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Increased association of CD38 with lipid rafts in T cells from patients with systemic lupus erythematosus and in activated normal T cells

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Abstract

In this study we have determined whether there is a relationship between CD38 expression on T cells, its distribution in different membrane microdomains, and T cell activation in SLE patients. The data show that CD38 expression is augmented in ex vivo $CD3^+$, $CD4^+$, $CD8^+$, and $CD25^+$ SLE T cells, which correlates with its increased insolubility in Brij 98 detergent, and its translocation into lipid rafts. Moreover, SLE T cells show an altered CD4:CD8 ratio, which is due to a decreased proportion of $CD4^+$ T cells and a concomitant increase in the proportion of $CD8^+$ T cells. These data are consistent with the increased CD38 expression and lipid raft formation, and the significant reduction in the CD4:CD8 ratio observed in mitogen-stimulated normal T cells as compared with that in *ex vivo* untouched normal T cells. Increased expression of CD38 in floating rafts from SLE T cells, or from activated normal T cells may modulate TCR signaling by providing or sequestering signaling molecules to the engaged TCR.

Keywords: CD38; TCR; Signaling; Lipid rafts; T cells; Lupus; Autoimmunity

1. Introduction

SLE is a multisystemic autoimmune disease characterized by the production of high levels of autoantibodies against nuclear antigens resulting, at least in part, from a dysregulated T lymphocyte response to autoantigens (Janeway et al., 2001; Kammer et al., 2002; Kong et al., 2003). Although SLE etiopathology is poorly understood, there is likely a role for environmental triggers, for instance viruses, acting in the context of susceptibility genes (Rozzo et al., 2001; Wakeland et al., 2001). In both, European-American, and Iceland populations it has been identified a SLE susceptibility locus on human region 4p15 (Gray-McGuire et al., 2000; Lindqvist and Alarcon-Riquelme, 1999). Located within human 4p15 region are a number of interesting candidate genes including CD38 and CD157, which are both ectoenzymes members of the ADP-ribosyl cyclase family that are directly involved with T and B lymphocyte activity (Deaglio et al., 2001; Ortolan et al., 2002). There is an association between a polymorphism located at position 182 of intron 1 of the CD38 gene, and the development of discoid rash in Spanish SLE patients (Gonzalez-Escribano et al., 2004).

Previous studies have defined B cell subsets from inflamed secondary lymphoid tissue, such as tonsil, or the periphery of active-SLE patients that are defined by very high expression of CD38 and the presence of intracellular immunoglobulin (Grammer and Lipsky, 2003). On the other hand, increased expression of CD38 on T cells has been found in SLE patients (Alcocer-Varela et al., 1991; al-Janadi and Raziuddin, 1993; Erkeller-Yuksel et al., 1997). In certain tumor types, up-regulation of CD38 in tumor-associated non-neoplastic T

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cells could be useful as a marker of persistent T cell activation as well as a surrogate marker of disease progression (Lin et al., 2004). Likewise, up-regulation of CD38 on $CD8^+$ T cells could be a marker of ongoing viral replication during acute or chronic viral infections (Belles-Isles et al., 1998; Carbone et al., 2000; Lynne et al., 1998; Ramzaoui et al., 1995).

Plasma membranes of many cell types, including T cells, contain specialized microdomains, or lipid rafts, enriched in sphingolipids, cholesterol, sphingomyelin and glycosylphophatidylinositol-anchored proteins. These membrane domains are characterized by detergent-insolubility at low temperatures and low buoyant density (Brown and London, 2000; Simons and Toomre, 2000). Lipid rafts play a central role in signal-transduction, in the immune response and in many pathological situations on the basis of two important raft properties, their capacity to incorporate or exclude proteins selectively, and their ability to coalesce into larger domains (Manes et al., 2003; Simons and Ehehalt, 2002). The densely packed, liquid-ordered environment of rafts excludes most integral membrane proteins. However, we have demonstrated that in T cells CD38 is constitutively associated with lipid rafts resistant to solubilization in 1% NP-40 at 4 °C (Zubiaur et al., 2002), or in 1% Brij 98 at 37 °C (Munoz et al., 2003). Furthermore, CD38 in rafts is able to initiate and propagate several activating signaling pathways, possibly by facilitating critical associations within other raft subsets, per example LAT rafts, via its capacity to interact with Lck and CD3-ζ (Munoz et al., 2003).

Changes in the properties or composition of lipid rafts may lead to inappropriate T lymphocyte signaling and ultimately to the development of pathological conditions, including autoimmunity (Gringhuis et al., 2000; Salojin et al., 1998). In this sense, in SLE T cells there are qualitative alterations in the protein composition of lipid rafts, alterations in actin dynamics, and increased calcium responses (Krishnan et al., 2004). Moreover, reduced expression of Lck in SLE T cells (Jury et al., 2003) is associated with increased expression of GM1 and increased localization of CD45, a molecule important in regulating Lck activity (Jury et al., 2004).

In this study we found that CD38 expression is augmented in $CD3^+$ T cells from patients with SLE, which correlates with its increased translocation into lipid rafts. These data are consistent with increased lipid raft formation in activated normal T cells, and the evidence that SLE T cells display activation phenotypes.

