation through BCR signaling. The decrease of Lyn and Syk, the expression of ZAP-70 by B cells and the increase in Calcium fluxes in response to BCR stimulation in active SLE patients, further support that B cells from SLE patients are under constant activation through BCR signaling, as has been proposed.

1. Introduction

T and B cells from patients with Systemic Lupus Erythematosus (SLE) exhibit alterations in their signaling activation pathways [1–5] Some of these alterations have been explained by changes in the molecules recruited into the lipid rafts (LR), which are small membrane domains formed by lipids and cholesterol where membrane receptors and other molecules involved in signal transduction are recruited upon cell activation [6]. After receptor crosslinking LR merge, occupying a larger and polarized area on the cell surface. Among the molecules present in T and B cells LR are the Protein Tyrosine Kinases (PTKs), including the Zeta-chain-associated protein of 70-kDa (ZAP-70), the Spleen tyrosine kinase (Syk), and the Lck/Yes-related novel protein tyrosine kinase (Lyn) [7–9].

In B cells, after BCR crosslinking, Lyn is the first activated kinase that phosphorylates the ITAM residues on the BCR, allowing the binding of Syk, which in turn autophosphorylates, resulting in new SH2 binding sites for the recruitment of downstream signaling molecules. However, ZAP-70 a characteristic PTK of T cells and which is structurally homologous to Syk may replace it functionally [10]. Kong et al.

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[11] showed that expression of ZAP-70 in Syk negative B cells reconstitutes BCR function and that both ZAP-70 and Syk can bind directly to the phosphorylated Igα and lgβ BCR subunits with affinities comparable to their binding to the TCR CD3ζ subunit.

Among the abnormalities described in SLE patients´ T cells [5] are decreased expression of the CD3ζ chain in most patients, which seems to be replaced by the Fc gamma receptor (FcγR) chain, that recruits Syk instead of ZAP-70, resulting in a higher expression of Syk in T cells´ LR. Since the FcRγ-Syk interaction is stronger than that of the ζ chain-ZAP-70, there is augmented phosphorylation of downstream signaling molecules with increased calcium flux and actin polymerization [5].

B cells from SLE patients also exhibit dysregulated signal transduction. Early reports from Liossis et al. [12] showed that after BCR crosslinking, there is an increase in calcium flux and PTKs phosphorylation. However, more recently, Fleischer et al. [1] reported diminished Syk phosphorylation and reduced intracellular calcium release after BCR activation in SLE, compared to healthy donors, but these changes were corrected by inhibition of tyrosine phosphatases. These authors also reported increased phosphorylation of Akt after BCR stimulation in B cells from SLE patients. Additionally, B cells from SLE patients have reduced translocation of Lyn into the lipid rafts [2] and enhanced expression of CD45 phosphatase in these structures [13].

Prior reports [14] in chronic lymphocytic leukemia (CLL) cells showed that leukemic B cells express ZAP-70 and that BCR stimulation of these cells induced high levels of tyrosine phosphorylation of PTKs, including Syk, resulting in augmented BCR-dependent signal transduction and poor clinical prognosis. More recently, Lafarge et al., [15] confirmed that ZAP-70 expression in CLL cells enhances BCR signaling.

Since in SLE, B cells have been shown to be hyperactivated and hyperphosphorylated, resembling the CLL cells, it is thus possible to hypothesize that ZAP-70, which is not normally expressed by B cells, may be abnormally recruited into B cell LRs in SLE patients, leading to enhanced BCR signaling. For these reasons, this study was aimed to better characterize the alterations in Lyn, Syk and Zap-70 recruitment into LR after BCR engagement in B cells from SLE patients, compared to healthy donors.

2. Materials and methods

2.1. Patients and controls

Fifteen women with a diagnosis of Systemic Lupus Erythematosus (SLE) were recruited at the Hospital San Vicente Fundación and the Sede de Investigación Universitaria (SIU) of the Universidad de Antioquia (Medellín, Colombia). SLE diagnosis was made using the criteria of the American College of Rheumatology [16] and the disease activity defined by the SLEDAI [17]. Ten healthy women, matched by age (range 20–55 years) with the patients, were included as controls. All patients and controls signed an informed consent previously approved by the Ethics Committee of the Hospital San Vicente Fundación. The main demographic and clinical characteristics of the SLE patients included in this study are shown in Table 1.

2.2. Reagents and antibodies

RossetteSep® was purchased from Stemcell Technologies (British Columbia, Vancouver, Canada); Fetal Bovine Serum (FBS), Phosphate buffered saline (PBS), and RPMI-1640 medium were obtained from Gibco (Grand Island, New York, USA); Histopaque, Cholera Toxin B subunit-HRP conjugate, Sucrose, Sodium Orthovanadate, Chloroform, sodium dodecyl sulfate, MNE buffer, morpholino ethane sulfonic acid and sodium chloride, saponin, sodium azide, antibiotics and tween from Sigma-Aldrich (St. Luis, Missouri, USA); EDTA from Promega (Madison, Wisconsin, USA), Sodium fluoride from ICN Biomedicals (Costa Mesa, California, USA); Tris base and Glycine from Invitrogen (Carlsbad, California, USA); Triton X-100 from J.T. Baker (Center Valley,

Table 1
Demographic and clinical characteristics of SLE patients.

Demographic characteristics	
Age in years, mean (range)	29.9 (20–56)
Disease characteristics	
Duration of disease/years mean (range)	7.5 (0,1–25)
Short duration (0–5 years), % (n)	47 (7/15)
Long duration (> 5 years), % (n)	53 (8/15)
SLEDAI, mean (range)	4.7 (0–16)
Inactive SLE (SLEDAI < 4), % (n)	47 (7/15)
Active SLE (SLEDAI > 4), % (n)	53 (8/15)
Severe (SLEDAI > 12), % (n)	13 (2/15)
Lupus nephritis history % (n)	60 (9/15)
Presence of ANAS, % (n)	90 (9/10)
Treatment	
Steroids < 10 mg, % (n)	54 (7/13)
Steroids > 10 mg, % (n)	46 (6/13)

Pennsylvania, USA,), protease inhibitor from Roche (Basel, Switzerland); Methanol from Merck (Darmstadt, Germany); Protein Dual Color molecular weight standards from Bio-Rad Hercules, California, USA); Pure F(ab´)2 goat anti-human IgM + IgG from Jackson Immunoresearch (West Grove, Pennsylvania, USA); goat anti mouse IgG (H + L) – Alexa Fluor® 488 (GAMAF488), monoclonal mouse anti-Flotillin-1 (FLO-1) (Clone 18) anti-CD3 APC-Cy7 (SK7), and anti-CD45 PeCy7 (cLON) from BD Biosciences (Franklin Lakes, New Jersey, USA); monoclonal mouse anti-Lyn (Clone H-6), monoclonal mouse anti-Syk (Clone G-2), rabbit polyclonal anti-ZAP-70 (sc-574), monoclonal anti-ZAP-70 (1E7.2), goat anti-mouse IgG-HRP and goat anti-rabbit IgG from Santa Cruz (Dallas, Texas, USA), anti CD19-PE (clone J3-119) Beckman Coulter (Atlanta, Georgia. USA); IRDye 800CW from LICOR (Lincoln, Nebraska, USA), 1-step Fix/Lyse Solution, Fetal Bovine Serum (FBS) and SuperSignal Chemiluminescent Substrates and Pluronic acid/Fluo-3 from Thermofisher (Waltham, Massachusetts, USA).

2.3. B cells isolation, culture and stimulation

B cells from SLE patients and healthy controls were isolated by negative selection of CD19+ cells using the RossetteSep®. Fifty mL of venous blood were mixed with 1.25 mL of the enrichment cocktail for 20 min at room temperature; thereafter the mixture was centrifuged on Histopaque at $1000 \times g$ for 30 min. Cells at the interface were collected, washed and resuspended in RPMI-1640, supplemented with 10% FBS and antibiotics. The percentage of B cells, as determined by flow cytometry using anti-CD19-FITC, varied between 89 and 95%. Cells were cultured overnight at 37 °C and 5% CO_2, followed by stimulation with 15 μg/mL of F(ab´)2 goat anti-human IgM + F(ab´)2 goat anti-IgG (anti-IgM + anti-IgG) for 10 min at 37 °C. Then, the cells were washed with cold PBS at 1500 rpm for 10 min at 4 °C. The cell pellets were used for total B cell lysates and lipid rafts preparations.