2. Material and methods

2.1. Patients and healthy controls

Fifty-one consecutive outpatients fulfilling the revised American College of Rheumatology (ACR) criteria for the diagnosis of SLE (Tan et al., 1982), and routinely followed in the Systemic Autoimmune Diseases Unit (Hospital Clínico San Cecilio, Granada, Spain) could participate in this study between December 2002 and May 2004. Thirty-six healthy, age-matched healthy volunteers served as controls. Disease activity was scored and the SLE Disease Activity Index (SLEDAI) was calculated (Bombardier et al., 1992). Our study included 22 inactive SLE patients, 29 active patients with SLEDAI ranging from one to 20, and 36 healthy control volunteers (Table 1). All of them were Caucasians. Patients who were being treated with prednisone were asked not to take this medication 24 h before blood was drawn. For each patient, data were recorded on age, duration of the disease defined as the period from the disease onset (time when patients fulfilled the ACR criteria) to the time of the study, current and cumulative gluco-corticoid doses by review of patient records. Complete blood cell count, serum complement and serum anti-nuclear and anti-DNA antibodies were measured in all patients. Because the paucity of cell numbers obtained from the disease group, many of whom were leukopenic, different sets of experiments were performed with different patient groups but care was taken to include patients of SLEDAI score ranging from low (0–4 range) to high (5–20) in each group. Within each group, patient samples were matched with normal samples of similar ages and gender. The study protocol was approved by the Hospital Clínico San Cecilio, and CSIC Review Board and Ethics Committees. Written informed consent was obtained from all participating patients and volunteers according to the Declaration of Helsinki.

Table 1
Clinical and demographic characteristics of the study subjects

	SLE ($N = 51$)	Healthy controls ($n = 36$)
Women	45 (88%)	21 (58%)
Age (y)	38.1 (20–77)	38.1 (20–77)
Caucasian	51 (100%)	36 (100%)
Disease duration (y)	4.1 (1–15)	NA
SLEDAI	3.35 (0–20)	NA
SLEDAI = 0	22 (43.1%)	NA
SLEDAI = 1–4	14 (27.4%)	NA
SLEDAI > 4	15 (29.4%)	NA

2.2. T lymphocyte isolation

PBMC were obtained by density gradient centrifugation over HISTOPAQUE®-1077 (Sigma-Aldrich Química, S.A., Spain). T cells were isolated from PBMC by magnetic separation after depletion of non-T cells by negative selection with the Pan T Cell Isolation Kit II (Miltenyi Biotec, GmbH, Germany) following the manufacturer's instructions. In all the cases, the percentage of T cells in the isolated subpopulation was >98% as determined by anti-CD3-ε mAb staining and fluorescence-activated cell sorter (FACS) analysis. T cells were stimulated with 5 μg/ml PHA-L and and 50 U/ml IL-2 (Sigma-Aldrich, Química, S.A.) for 3 days.

2.3. Antibodies and reagents

Anti-human CD3-ε mAb OKT3 (IgG_{2a}), or the CD38 mAbs HB136 (IgG_1) and OKT10 (IgG_1) were prepared, pu-

rified, and labeled with fluorescein isothiocyanate (FITC) as previously described (Zubiaur et al., 1999). Affinity purified anti-human CD4-PE and CD8-FITC antibodies, the CTB-FITC conjugate and the affinity purified mouse mAb to α-Actin (clone AC40) were obtained from Sigma-Aldrich. Affinity purified anti-human mAb CD19-PE ($IgG_{1\kappa}$), anti-human mAb CD38- PE ($IgG_{1\kappa}$), and mouse immunoglobulin isotype controls were purchased from BD Biosciences (San Jose, CA). Affinity purified mAb anti-human CD56-PE (IgG_1) was purchased from Miltenyi Biotec, GmbH (Germany). Affinity purified, FITC-conjugated, $F(ab')_2$ fraction of rabbit antibody to mouse immunoglobulins was purchased from DAKO (Glostrup, Denmark). The affinity-purified rabbit polyclonal antibody anti-Fyn was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An affinity purified rabbit antibody to CD3-ε was purchased from Dako (Denmark). Polyclonal antibody anti-LAT was from UBI Upstate Biotechnology (Upstate, NY). Anti-CD3-ζ antiserum 448 was a gift from Dr. B. Alarcón (Centro de Biología Molecular, CSIC, Madrid, Spain). Affinity purified goat anti-rabbit IgG (Fc) HRP Conjugate, and goat anti-mouse IgG (H + L) HRP Conjugate were from Promega (Madison, WI). Prestained SDS-PAGE Standards (broad and precision range), and ImmunoStart reagents were from Bio-Rad (Hercules, CA).

2.4. FACS analysis

PBMC or purified T cells were analyzed for surface expression of CD3, CD4, CD8, CD38, CD19 and CD56 by single or double-staining using FITC-, or PE-labeled anti-CD3, anti-CD38, anti-CD19, and anti-CD56 mAbs in the relevant combinations. Compensation settings were adjusted using single stained PBMC, or T cell samples. Isotype-matching labeled antibodies were used to calculate the non-specific staining. PBMC and lymphocytes were gated according with their forward and scatter characteristics (Munoz et al., 2003; Zubiaur et al., 1997). Two color immunofluorescence analysis was performed on a FACScan flow cytometer (BD Biosciences, San Jose, CA), using the CellQuest Pro (BD Biosciences), and FlowJo (Tree Star, Inc. San Carlos, CA) software.

2.5. Detergent solubilization of cells at 37 °C

Cells ($1–5 \times 10^6$) were lysed in 1% Brij 98 at 37 °C as described previously (Munoz et al., 2003). Lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, and supernatant (SN) and pellet (P) were diluted in 3× non-reducing Laemmli sample buffer. Both fractions were resolved on a 12.5% SDS-PAGE, transferred to PVDF and immunoblotted with specific antibodies, followed by the ECL system, as previously described (Munoz et al., 2003; Zubiaur et al., 2002).

2.6. Fractionation of floating rafts by sucrose gradient ultracentrifugation

Detergent-insoluble and -soluble fractions were separated in a discontinuous sucrose gradient as described (Munoz et al., 2003; Zubiaur et al., 2002) with minor modifications. Two pools of the sucrose gradient fractions were then collected. First, the low-density fractions corresponding to the 5/30% interface (fractions 2 and 3, along fraction 4) and referred to as *Floating Rafts* (R). Second, the high-density soluble material corresponding to fractions 7 and 8 of the gradient and referred to as *Soluble* (S). The two pools were diluted with 3× non-reducing Laemmli sample buffer and resolved on 12.5% SDS-PAGE, transferred to PVDF and immunoblotted with specific antibodies, followed by the ECL system, as previously described (Zubiaur et al., 1999). Except where otherwise noted, 27 μl of each sample was loaded onto gels.

2.7. Densitometric and statistical analysis

Densitometric analysis was performed on a PC using the Quantity One 1-D Analysis Software Version 4.4 (Bio-Rad Laboratories, Inc., USA). To compare sample groups statistical analysis were performed using the Student's *t*-test, the Mann–Whitney U test, or the Fisher's exact test when appropriate. *P* values less than 0.05 were considered significant. The tests were performed using the GraphPad Prism software version 4.02 (GraphPad Software, Inc. San Diego, CA).

3. Results

3.1. A significant percentage of CD38 is insoluble in Brij 98 at 37 °C in PBMC from SLE patients

The non-ionic detergent Brij 98 has been successfully used to selective isolate, at physiological temperatures, detergent-insoluble microdomains with the biochemical characteristics of rafts (Drevot et al., 2002; Munoz et al., 2003; Schuck et al., 2003). Therefore, we first investigated in PBMC whether CD38 was associated with the membrane raft vesicles that are recovered as detergent-insoluble complexes upon cell lysis in Brij 98 detergent at 37 °C and centrifugation at $13,000 \times g$ for 15 min at 4 °C. After this treatment, cytosolic and fully solubilized membrane proteins were found in the supernatant (referred to as SN), whereas lipid rafts and associated proteins, as well as cytoskeletal components remained in the pellet (referred to as P) (Munoz et al., 2003). The presence of CD38 in the P and SN fractions was determined by Western blotting. Equal number of cell equivalents (0.5×10^6) were loaded in patients and control samples, and the proportion of CD38 present in the P fraction relative to the total amount of CD38, (SN + P) was calculated. CD38 was present in the P fraction from either the SLE patients and the healthy controls (Fig. 1A, lanes 2 and 4), although its proportion, relative to the total amount of CD38 was significantly higher in SLE than in controls ($P = 0.0090$, Fig. 1B). In contrast, similar proportions of Fyn, or actin relative to controls were detected when the same membranes were re-blotted with anti-Fyn, or anti-α-actin antibodies, respectively (Fig. 1A). Both SLE patients with high or low SLEDAI scores displayed a sta-

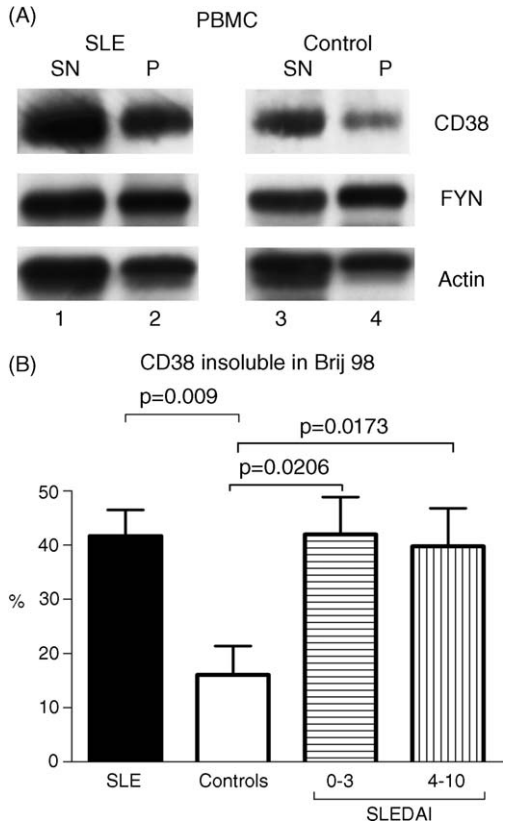

Fig. 1. A significant proportion of CD38 is insoluble in 1% Brij 98 in PBMC from SLE patients. (A) PBMC from SLE patients, or healthy controls were lysed in 1% Brij 98 at 37 °C as described in Section 2, and the detergent-insoluble fraction was separated from the soluble fraction by centrifugation at 13,000 × *g*. Supernatant (SN) and pellet (P) were diluted in 3× SDS non-reducing sample buffer and proteins were separated by 12% SDS-PAGE, transferred, and immunoblotted for the indicated proteins on the right of each panel. (B) bands intensities were quantified by densitometry, and the densitometric units on CD38 in pellet were presented as percentage of the sum of the densitometric units of supernatant + pellet. Values are the mean and S.E.M. The mean percentage of CD38 insoluble in Brij 98 was significantly increased in 29 SLE patients (black histograms) compared with nine healthy controls (open histograms). Likewise, SLE patients segregated according their SLEDAI scores as low (0–3) (horizontal stripped histograms, $n = 18$), or high (4–10) (vertical stripped histograms, $n = 11$), showed significantly increased percentages of CD38 insoluble in Brij 98 compared with controls (two-tailed unpaired *t*-test).

tistically significant higher proportion of CD38 in the Brij 98-insoluble fraction as compared with that in healthy controls ($P = 0.0173$ and 0.0206, respectively, Fig. 1B). These results strongly suggested that in SLE PBMC a large fraction of CD38 was associated with the membrane raft vesicles recovered as Brij 98-insoluble complexes.