2.4. Isolation and analysis of lipid rafts enriched-fractions

Lipid rafts (LR) were isolated in non-ionic detergent by ultracentrifugation on sucrose discontinuous gradients as described by Abdoel et al. [18]. Briefly, the cell pellet ($2–4 \times 10^6$ CD19 + cells) were mixed with 750 μL of lysis buffer (Triton X-100) in MNE buffer (25 mM morpholino ethane sulfonic acid), pH 6.5, 2 mM EDTA, 150 mM NaCl supplemented with proteases inhibitors (1×), sodium orthovanadate (2 mM), sodium fluoride (10 mM) for 20 min. After that, an equal volume of 90% sucrose MNE buffer with proteases/phosphatases inhibitors was added. The final mixtures (1.5 mL) at 45% sucrose concentration, were transferred to ultracentrifuge tubes (Beckman, Fullerton, California, USA) where 1.5 mL of 35% sucrose and a 1.5 mL
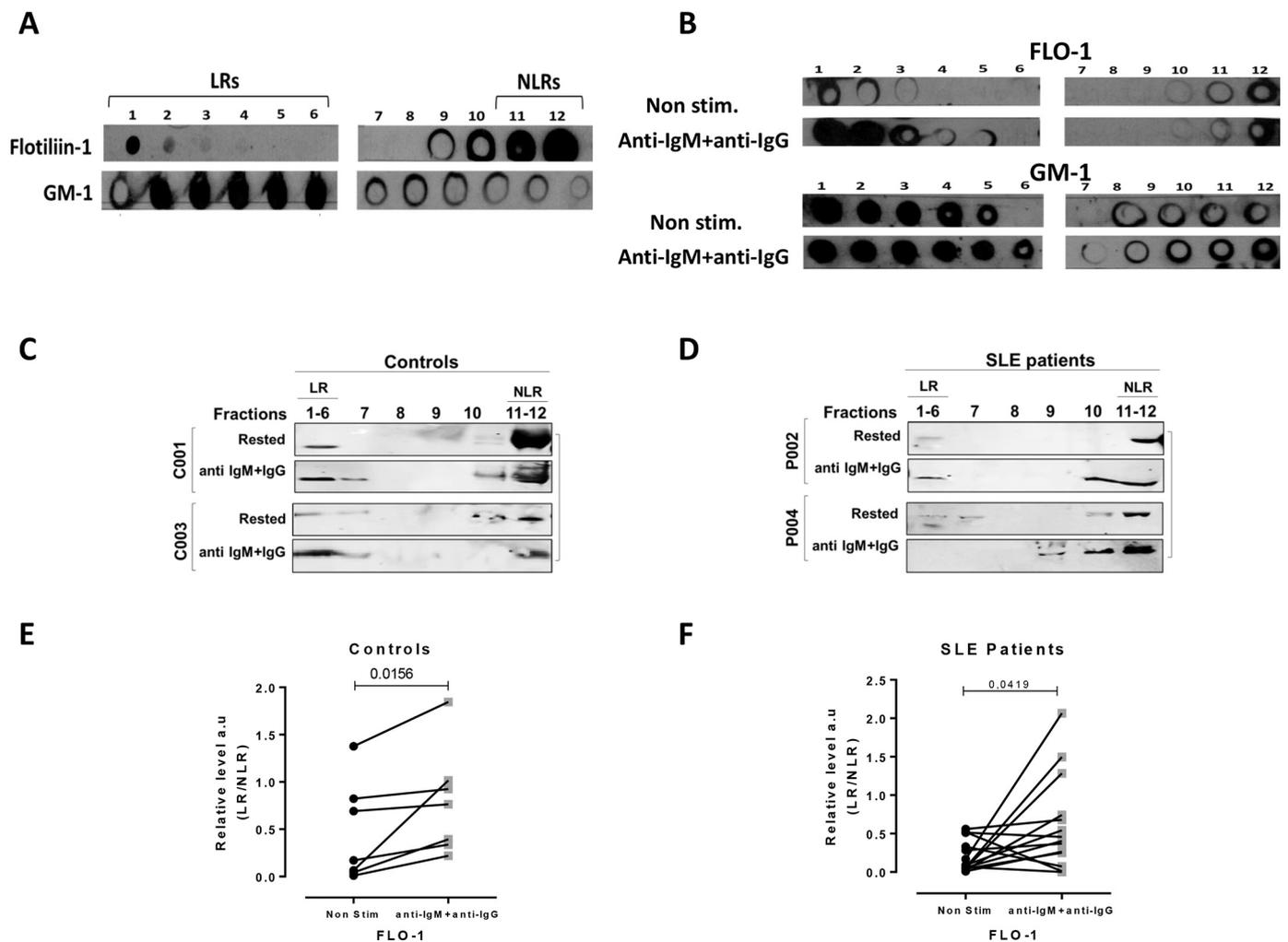
A

B

C

D

E

F

Fig. 1. Treatment with anti-IgM + anti-IgG induces the formation of LR in B cells from healthy controls and SLE patients.
B cell lysates were fractionated on discontinuous sucrose gradients, and the fractions dot blotted onto nitrocellulose membranes. A, B. FLO-1 and GM-1 were detected as described in Materials and Methods. Fractions 1–6 correspond to lipid rafts (LR) and fractions 11 and 12 correspond to non-lipid rafts (NLR) cytosolic fractions. A. Representative dot blots showing the distribution of FLO-1 and GM-1 in the 12 fractions obtained with the sucrose gradients from non-stimulated B cells of a healthy control. B. Representative dot blots showing the distribution of FLO-1 and GM-1 in the 12 fractions obtained with sucrose gradients from non-stimulated and anti-IgM + anti-IgG stimulated B cells of a healthy control. C–F. Fractions were electrophoresed on polyacrylamide gels, and the presence of FLO-1 was detected by Western Blot (WB), as described in Materials and Methods. C, D. Representative WB membranes showing FLO-1 in the fractions obtained with sucrose gradients from non-stimulated and anti-IgM + anti-IgG stimulated B cells of two healthy controls and two SLE patients, respectively. E, F. Consolidated results comparing the relative levels of FLO-1 (LR/NLR) in non-stimulated and anti-IgM + anti-IgG stimulated B cells of healthy controls (n = 10) and SLE patients (n = 15), respectively. Paired two-tailed t-student test.

of the second solution of 5% sucrose were added. The lysates were centrifuged at 38000 rpm (Beckman Coulter Optima xl-100 k Ultracentrifuge) for 20 h at 4 °C using a Beckman SW41 rotor. Twelve fractions of 375 μL each were collected starting at the upper part of the sucrose gradient.

2.5. Characterization of lipid rafts

One hundred and fifty microliters of each sucrose gradient fraction were vacuum transferred to nitrocellulose membranes (0.2 μm) using a Dot Blot vacuum equipment (Bio-Rad, Hercules, California, USA). Membranes were air-dried and then incubated with 5% non-fat milk in PBS for 1 h at room temperature.

Ganglioside GM-1 (GM-1) and flotillin-1 (FLO-1) were used as LR markers [19]. These molecules are typically found in fractions 1–6, whereas fractions 11–12 correspond to cytosolic fractions or non-LR (NLR). For detection of GM-1, the membranes were incubated with the Cholera Toxin B subunit-HRP (HRP-CTxB) diluted at 1:3000 in PBS

supplemented with 1% BSA for 1 h at room temperature. For detection of FLO-1, Syk and Lyn, the membranes were incubated with their respective antibodies (at 1:1000 for anti-FLO-1 and 1:500 for anti-Syk and anti-Lyn) in staining solution (5% non-fat milk/PBS 1 ×/Tween 0.05%) for 10 min; then the membranes were washed and incubated with goat anti-mouse IgG-HRP at 1:3000 in PBS 1 ×/Tween 0,05% for 10 min, and finally washed with PBS/Tween 0.05% for 10 min. The reaction was developed with SuperSignal™ (Thermo Scientific, Waltham, Massachusetts, USA) on X-Ray films (Kodak, Rochester, Nueva York, USA).