3.2. Increased association of CD38 with floating rafts in PBMC from SLE patients

Insolubility of a membrane protein in a detergent as Brij 98 can be due to its association with detergent-resistant lipid rafts and/or its anchoring to cytoskeletal elements (Munoz et al., 2003). Proteins associated with detergent-insoluble lipid rafts float in a sucrose density gradient, while cytoskeleton-associated proteins remain at the bottom of the ultracentrifuge tube (Brown and Rose, 1992; London and Brown, 2000; Simons and Ikonen, 1997). Therefore, if CD38 is associated with Brij 98-insoluble lipid rafts, this CD38 should also float in a sucrose density gradient (Drevot et al., 2002; Munoz et al., 2003). To address this issue, rafts and non-raft fractions were isolated by sucrose gradient ultracentrifugation upon lysis of PBMC in Brij 98 at 37 °C (see Section 2, and (Munoz et al., 2003)). In floating rafts (referred to as R), five out of nine lupus patients studied showed percentages of CD38 above the upper 99% confidence interval (upper CI = 9.9%) of the mean values for six normal individuals (Fig. 2A, *left panel* 1 and 3). Application of the Fisher's exact test revealed a statistically significant difference (Fig. 2A, *right panel*, $13.7 \pm 4.6\%$ in SLE, $n = 9$ versus $3.6 \pm 1.6\%$ in controls, $n = 6$, $P = 0.0440$). In contrast, five out of nine patients studied showed percentages of CD3-ζ below the lower 95% confidence interval (lower CI = 4.2%) of the mean values for six normal healthy controls (Fig. 2A, *left panel*, lanes 1 and 3), although these differences were not statistically significant (Fig. 2B, *left panel*, $7.0 \pm 2.6\%$ in SLE, $n = 9$ versus $12.7 \pm 3.0\%$ in controls, $n = 6$, $P = 0.2867$, Fisher's exact test). Likewise, LAT that is constitutively associated with lipid rafts (Zhang et al., 1998), was always detected in floating rafts from both SLE patients and controls (Fig. 2A, *left panel*, lanes 1 and 3), and the minor differences observed between them were not statistically significant (Fig. 2B, *right panel*, $20.7 \pm 5.9\%$ in SLE, $n = 9$ versus $26.9 \pm 4.1\%$ in controls, $n = 6$). Therefore, these results demonstrate that SLE PBMC express an increased proportion of CD38 associated with floating lipid rafts, while similar proportions of CD3-ζ and LAT were present compared with that in PBMC from healthy controls.

3.3. Increased CD38 expression in SLE T cells and T cell subsets

Highly purified untouched T cells were obtained from PBMC by depletion of non-T cells by magnetic separation (see Section 2). T lymphocytes from eight patients with SLE and seven healthy controls were analyzed for expression of CD38 in $CD3^+$, $CD4^+$, $CD8^+$, and $CD25^+$ cells. The proportion of $CD38^+$ cells in the whole T cell population ($CD3^+$), as well as in the three T cell subsets studied ($CD4^+$, $CD8^+$, and $CD25^+$), was significantly increased in SLE patients as compared with that in healthy controls (Fig. 3). These increases seemed to be specific for T cells since neither the B or the NK cell compartments showed significant differences in CD38 expression between SLE patients and healthy controls when evaluated in PBMC by dual staining for CD38 and CD19 (B cells), or CD38 and CD56 (NK cells) (data not shown). No significant differences were observed in the proportion of CD3, CD19, or CD56 subsets within the PBMC population (data not shown). These results support the observation that the altered distribution and expression

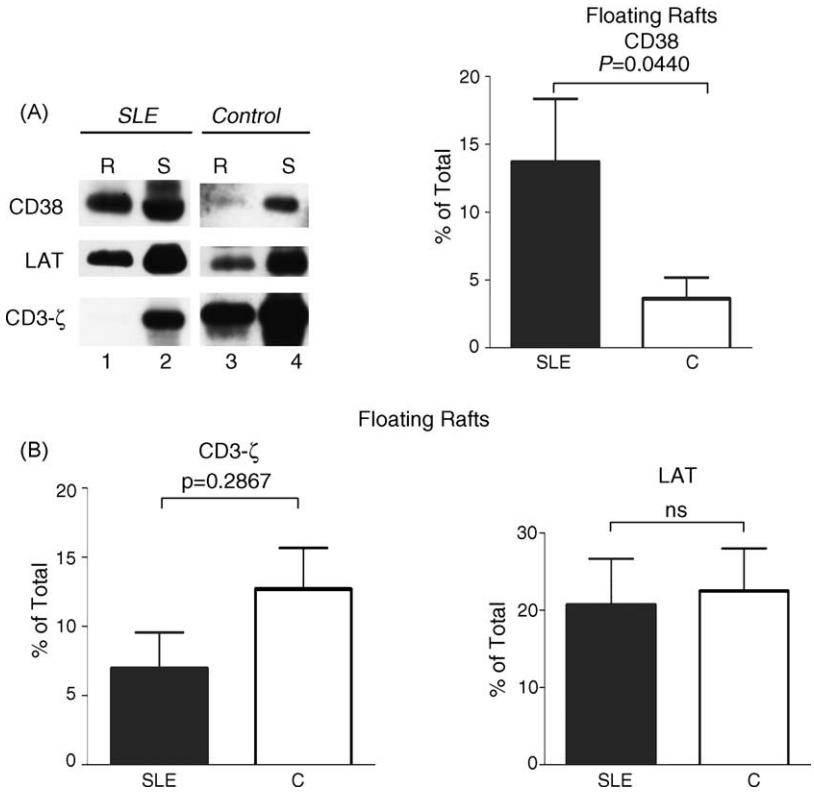

Fig. 2. Increased association of CD38 with floating rafts in PBMC from SLE patients. (A) *Left panel*, floating rafts (R) and soluble (S) fractions from one SLE (lanes 1 and 2) and one healthy control (lanes 3 and 4) PBMC were isolated as described in Section 2. Proteins were separated on SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-CD38, anti-LAT, or anti-CD3-ζ antibodies. *Right panel*, graph displaying cumulative results for CD38 expression in PBMC floating rafts from nine SLE patients (closed histograms), and five healthy controls (open histograms). Densitometric data on CD38 in floating rafts (R) are presented as percentage of the sum of all sucrose gradient fractions ($P = 0.0440$, Fisher's exact text). (B) The mean percentages for CD3-ζ ($P = 0.2867$, not significant, Fisher's exact text) and LAT expression (*left and right panels*, respectively) in PBMC floating rafts from SLE patients and healthy controls was calculated as in A.

of CD38 in PBMC from lupus patients are unique to SLE T cells.