2.6. Western blot analysis of B cell lysates and ultracentrifugation fractions

The presence of FLO-1, Lyn, Syk and ZAP-70 in B cell lysates and ultra-centrifuged fractions was determined by SDS-PAGE electrophoresis and Western Blot. Fractions 1–6 (LR) and 11–12 (Non-LR) were pooled to facilitate their analysis. For this purpose, proteins were precipitated with methanol (1:1) and chloroform (1:1/4), and centrifuged at 4.200 rpm for 15 min at 4 °C; the supernatants were removed, 300 μL
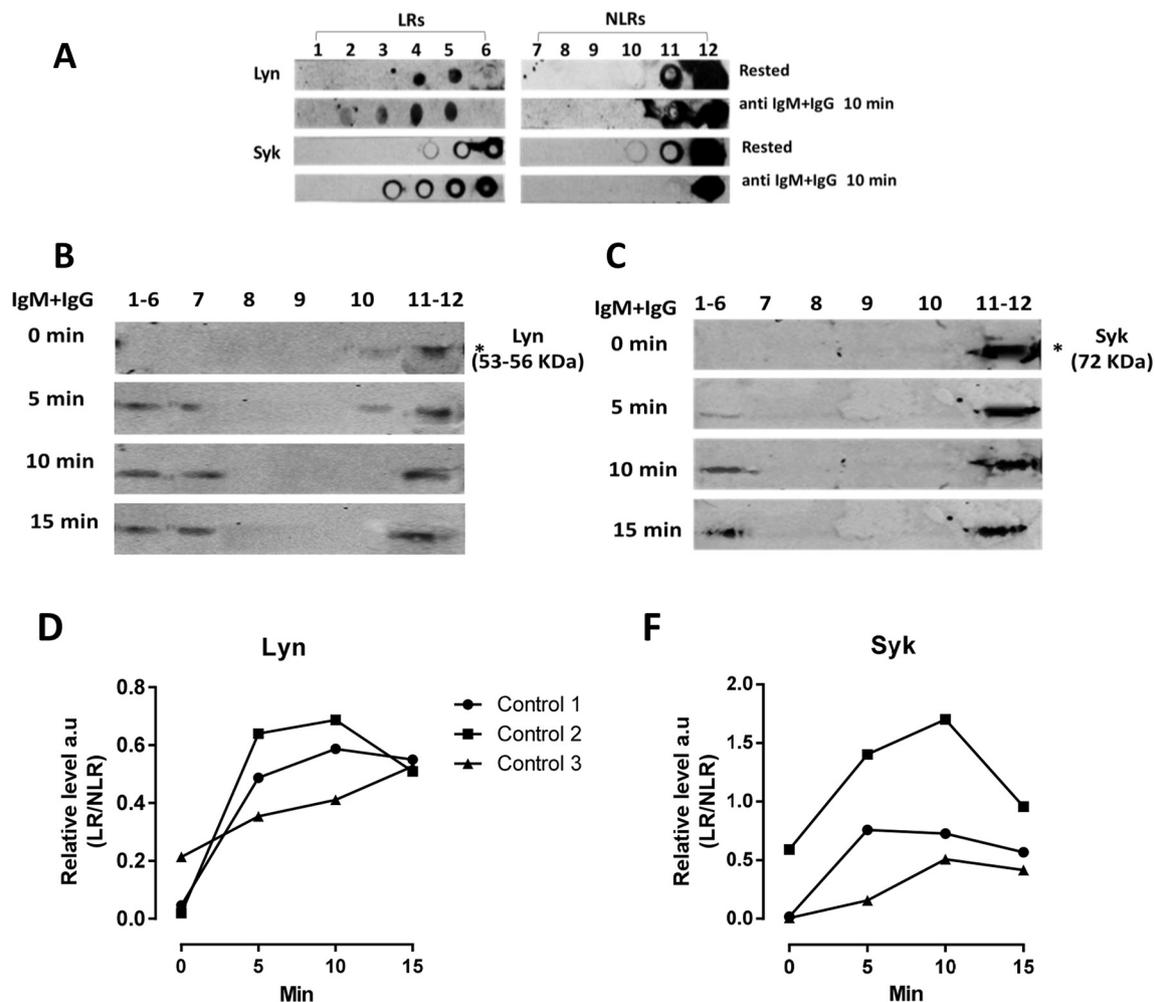
Fig. 2. Treatment with anti-IgM + anti-IgG induces the recruitment of Lyn and Syk into B cells LR from healthy controls.
B cell lysates were fractionated on discontinuous sucrose gradients, and the fractions dot blotted onto nitrocellulose membranes. Lyn and Syk were detected as described in Materials and Methods. Fractions 1–6 correspond to lipid rafts (LR) and fractions 11 and 12 correspond to non-lipid rafts (NLR) cytosolic fractions. A. Representative dot blots showing the distribution of Lyn and Syk in the 12 fractions obtained with sucrose gradients from non-stimulated and anti-IgM + anti-IgG stimulated B cells of a healthy control. Representative WB membranes showing Lyn (B) and Syk (C) in the fractions obtained with sucrose gradients from the B cells non-stimulated or stimulated with anti-IgM + anti-IgG stimulated for 5, 10 and 15 min of a healthy control. Kinetics of Lyn (D) and Syk (E) recruitment into LR in anti-IgM + anti-IgG stimulated B cells of 3 healthy controls.

of methanol was added and the mixture centrifuged again at 4.200 rpm for 15 min at 4 °C. The pellets were resuspended in Laemmli's buffer (5×).

2.7. Electrophoresis and WB

Ten microliters of each sample were loaded on 10% SDS-PAGE gels and electrophoresed at 100 mv for 40 min. Proteins were transferred to nitrocellulose membranes and incubated with blockade buffer (5% non-fat milk in PBS 1 ×) for 1 h at room temperature. Membranes were then incubated with each of the following antibodies: anti-FLO-1-1 at 1:1000, anti-Lyn at 1:500, anti-Syk at 1:500, anti-ZAP-70 at 1:500, and anti-B-actin at 1/10000, for 2 h. The membranes were then washed 3 × with PBS 1 ×/Tween 0,05% for 10 min and incubated with the secondary IRDye 800CW-labeled antibodies at 1:3000 for 1 h the reaction was developed using a chemiluminescence system and read in a LICOR Odyssey Fluorescent Imager (Surplus Solutions LLC, Lincoln, Nebraska USA). In the case of ZAP-70, the secondary antibody was goat anti-mouse IgG-HRP at 1:3000 and the reactions was determined by chemiluminescence, as described above.

2.8. Densitometric analysis

The quantitative densitometric analysis was done using Odyssey Infrared Imaging System application software, version 3.0 (Li-COR Miami, FL, USA). Background subtraction was automatically done by the software. Also, a second correction was done by measuring the signal in 10 different areas of the membrane chosen at random. To estimate the relative signal strength of the LR-associated proteins the ratio of the intensity of the bands corresponding to fractions 1–6 over the intensity of the bands corresponding to fractions 11 and 12 was calculated.

2.9. Detection of ZAP-70 in T and B cells by flow cytometry

Blood samples from one healthy control and four SLE patients were studied to determine the expression of ZAP-70 in T and B cells. For this purpose, 150 μL of venous blood were mixed with 2 μL de anti-CD45-Pe-Cy7, 15 μL of anti-CD19-PE, and 10 μL of anti-CD3-APC-Cy7 and incubated for 20 min at room temperature in the dark; and then treated with 2 mL of 1-step Fix/Lyse Solution. Thereafter, the cells were washed and resuspended in 2% FBS – DPBS, containing 0.09% sodium azide. Half of the cell suspension was kept as a control and the other half
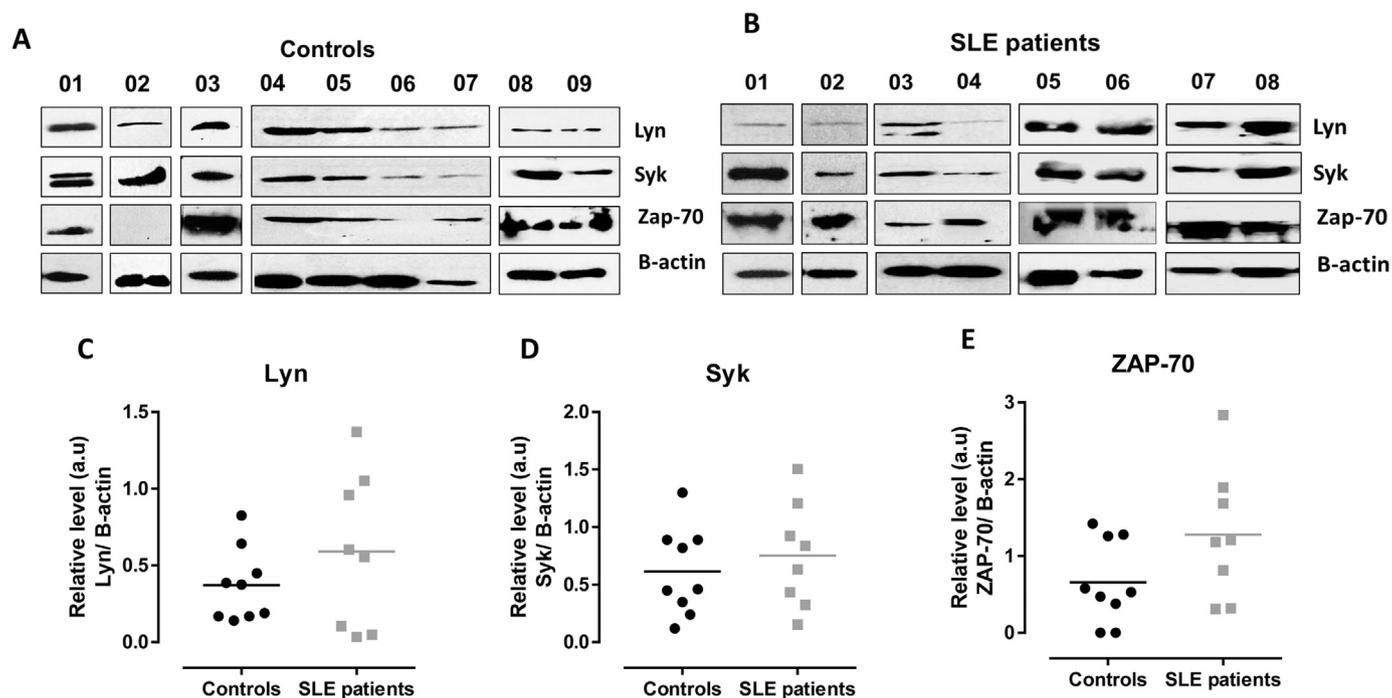
Fig. 3. Levels of Lyn, Syk, and ZAP-70 in whole B cell extracts from healthy controls and SLE patients.
A, B. Lysates of non-stimulated B cells were electrophoresed on polyacrylamide gels, transferred to nitrocellulose membranes and the presence of Lyn, Syk, ZAP-70 and β-actin detected by WB. Consolidated results of (C) Lyn, (D) Syk, and (E) ZAP-70 normalized with β-actin signal in healthy controls ($n = 9$) and SLE patients ($n = 8$). Non-paired t-student test.

permeabilized for the detection of intracellular ZAP-70. For permeabilization, the cells were washed and resuspended in 2% FBS – DPBS, containing 0.2% saponin; then, half of the permeabilized cells were incubated with 0.1 μg of anti-ZAP-70 (1E7.2) for 1 h on ice. The rest of the cells were not stained with anti-ZAP-70. After incubation, both cells fractions were washed and resuspended in 2% FBS – DPBS, containing 0.2% saponin and stained with 0.01 μg/mL of goat anti – mouse IgG (H +L) – Alexa Fluor® 488 (GAMAF488) for 60 min on ice. Finally, the cells were extensively washed and analyzed in a LSR Fortessa II flow cytometer (BD Biosciences, San Diego, CA). CD45$^+$CD3$^+$ and CD45$^+$CD19$^+$ cells were gated according to the following conditions: non-permeabilized cells treated with anti-ZAP-70 plus GAMAF4880, permeabilized cells treated with GAMAF488, and permeabilized cells treated with anti-ZAP-70 plus GAMAF488.

2.10. Measurement of calcium fluxes

PBMCs from three active SLE patients and three healthy women, were suspended at 1×10^6 cells/mL in 2 mL of loading buffer (Hans Balanced Solution with 0.1 μM pluoronic acid and 1 μg/mL of Fluo-3) and incubated at 37 °C, 10 min later 5 μg/mL anti-CD19-PE were added and the mixture incubated for additional 20 min. Calcium fluxes were measured by flow cytometry in a FACS Canto™ II flow cytometer (Becton Dickinson, San Jose, CA) with FACS DIVA software. Baseline was measured for 120 seg; thereafter, 5 μg/mL of anti-IgM + anti-IgG were added and mixed well; measurement continued until a total of 840 s. The Areas Under the Curve (AUC) were calculated using the FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR).

2.11. Statistical analysis

Differences in FLO-1, Lyn, Syk and ZAP-70 levels in LR between controls and patients were assessed using non-paired two tailed t-student test. Comparison of the relative signal strengths in non-stimulated and anti-IgM + anti-IgG stimulated B cells was done using the paired

two-tailed t-student test. The associations of Lyn, Syk and ZAP-70 alterations and SLE activity, as measured by the SLEDAI, were analyzed with the chi-square test. The differences in Calcium fluxes between stimulated and non-stimulated cells from active SLE patients and healthy controls were analyzed by Two-way ANOVA. *P* values < .05 were considered significant. Statistical analyses were done using the Prism 6 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Lipid rafts markers in resting and stimulated B cells from SLE patients and healthy controls

To characterize the composition of the fractions obtained in the sucrose gradients, the presence of the lipid rafts markers FLO-1 and GM-1 was determined in each fraction. As expected, these molecules were found in the fractions that correspond to LR. FLO-1 was found in fractions 1 and 2 of resting B cells (Fig. 1A, B), while in anti-IgM + anti-IgG activated B cells it exhibited a stronger signal that was present up to the fifth fraction (Fig. 1B). GM-1 was found mainly in fractions 1 to 6 of resting cells and stimulated B cells (Fig. 1A, B). FLO-1 and GM-1 were also found in cytosolic fractions 11 and 12 from resting and stimulated B cells.

Comparison of FLO-1 levels in LR (pooled fractions 1–6) of B cells from healthy controls and SLE patients, stimulated or not with anti-IgM + anti-IgG (Fig. 1C–E), showed that in both groups there was a significant increase in response to the BCR stimulation ($p = .029$ and $p = .038$, respectively); however, in all healthy controls the relative levels of FLO-1 increased (Fig. 1E), whereas 4/14 SLE patients these levels decreased (Fig. 1F) in response to anti-IgM + anti-IgG stimulation.