3.4. CD38 is associated with floating rafts in ex vivo SLE T cells

3.4.1. Increased CD38 expression and insolubility in Brij 98 upon mitogenic stimulation

Next, we analyzed CD38 distribution in raft and non-raft compartments in untouched T cells in isolation. Floating rafts were isolated by sucrose gradient ultracentrifugation as described for PBMC. A representative experiment is shown in Fig. 4A (*upper panels*, lane 1 versus lane 3). CD38 was readily detected in the floating raft pool from SLE T cells in five out of eight SLE patients ($16.9 \pm 7.5\%$), whereas very little, if any, was detected in eight healthy control T cells ($0.0 \pm 0.0\%$). In contrast, the mean values for the percentages of CD3-ζ ($13.7 \pm 4.8\%$, in SLE versus $17.9 \pm 5.6\%$, in controls), and CD3-ε ($7.0 \pm 1.8\%$ in SLE versus $10.8 \pm 4.6\%$ in controls) in floating rafts were somewhat lower in SLE than in normal T cells, although the differences were not statistically significant (either by the Fisher's exact test, or unpaired *t*-test).

PHA acts as a potent and specific T cell activator by binding to cell membrane glycoproteins including the TCR/CD3 complex and the CD2 co-receptor, and exogenous IL-2 provides further T cell expansion (Kay, 1991). Moreover, stimulation of resting human T cells with PHA results in a significant increase in proportion of the plasma membrane that adopts a raft structure as judged by the dramatic increase on the cell surface expression of the raft-associated glycosphingolipid GM1 (Tuosto et al., 2001), which has previously been shown a reliable marker for detection of lipid raft domains (Janes et al., 1999). We next explored whether mitogenic stimulation could affect raft CD38 expression and distribution in SLE T cells compared with normal T cells. Thus, T cells were stimulated in vitro with PHA and IL-2 for 3 days (see Section 2) and then analyzed for CD38 expression by Western blotting of Brij 98-insoluble and -soluble fractions from whole cell extracts. Equal number of cell equivalents (0.5×10^6) were loaded in patients and control samples, and the proportion of CD38 present in the P fraction relative to the total amount of CD38, (SN + P) was calculated. As shown in Fig. 4B, there was a dramatic increase in the total amount of CD38 (SN + P) in PHA-stimulated T cells from both SLE patients (lanes 3 and 4) and healthy controls (lanes 7 and 8)

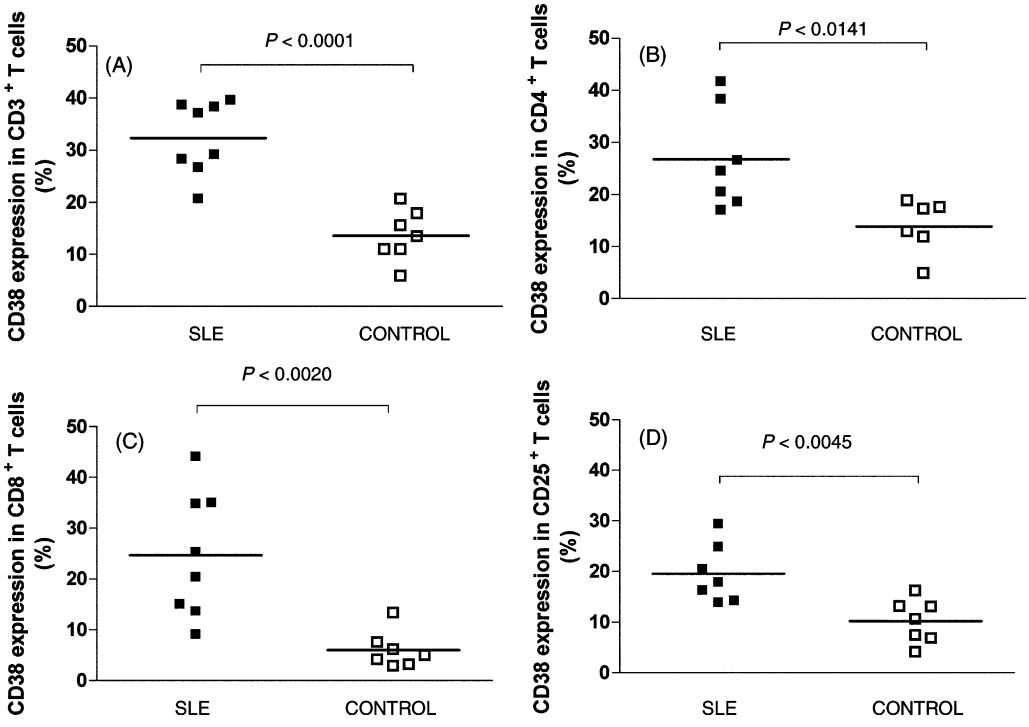

Fig. 3. Increased CD38 expression in SLE T cells and SLE T cell subsets. (A) Percentage of purified $CD3^+$ T cells expressing CD38. (B) Percentage of $CD4^+$ T cells expressing CD38. (C) Percentage of $CD8^+$ T cells expressing CD38. (D) Percentage of $CD25^+$ T cells expressing CD38. Scattered plots of percent of double positive cells are shown for SLE patients (filled squares), and healthy controls (open squares). Each symbol represents one patient or control, and the bar within each group represents the mean percentage value. The *P* values are indicated in each panel (two-tailed unpaired Student's *t*-test).

as compared with that in ex vivo untouched T cells (lanes 1, 2, 5, and 6). Moreover, in PHA-stimulated SLE and control T cells there was a significant shift of CD38 into the Brij 98-insoluble fraction (lanes 3 and 7) as compared with that in ex vivo cells (lanes 1 and 5). In contrast, the amount of Fyn, or actin and its distribution between soluble and insoluble fractions remained constant upon PHA stimulation, which was indicative of equal protein loading (Fig. 4B). Therefore, PHA stimulation induced quantitative and qualitative changes in the amount and distribution of CD38 between raft and non-raft fractions.