3.2. Lyn, Syk in B cells lipid rafts from SLE patients and healthy controls

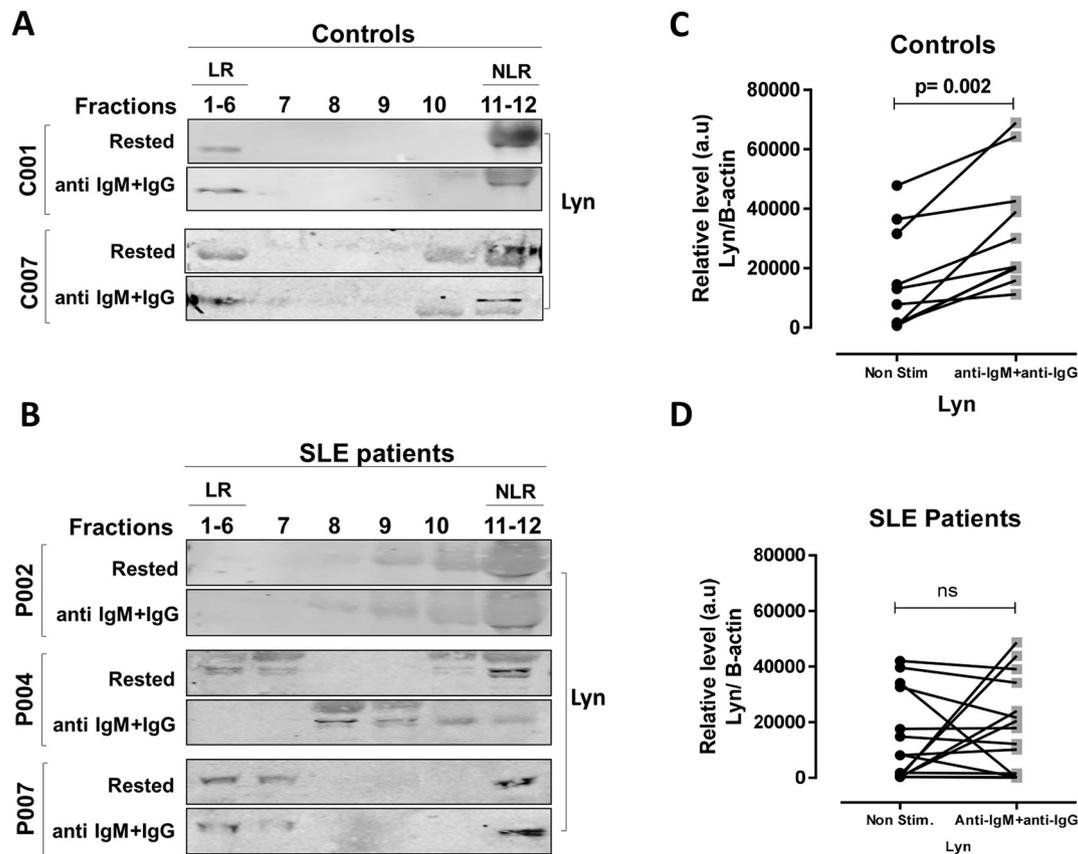The kinases Lyn and Syk were found in LR fractions, and their

Fig. 4. Effect of B cell activation on the recruitment of Lyn into LR in healthy controls and SLE patients.
B cells were stimulated or not with anti-IgM + anti-IgG for 10 min and their lysates fractioned with discontinuous sucrose gradients; fractions were electrophoresed on polyacrylamide gels. The presence of Lyn was detected by WB as described in Materials and Methods. A, B. Representative WB membranes showing Lyn in the fractions obtained from the non-stimulated and anti-IgM + anti-IgG stimulated B cells of two healthy controls and three SLE patients, respectively. C, D. Consolidated results comparing the relative levels of Lyn (LR/NLR) in non-stimulated and anti-IgM + anti-IgG stimulated B cells of healthy controls ($n = 10$) and SLE patients ($n = 15$), respectively. Paired two-tailed t-student test.

signals increased after the treatment with anti-IgM + anti-IgM (Fig. 2A) suggesting that the BCR stimulation induced their translocation to the LR. The kinetics of Lyn and Syk translocation to LR was studied by Western Blot (WB) (Fig. 2B). For these experiments, the fractions corresponding to the LR (fractions 1–6), as well as those corresponding to the cytosol (fractions 11–12), were pooled. Lyn and Syk were detected 5 min after treatment with anti-IgM + anti-IgM, and their signals decreased after 10 min, although they were still detected at 15 min (Fig. 2B, C). Of note, Lyn was also detected in fraction 7 of BCR stimulated cells (Fig. 2B). The densitometric results of the kinetics done in 3 healthy donors (Fig. 2D and F) showed albeit differences among the individuals, they have the same kinetics profile with maximal signals after 10 min of stimulation with anti-IgM + anti-IgG.

To determine whether the changes in the kinases recruitment to the LR may be due to differences in the total cellular levels of these molecules, the presence of Lyn and Syk in whole B cell extracts from SLE patients and controls was studied by WB. There were no significant differences in the relative levels of Lyn (Fig. 3A, B, and C) and Syk (Fig. 3A, B, and D) between SLE patients and controls.

Comparison of Lyn relative levels in LR (pooled fractions 1–6) of B cells from SLE patients and healthy controls, stimulated or not with anti-IgM + anti-IgG (Fig. 4) showed that while healthy controls exhibited a significant increase in response to BCR stimulation ($p = .007$) (Fig. 4A and C), the response in SLE patients was not significant (Fig. 4B and D). Similarly, Syk expression increased in B cell LR from healthy controls ($p = .001$) in response to BCR stimulation (Fig. 5A and C), whereas such increase was not observed in LR from SLE patients (Fig. 5B and D). These results, support that even though in B cells from

SLE patients the formation of LR in response to BCR stimulation was normal, the recruitment of Lyn and Syk to these structures was diminished.

3.3. ZAP-70 in B cells LR from SLE patients and healthy controls

To test the hypothesis that ZAP-70 may be present in B cells LR from SLE patients, its presence was determined in whole B cells extracts and sucrose gradients fractions of non-stimulated and BCR-stimulated B cells from SLE patients and healthy controls. The relative levels of ZAP-70 in whole B cell extracts was not different between controls and SLE patients (Fig. 3A, B, and E); however, LR (fractions 1–6) from BCR stimulated B cells from SLE patients (Fig. 6A, B) showed an increase in ZAP-70 ($p = .033$), not observed in LR from BCR stimulated B cells from healthy controls (Fig. 6C). Of note, in 4 SLE patients (P004, P005, P006 and P011) in whom Syk was absent in the LR of stimulated B cells, there was a strong increase of ZAP-70 signal in response to BCR stimulation (Fig. 6A).