3.5. Increased lipid raft expression and altered CD4:CD8 ratio in ex vivo SLE T cells

3.5.1. Recapitulation in normal T cells upon mitogenic stimulation

Next, to visualize rafts on the cell surface, ex vivo and mitogen-stimulated T cells were analyzed for GM1 surface expression with fluorescent-labeled CTB and flow cytometry. CTB binds the raft-associated glycosphingolipid GM1, previously shown to be a reliable marker for detection of lipid raft domains (Janes et al., 1999). The analysis (Fig. 5A) revealed an increased proportion of ex vivo SLE T cells that bound CTB compared with that in normal T cells ($2.5 \pm 0.4\%$, $n = 18$, in SLE versus $0.9 \pm 0.1\%$, $n = 10$, in controls, $P = 0.0095$). PHA stimulation resulted in dramatic increases in the percentages of $GM1^+$ cells in both SLE and normal T cells (Fig. 5A). However, although the percentages of $GM1^+$ SLE T cells were increased as compared with that in normal T cells, these differences were not statistically significant ($18.5 \pm 3.7\%$, $n = 6$, in SLE versus 13.0 ± 2.4, $n = 8$, in controls, $P = 0.2180$). Interestingly in PHA-stimulated T cells about 35–40% of the $CD38^+$ cells were also $GM1^+$ (Fig. 5B), which suggested the selective expansion of a subset that expressed both markers. Moreover, these data were indicative that in both SLE and normal T cells an increased proportion of the plasma membrane adopts a lipid raft structure upon mitogenic stimulation.

Ex vivo and PHA-stimulated T cells were analyzed for CD4:CD8 ratio by flow cytometry. Patients with SLE had a significantly reduced CD4:CD8 ratio compared with that of healthy controls (Fig. 5C, $P = 0.0022$), as described previously (Jury et al., 2004). This was due to a significant reduction in the number (%) of $CD4^+$ T cells ($P < 0.0001$, Fig. 5D), and to a significant augmentation in the proportion of $CD8^+$ T cells ($P = 0.0447$, Fig. 5E). Interestingly, in healthy controls PHA-stimulated T cells had a significantly reduced CD4:CD8 ratio compared with that of ex vivo T cells (Fig. 5C, $P = 0.0052$), which was caused by a significant reduction in the percentage of $CD4^+$ T cells (Fig. 5D, $P = 0.0145$), and an increase in the percentage of $CD8^+$ T cells (Fig. 5E, $P = 0.0156$). This altered phenotype paralleled the one obtained in untouched *ex vivo* SLE T cells. In contrast, in SLE patients, PHA-stimulation did not induce significant changes in the CD4:CD8 ratio, neither in the number

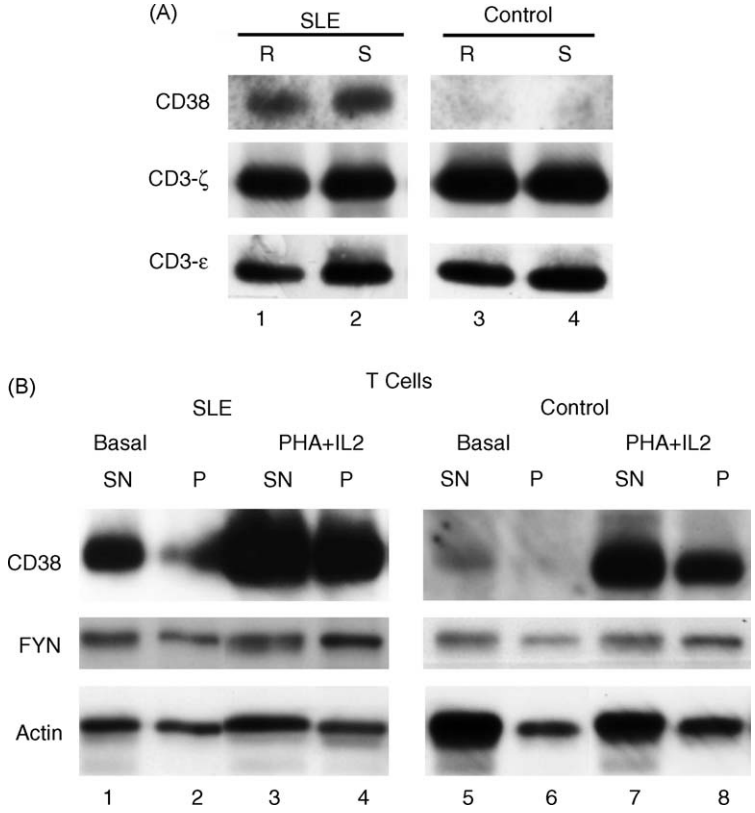

Fig. 4. (A) CD38 is associated with floating rafts in ex vivo SLE T cells. Floating rafts (R), and Soluble fractions (S) were prepared from untouched T cells purified by immunomagnetic depletion of non-T cells (see Section 2) from one SLE patients (lanes 1 and 2) and one healthy control (lanes 3 and 4) without any ex vivo stimulation. Proteins were separated on SDS-PAGE and analyzed by Western blotting with anti-CD38, anti-CD3-ζ, and anti-CD3-ε antibodies. This is a representative experiment out of eight experiments done in eight different SLE patients and eight healthy controls. Densitometric data on CD38, CD3-ζ, and CD3-ε in floating rafts (R) were calculated as in Fig. 2A (see Section 3 for specific numbers). (B) Increased CD38 expression and translocation into the Brij 98-insoluble fraction in T cells upon PHA stimulation. SN and P fractions were prepared as in Fig. 1A from: ex vivo untouched SLE T cells (lanes 1 and 2), ex vivo normal healthy control T cells (lanes 5 and 6), PHA + IL-2-stimulated SLE T cells (lanes 3 and 4), or PHA + IL-2-stimulated normal T cells (lanes 7 and 8). Proteins were separated by 12% SDS-PAGE, transferred, and analyzed by Western blotting with anti-CD38, anti-Fyn, and anti-α-actin antibodies. This is a representative experiment out of eight experiments done with cells from eight different SLE patients and eight healthy controls.

of $CD4^+$, nor $CD8^+$ T cells compared with that of ex vivo SLE T cells (Fig. 5C–E, respectively).

Taken together, these results on Figs. 4 and 5 indicate that ex vivo SLE T cells show an increased raft expression and altered phenotype, which can be recapitulated by PHA stimulation of normal T cells. PHA stimulation also induces increased CD38 expression, and active recruitment of CD38 to lipid rafts. This may be associated with an augmentation in the proportion of the plasma membrane adopting a lipid raft structure, as judged by the increased expression of GM1 on the cell surface, and the distinct expansion of the $CD38^+GM1^+$ T cells.

4. Discussion

In this study, we provide evidence of increased insolubility of CD38 in the non-ionic detergent Brij 98 at physiological temperatures in SLE PBMC as compared with normal PBMC, which correlated with an increased proportion of CD38 in floating rafts in both SLE PBMC and T cells. Consistent with previous reports (Alcocer-Varela et al., 1991; al-Janadi and Raziuddin, 1993), the percentage of $CD3^+$ T cells that express CD38 is significantly higher in SLE patients than in healthy controls. Moreover, we have found a significant increased percentage of $CD8^+CD38^+$, $CD4^+CD38^+$, and $CD25^+CD38^+$ T cells in patients with SLE in comparison to normal controls. $CD8^+CD38^+$ T cells are increased in advanced viral infections (Belles-Isles et al., 1998), and $CD8^+CD38^+$ lymphocyte percent is a useful immunological marker for monitoring HIV-1-infected patients (Mocroft et al., 1997). In this regard, PHA-stimulation of normal T cells results in reduced CD4:CD8 ratio and increased CD38 expression, which paralleled the altered phenotype of ex vivo SLE T cells. The contribution of the increased $CD8^+CD38^+$ T cells to the pathology of the SLE disease remains unclear (Erkeller-Yuksel et al., 1997), but together with increased $CD4^+CD38^+$ and $CD25^+CD38^+$ T cells could be indicative of persistent T cell activation.