In order to determine whether the detection of ZAP-70 in enriched B cell lysates could be due to the contamination with the remaining non-B cells, that may include ZAP-70^{+} T cells, its presence was studied by intracellular staining of circulating T and B cells from one healthy control and four SLE patients and detected by Flow Cytometry (Fig. 7). Non-permeabilized and permeabilized T and B cells stained only with the secondary antibody did not show any significant fluorescent signal. Contrariwise, permeabilized T and B cells stained with anti-ZAP-70 plus the secondary antibody exhibited an increased fluorescent signal, compared with the cells permeabilized but stained only with the
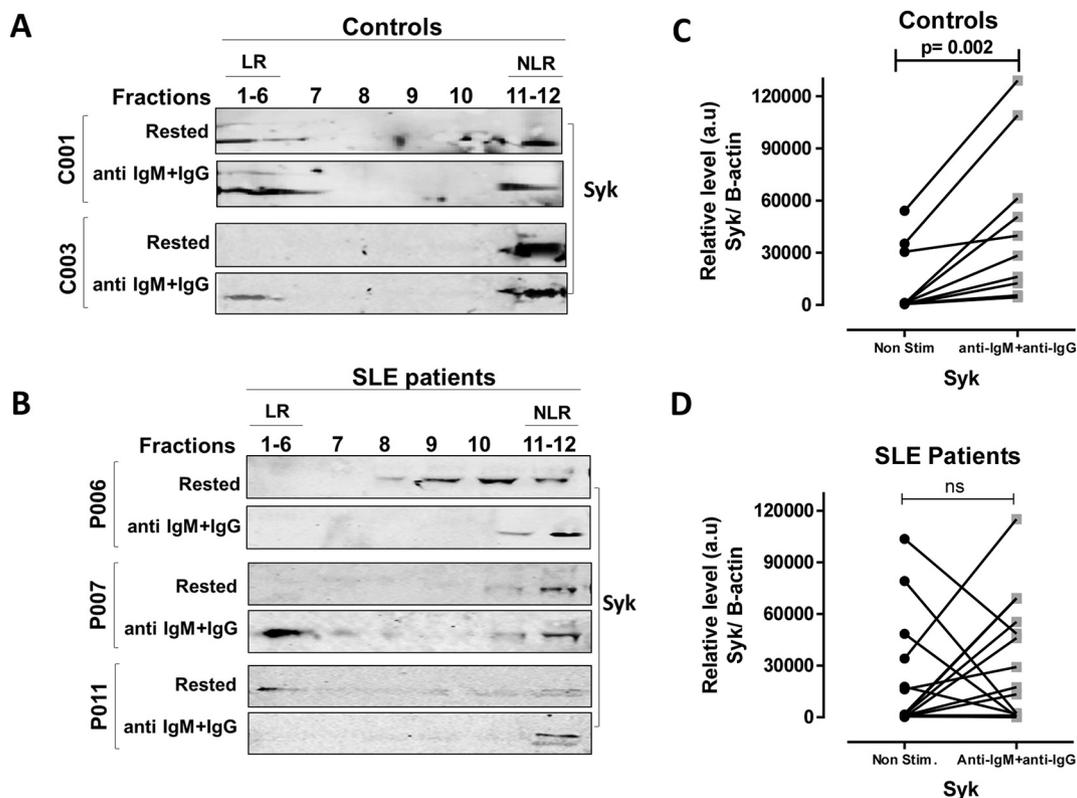
Fig. 5. Effect of B cell activation on the recruitment of Syk into LR in healthy controls and SLE patients.
B cells were stimulated or not with anti-IgM + anti-IgG for 10 min and their lysates fractioned with discontinuous sucrose gradients; fractions were electrophoresed on polyacrylamide gels. The presence of Syk was detected by WB as described in Materials and Methods. A, B. Representative WB membranes showing Syk in the fractions obtained from the non-stimulated and anti-IgM + anti-IgG stimulated B cells of two healthy controls and three SLE patients, respectively. C, D. Consolidated results comparing the relative levels of Syk (LR/NLR) in non-stimulated and anti-IgM + anti-IgG stimulated B cells of healthy controls (n = 10) and SLE patients (n = 15), respectively. Paired two-tailed t-student test.

secondary antibody. These findings are strong evidence that circulating B cells express ZAP-70, albeit at a lower level than T cells.

3.4. Lipid rafts profiles in SLE patients

Table 3 summarizes the findings of the marker molecule FLO-1 and activation-associated molecules Lyn, Syk and ZAP-70 in SLE patients. In only one (P002) of the inactive patients FLO-1 was found in resting cells, and in all of them (P002, 003, 006, 007, and 010) FLO-1 was recruited in response to BCR stimulation. In contrast, in 6/9 of active SLE patients FLO-1 was already present (P004, 008, 011, 012, 013, and 014), suggesting that their cells were pre-activated *in vivo*. Also, in 4/9 (P004, 008, 011, and 013) a reduction in FLO-1 was observed upon BCR stimulation, suggesting alterations in the conformation or maintenance of their lipid rafts after BCR stimulation.

Regarding the signaling molecules Lyn and Syk, it was observed that in inactive SLE patients these molecules increased or remained at the same level upon BCR stimulation; while in active SLE patients there was a great heterogeneity in the recruitment of these molecules, with a reduction or no change of Lyn in 7/9 and Syk in 4/7.

Regarding ZAP-70, although the number of samples was smaller, its presence was observed in stimulated cells of 8/9 patients evaluated. Of note, in 4 patients, 3 active and one inactive, who had reduction or no change in the Syk molecule, the presence of ZAP-70 was detected.

3.5. Association of LR changes with SLE activity

Since the levels of Lyn and Syk in LR from SLE patients in response to BCR stimulation was not homogeneous, being absent or decreased in some of them, compared to the response observed in healthy controls

that always exhibited increased signals of these kinases, the possible association of such alterations with SLE activity, measured by the SLEDAI, was calculated (Table 2). Indeed, the altered recruitment of FLO-1 and Lyn was associated with SLEDAI ≥4 ($p = .038$ and $p = .042$, respectively), which is considered an indicator of disease activity.

3.6. Calcium fluxes in B cells

Since the altered BCR-induced cell signaling found in SLE patients, involving changes in lipid rafts recruitment of Lyn, Syk and the possible ectopic expression of ZAP-70, may result in functional B cell hyperactivity, calcium fluxes were measured in B cells from 3 healthy controls and 3 patients with active SLE, after stimulation or not with anti-IgM + anti-IgG. As shown in Fig. 8, active SLE patients and healthy controls had very different response to BCR stimulation. While B cells from SLE patients responded immediately to the stimulus and exhibited a rapid and steady increase in intracellular calcium, B cells from healthy controls showed a lag of approximately 2 min to initiate Calcium fluxes, that reached a plateau almost immediately and remained at levels well below those observed in SLE patients (Fig. 7A). Consolidated results of SLE patients and healthy controls show that even though both controls and patients responded to BCR stimulation, the response by B cells from SLE patients was higher (Fig. 7B), supporting that indeed B cells from active SLE patients are functionally hyperactivated.

4. Discussion

It is well known that upon cell activation different immunoreceptors are recruited to LR [3,5,13,20]. Receptor-LR association appears to be a regulatory step for the signaling mechanisms used by many
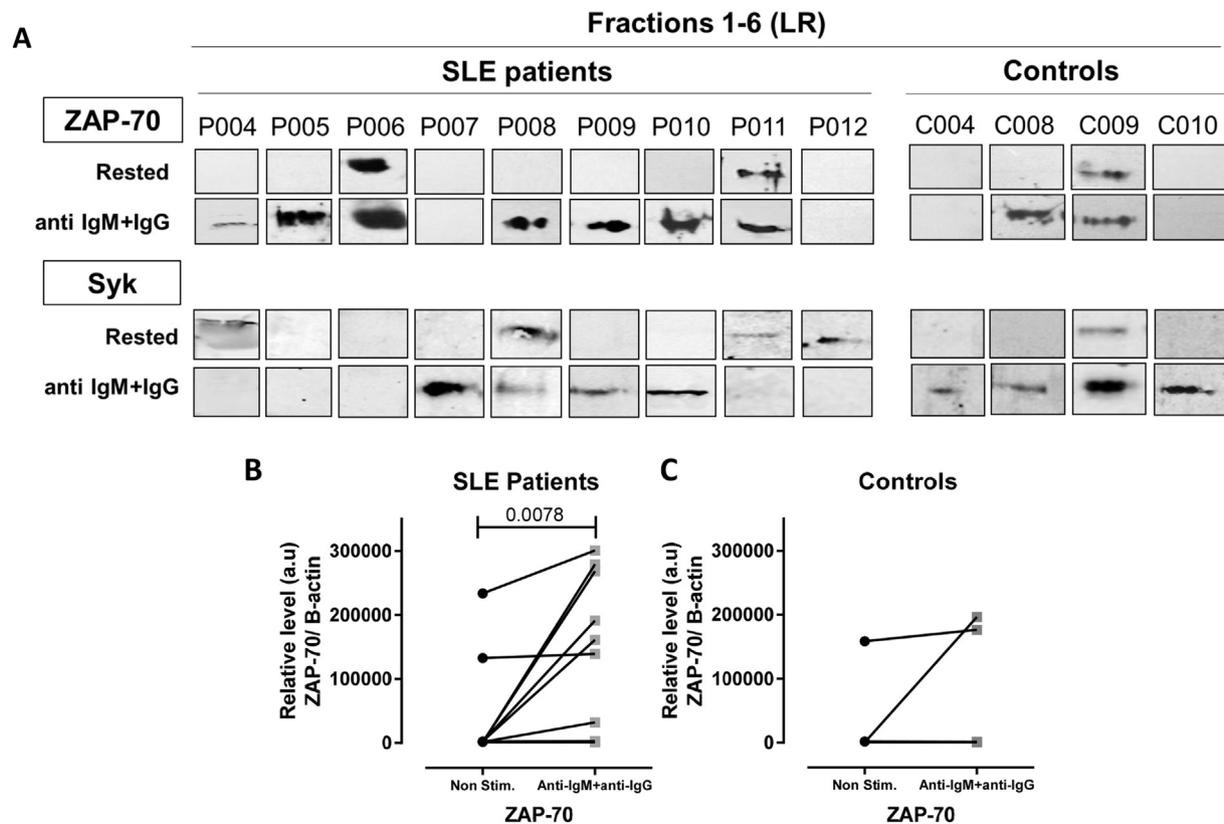
Fig. 6. Effect of B cell activation on the recruitment of ZAP-70 into LR in healthy controls and SLE patients.
B cells were stimulated or not with anti-IgM + anti-IgG for 10 min and their lysates fractioned with discontinuous sucrose gradients; fractions were electrophoresed on polyacrylamide gels. The presence of ZAP-70 was detected by WB as described in Materials and Methods. A. Representative WB membranes showing ZAP-70 and Syk in fractions obtained from non-stimulated and anti-IgM + anti-IgG stimulated B cells of nine SLE patients and four healthy controls, respectively. B, C. Consolidated results comparing the relative levels of ZAP-70 (LR/NLR) in non-stimulated and anti-IgM + anti-IgG stimulated B cells of healthy controls ($n = 4$) and SLE patients ($n = 9$), respectively. Paired two-tailed t-student test.