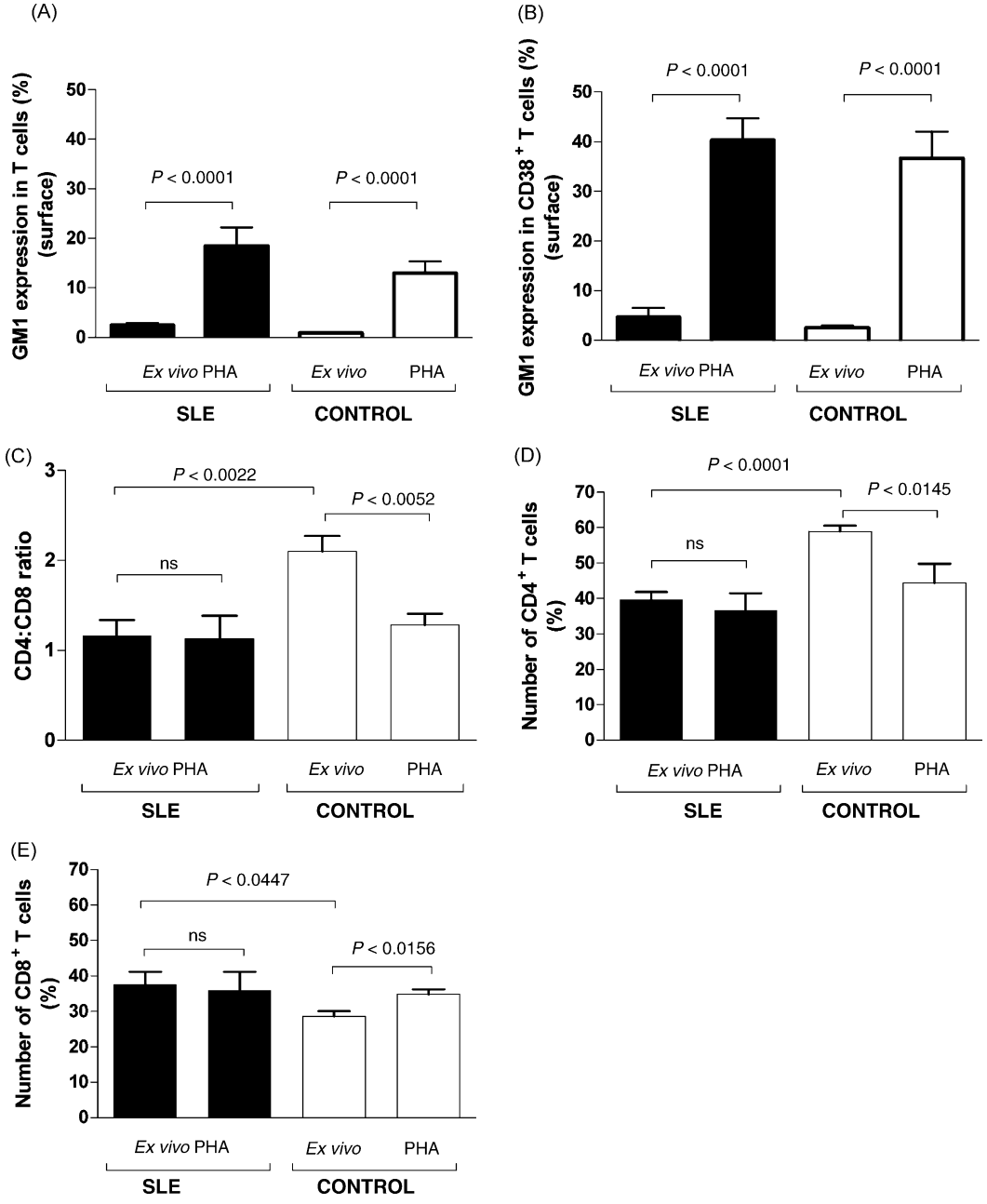

Fig. 5. Increased lipid raft expression and altered phenotype in ex vivo SLE T cells. Effect of PHA stimulation. Number (%) of $CD3^+$ cells (panel A), or $CD38^+$ cells (panel B) expressing GM1 on the cell surface in ex vivo or PHA-stimulated T cells from 18 SLE patients (black histograms) and 10 healthy controls (open histograms). The relative proportions of T cells expressing CD4 or CD8 (panel C) were expressed as a ratio (CD4:CD8) on ex vivo or PHA-stimulated T cells from eight SLE patients (black histograms) or from seven healthy controls (open histograms). The number (%) of $CD4^+$ (panel D), or $CD8^+$ (panel E) T cells. All results are expressed as mean + S.E.M. The *P* values are indicated in each panel (two-tailed unpaired Student's *t*-test).

CD38 function in T cells is mediated by cell-surface association with the TCR/CD3 complex (Morra et al., 1998; Zubiaur et al., 1997, 1999), and the localization of CD38 to lipid raft domains is essential for CD38-mediated signaling in CD38-transfected murine T cell lines (Zubiaur et al., 2002), or in Jurkat T cells, which constitutively express CD38 (Munoz et al., 2003). In these T cell lines, CD38 appears to localize to the rafts without the need for ligation (Munoz et al., 2003; Zubiaur et al., 2002). Therefore, it was to some extent surprising that in normal resting T cells CD38 was detected exclusively in soluble fractions. The mechanism by which CD38 in T cells is included or excluded from rafts is not known, but it may be related rather with the significant differences in the lipid raft composition and dynamics between resting and activated/effector T cells, and not with the level of expression of CD38. Thus, the organization of GM1-lipid rafts on the T cell membrane appears developmentally regulated (Hare et al., 2003). Interestingly, in rest-

ing human T cells from peripheral blood Lck and the raft glycosphingolipid GM1 reside in intracellular membranes, while in activated/effector T cells the amount of these markers at the plasma membrane increases significantly (Tuosto et al., 2001). These dramatic increases in GM1 cell surface expression can be recapitulated in vitro by a number of different stimuli including PHA (Tuosto et al., 2001), TCR/CD3 cross-linking with a soluble CD3 ligand (Thomas et al., 2003b), or with anti-CD3/CD28 beads (Slaughter et al., 2003; Tuosto et al., 2001). Likewise, murine and human T cell lines, which have an effector phenotype, express high levels of GM1 on the cell surface, and CD38 is readily detected in lipid rafts (Zubiaur et al., 2002; Munoz et al., 2003). Our present findings are significant in this regard, as the dramatic changes in the distribution of CD38 into Brij 98-insoluble and -soluble fractions upon mitogenic stimulation of normal T cells (Fig. 4B) correlated with increased GM1 expression on the cell surface (Fig. 5A), and a distinct expansion of $GM1^{+}CD38^{+}$ T cells (Fig. 5B). Likewise, in untouched SLE T cells a significant proportion of CD38 is associated with lipid rafts, which again correlates with increased basal expression of GM1 on the cell surface (Fig. 5A). Note that we have just measured the GM1 pool that is on the cell surface, which is only a fraction of the total GM1 pool (surface + intracellular). Increased basal expression of total GM1 has been reported previously in SLE T cells compared with normal T cells (Jury et al., 2004; Krishnan et al., 2004). Therefore, SLE T cells possess more extensive basal levels of lipid rafts than normal T cells, which may be indicative of their "activated" phenotype that could result from the exposure of SLE T cells to the abnormal frequency of differentiated dendritic cells found in these patients (Blanco et al., 2001).

Increased lipid raft expression on the plasma membrane may constitute a means by which effector T cells acquire an improved signaling machinery (Tuosto et al., 2001). In contrast, displacement of the CD4:Lck signalosome from the lipid rafts by a soluble, dimeric peptide-MHC Class II chimera induces Ag-specific T cell anergy (Thomas et al., 2003a), suggesting that the defective partitioning of signaling molecules in lipid rafts is an early, negative signaling event in T cells. The study of raft protein composition in the Jurkat T cell line have revealed that CD38, Lck and CD3-ζ reside in a subset of lipid rafts separately from LAT and other key signaling molecules (Munoz et al., 2003), which is compatible with other studies showing that in resting T cells Lck and LAT are located in different raft subsets (Drevot et al., 2002; Schade and Levine, 2002; Slaughter et al., 2003). After cellular activation, these rafts coalesce, leading to Lck and LAT colocalization in the same raft population (Drevot et al., 2002; Schade and Levine, 2002; Slaughter et al., 2003). CD38 residency in a subset of lipid rafts together with Lck and CD3-ζ provides a structural basis for initiating CD38-mediated signaling in this compartment, where fully phosphorylation of CD3-ζ, CD3-ε, Lck and LAT occurs, as well as the translocation of key signaling molecules as Sos and p85-phosphatidylinositol 3-kinase into rafts (Munoz et al., 2003).

The low number of cells used in this study precluded the analysis of the protein composition of CD38-containing rafts in either resting or activated SLE T cells but it is likely that the anomalous expression or changes in membrane location of signaling molecules as CD3-ζ, or Lck found in these patients (Jury et al., 2003; Krishnan et al., 2004) may lead to anomalous lipid raft-mediated signaling. It has been shown that ligand-mediated cross-linking of GM1 moieties in lipid rafts facilitates an exchange of proteins including the redistribution of the TCR and adhesion molecules in T cells (Mitchell et al., 2002). We speculate that the increased expression of CD38 in floating rafts from SLE T cells, or from activated T cells may modulate TCR signaling by providing or sequestering signaling molecules to the engaged TCR. This aspect will require further study and may help in searching for new molecular mechanisms of positive or negative regulation in T cells.

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