immunoreceptors, although the molecular requirements that regulate these associations remain unclear [13]. Alterations in the recruitment of such receptors into LR occurs in different immune-mediated diseases and may play an important role in their pathogenesis [21]. Hyperactivity of B cells is a central feature of SLE [1]. Some abnormalities of BCR signaling in human SLE have been described; among them, decreased Lyn and enhanced CD45 recruitment in B cells LR and reduced levels of the phosphatase and tensin homologous (PTEN), resulting in enhanced Akt activation and a general increase of tyrosines phosphorylation [2,22].

The results presented herein show that B cells from SLE patients have an altered capacity to structure the LR in response to BCR stimulation; particularly, active SLE patients showed a great heterogeneity in the recruitment of signaling molecules Lyn and Syk and a significantly enhanced of recruitment of ZAP-70 to LR, in contrast in inactive SLE patients these molecules increased or remained at the same level upon BCR stimulation;. Importantly, these alterations do not seem to be due to changes in the total content of these molecules, since whole extracts of resting B cells did not show differences with healthy controls.

Previous studies revealed that B cells from most patients with SLE have an altered translocation of Lyn to LR [2], and there is evidence that Lyn reduction may be due to increased ubiquitination [2]. Studies of B cell responses in SLE patients reported that decreased Lyn expression might be associated with heightened spontaneous proliferation, anti-dsDNA autoantibodies, and increased interleukin-10 production [12].

Regarding the decrease in Syk, an important PTK in the BCR-signaling pathway, it has been reported that in T cells from SLE patients,

upon stimulation of the FcγR chain, there is the recruitment of Syk, instead of the normally recruited ZAP-70 [5]. The FcγR-Syk interaction is exponentially (> 100-fold) stronger than that of the ζ chain-ZAP 70 combination, rendering a stronger downstream intracellular signal [5].

The ZAP-70 expression in B cells is not clearly defined; there is evidence of the presence of this molecule in chronic lymphocytic leukemia (CLL) B cells [23–25], but the functional significance of ZAP-70 expression in CLL B cells is still uncertain. ZAP-70 was shown to undergo tyrosine phosphorylation and to associate with the BCR complex in antigen-stimulated CLL B cells, which suggested that ZAP-70 might be involved in BCR signal transduction even when Syk is present [11]. Furthermore, experiments with avian lymphoma B cells have shown that ZAP-70 can restore some BCR-signaling events when Syk is not expressed, indicating a certain degree of functional homology between the both PTKs [11].

In the past, ZAP-70 was considered a T cell specific kinase, and its ectopic expression in B-CLL cells was interpreted as a sign of malignant transformation and de-differentiation [25–27]. However, using more sensitive and specific antibodies, ZAP-70 was recently detected in normal human B cells [25] and in pro-B, pre-B and mature murine B cells suggesting that it may participate in the transition of pro-B to pre-B cells in the bone marrow. Also, recent reports in human B cells indicated that ZAP-70 is expressed at the mRNA and protein level in some human B-cell lines [22]. Others reports confirmed that a subgroup of normal B cells expresses ZAP-70 and this expression may correlate with the state of B-cell activation [28]. Furthermore, ZAP-70 expression can be induced *in vitro* upon stimulation of blood and tonsillar B cells [28].

In this study, we found expression of ZAP-70 in B cells of SLE patients and healthy controls; however, since the enriched B cell fractions
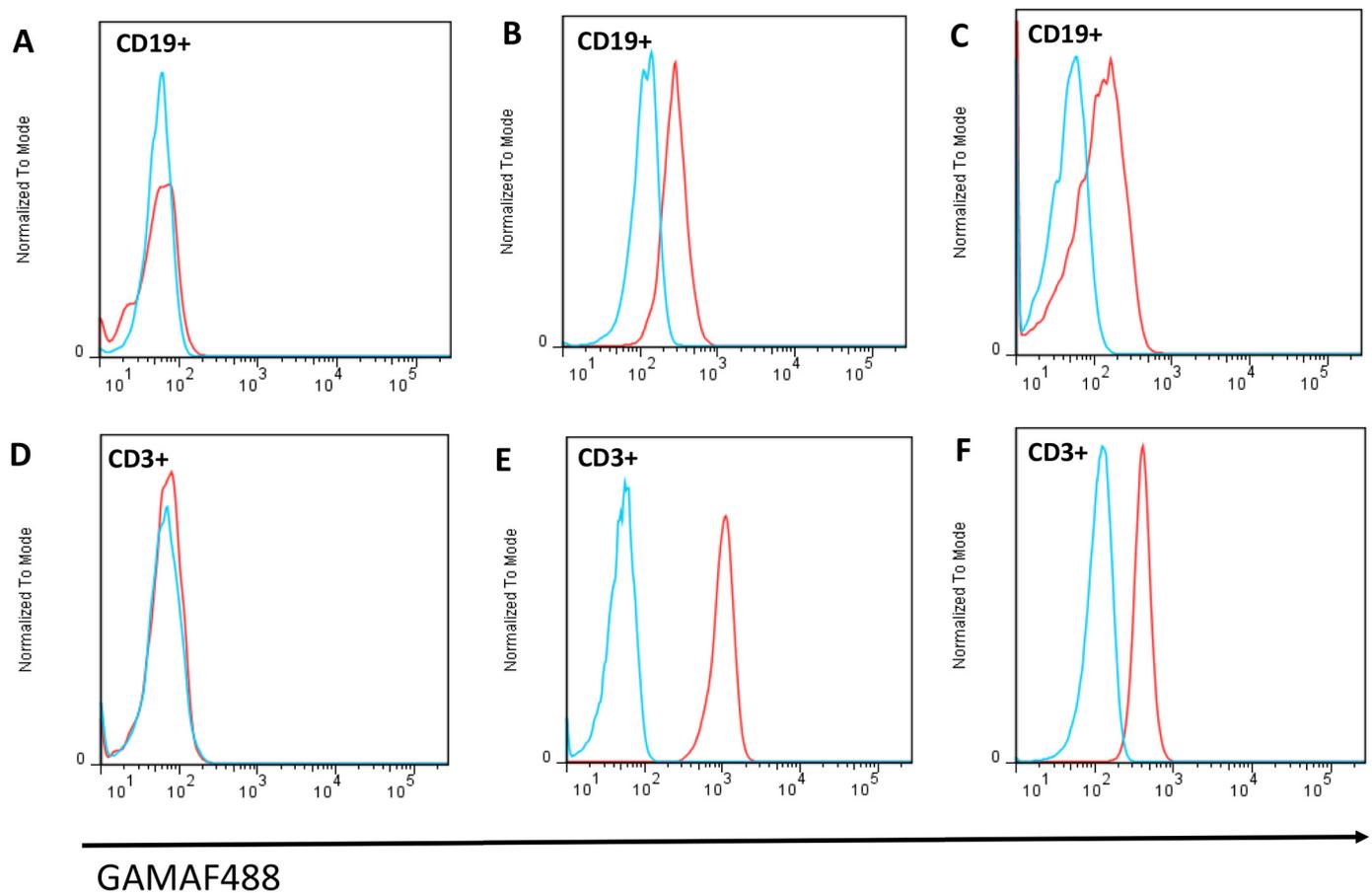
Fig. 7. Expression of ZAP-70 in circulating T and B cells as determined by Flow Cytometry.

Samples of venous blood were stained with anti-CD45-Pe-Cy7 plus anti-CD19-PE plus antiCD3-APC Cy7, followed by permeabilization with saponin and intracellular staining with GAMAF488 ± anti-ZAP-70 and analyzed by Flow cytometry as described in Materials and Methods. (A–C) CD19^{+} B cells; (D–F) CD3^{+} T cells; (A, D) Staining controls of non-permeabilized CD19^{+} and CD3^{+} cells; (B, E) CD19^{+} and CD3^{+} permeabilized cells from one healthy control; (C, F) CD19^{+} and CD3^{+} permeabilized cells from one SLE patient, representative of four SLE patients studied. Blue histograms: cells stained with GAMAF488; red histograms: cells stained with anti-ZAP-70 plus GAMAF488.

Table 2

Association of SLE activity and alterations in LR of anti-IgM + anti-IgG stimulated B cells.

Absence/reduction in LR	SLEDAI < 4 (n = 6)	SLEDAI ≥ 4 (n = 9)	P value
FLO-1	0/5 (0%)	4/9 (44%)[a]	0.038
Lyn	1/6 (17%)	7/9 (78%)	0.042
Syk	2/6 (33%)	5/9 (56%)	0.2
ZAP-70	1/3	2/6	

[a] Chi^2 test.

used to prepare the cell extracts still contained a small percentage (< 10%) of non-B cells that may explain the presence ZAP-70 positive signals detected by western blot, further experiments were done to detect it by flow cytometry after intracellular staining of circulating T and B cells. The results strongly support that circulating B cells indeed express ZAP-70, albeit at lower levels of T cells, as expected. After BCR stimulation, a significant increase of ZAP-70 levels in LR of SLE patients was found, and additionally, a possible association between increased ZAP-70 recruitment into LR with decreased recruitment of Syk in activated B cells of SLE patients was also observed. These findings suggest that ZAP-70 could restore some BCR-signaling events when Syk is decreased and even when both molecules are simultaneously present. The presence of both molecules in B cell LR suggests a possible mechanism of potentiation of BCR signals, which could explain the higher Calcium fluxes observed in BCR-stimulated cells in active SLE patients and the B

cell activation previously described in patients with SLE.

The classification of patients according to the disease activity allowed to establish some patterns of alterations in LR. Whereas inactive patients homogenously structure the LR and recruit the signaling molecules in response to BCR-stimulation, active SLE was associated with a high heterogeneity in their LRs. These alterations may be due not only to the immunological alterations associated with this disease, but also to the different immunosuppressants used in the management of the active disease.

The association of altered translocation of FLO-1 and Lyn with disease activity contrast with previous findings of Borja and Liossis [2,12] who found similar disease activity, establish by SLEDAI or by the British Isles Lupus Assessment Group score, in patients with decreased levels of Lyn in LR; however, a larger number of patients should be studied in order to further strengthen our observation.

5. Conclusions

- Signaling through the BCR and the structuring of LR in B cells of patients with SLE seem to be abnormal.
- The finding of reduced recruitment of Lyn, a negative regulatory signal, coupled with the absence of Syk and presence of ZAP-70 suggests that the activation pathway of B cell through the BCR is dysregulated.
- Altogether, these may partially explain the constant activation of B cells, extensively described in SLE.

Table 3
Summary of changes observed in lipid rafts of B cells from SLE patients treated with anti-IgM + anti-IgG.

SLE patients	FLO-1			Lyn			Syk			ZAP-70		
	Rested	Treated	Response	Rested	Treated	Response	Rested	Treated	Response	Rested	Treated	Response
P002	+	+	↑	−	+	↑	−	−	→	nd	nd	nd
P003	−	+	↑	−	−	→	−	+	↑	nd	nd	nd
P006	−	+	↑	+	+	↑	−	−	→	+	+	↑
P007	−	+	↑	+	+	↑	−	+	↑	−	−	→
P010	−	+	↑	−	+	↑	−	+	↑	−	+	↑
P015	nd	nd	nd	−	+	↑	+	+	↑	nd	nd	nd
P001**	−	+	↑	−	−	→	−	+	↑	nd	nd	nd
P004**	+	−	↓	+	−	↓	+	−	↓	−	+	↑
P005**	−	+	↑	−	−	→	−	−	→	−	+	↑
P008**	+	+	↓	+	+	↓	+	+	↓	−	+	↑
P009**	−	+	↑	−	+	↑	−	+	↑	−	+	↑
P011**	+	−	↓	+	+	↓	+	−	↓	+	+	↓
P012**	+	+	↑	+	+	↑	+	−	↓	−	−	→
P013**	+	+	↓	+	+	↓	+	+	↑	nd	nd	nd
P014**	+	+	↑	+	−	↓	+	+	↑	nd	nd	nd

Dashed line separates the inactive (above) and active (below) SLE patients.

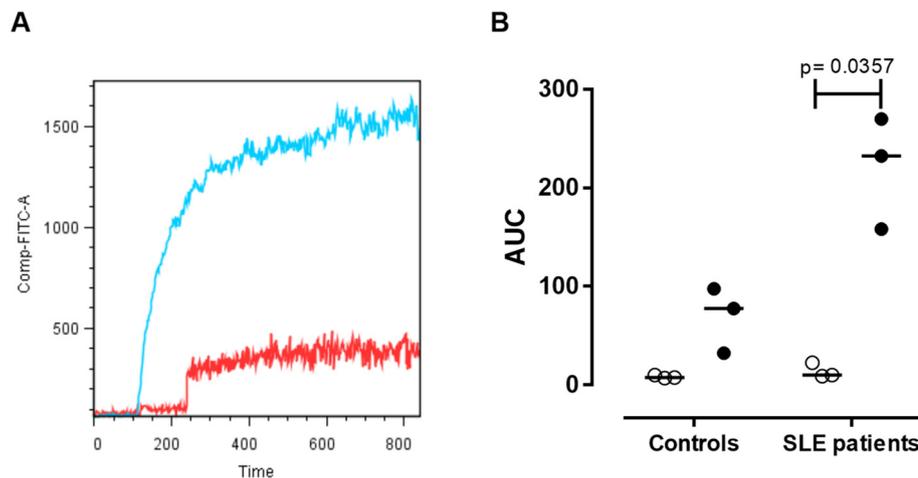** : SLEDAI ≥ 4; −: Absent; +: Present; →: no change; ↑: increase; ↓: decrease; nd: not determined.

Fig. 8. Calcium fluxes in B cells from healthy controls and SLE patients.
PBMCs were isolated and B cells were loaded with pluoronic acid/Fluo-3 and stained with anti-CD19-PE; Calcium fluxes were measured by flow cytometry up to 840 s as described in Materials and Methods. A. Calcium fluxes in a healthy control (red) and an active SLE patient (blue) run in parallel. B. Consolidated results of the Area Under the Curve (AUC) of Calcium fluxes in B cells from 3 healthy controls and 3 active SLE patients stimulated or not with anti-IgM + anti-IgG. Open circles: non-stimulated B cells; Closed circles: anti-IgM + anti-IgG stimulated B cells. Two-Way ANOVA.

Funding

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