Quantitative Abnormalities of Peripheral Blood Distinct T, B, and Natural Killer Cell Subsets and Clinical Findings in Obstetric Antiphospholipid Syndrome

JAVIER CARBONE, ANTONIO GALLEGO, NALLIBE LANIO, JOAQUIN NAVARRO, MARIA ORERA, ANGEL AGUARON, EDUARDO FERNANDEZ-CRUZ, and ELIZABETH SARMIENTO

ABSTRACT. *Objetive.* Few studies have assessed immunophenotypic abnormalities on lymphocyte subsets in patients with antiphospholipid syndrome (APS). We performed an extended immunological study to define alterations of distinct T, B, and natural killer (NK) cell subsets in obstetric patients with APS and their relationship with APS–associated complications.

Methods. Patients and controls: 36 women with APS [Sydney criteria, Group A1 without thrombosis (n = 26), Group A2 with thrombosis (n = 10)]; and 36 age matched women with recurrent abortion without antiphospholipid antibodies (disease controls; Group B), 36 healthy parous women (healthy controls; Group C), and 36 healthy nonparous women (healthy controls; Group D). Thrombotic events occurred after history of abortions in all A2 women. Three-color whole-blood flow cytometry was used to characterize the distinct immunophenotypes.

Results. A1 patients had significantly higher percentages of CD4+CD45RA–CCR7+ central memory cells (A1 vs D), higher percentages of activated CD4+CD25+ T cells (A1 vs D), and lower percentages and absolute counts of CD4+CD45RA–CCR7– effector memory cells (A1 vs D). Group A2 patients had higher percentages and absolute numbers of CD19+CD27–IgD+ naive B cells (A2 vs A1 vs all controls), lower percentages and absolute numbers of CD3–CD56+CD16+ NK cells (A2 vs all controls), and higher percentages of activated CD4+DR+ (A2 vs all controls), CD8+DR+ (A2 vs A1 vs C vs D), CD4+CD38+DR+ (A2 vs D), and CD4+CD25+DR+ T cells (A2 vs all controls). Increased percentages of CD8+DR+ T cells [relative risk (RR) 2.43, 95% CI 1.09–5.44, p = 0.02] and of naive B cells (RR 3.05, 95% CI 1.30–7.11, p = 0.009) were associated with development of thrombosis.

Conclusion. In obstetric patients with APS we documented significant changes in T, B, and NK cell homeostasis. Increased levels of CD8+DR+ and CD19+CD27–IgD+ cells might identify obstetric patients with APS at risk of having thrombosis. (First Release April 1 2009; J Rheumatol 2009;36:1217–25; doi:10.3899/jrheum.081079)

Key Indexing Terms:
ANTIPHOSPHOLIPID SYNDROME   IMMUNE SYSTEM   B LYMPHOCYTES   OBSTETRICS

Antiphospholipid antibodies (aPL) are a risk factor for recurrent abortion and obstetrical complications[1]. Twenty-five years after the description of the disease, the mechanisms of aPL-mediated pregnancy failure are incompletely understood and some issues of management of pregnant women remain unsolved[2]. It has been suggested that antiphospholipid syndrome (APS) has some pathogenic features of an inflammatory disease, rather than being only a prothrombotic state[3]. aPL are associated with thrombotic events but these cannot explain all the abortion events. Recent experimental and clinical evidence of an associated inflammatory condition in APS include adhesion molecule expression, complement activation, or proinflammatory cytokine/chemokine secretion[4-6]. Autoreactive CD4+ T cells to β_2-glycoprotein I (β_2-GPI) that promote production of pathogenic aPL have also been identified[7-9].

Few studies have assessed the presence of immunophenotypic abnormalities on peripheral blood lymphocyte subsets in APS[10-14]. In a B cell-induced autoimmune disease we still lack basic information such as the distribution of naive

and memory B cells. No single previous study has simultaneously analyzed T, B, and natural killer (NK) cell subsets using newer definitions of T and B cell subpopulations, which might provide a more complete picture of the peripheral blood lymphocyte dynamic in these patients.

Thus, our aim was to perform an extended cellular immunological analysis to better define alterations of functionally distinct lymphocyte subsets in patients with obstetric APS and its relationship with APS–associated clinical complications.

MATERIALS AND METHODS

In a transversal case-control study we investigated 144 women in the Immunology Department at the University Hospital Gregorio Marañon. Patients and controls consisted of 36 women with obstetric APS (Group A), 36 age matched women with recurrent abortion without aPL (disease controls, Group B), 36 healthy parous women (parous controls, Group C), and 36 healthy nonparous women (nonparous controls, Group D). The patients, with a history of spontaneous recurrent abortion, were admitted to the hospital at the outpatient clinic for other departments (genetics, infertility, or obstetrics). All patients with recurrent abortion were referred to the Clinical Immunology outpatient clinic for an immunological study. Control women were drawn from a normal population after completion of a questionnaire.

APS group: Clinical and laboratory manifestations of APS were considered according to a recent consensus document[1]. Briefly, women with either a history of one or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, or with 3 or more unexplained consecutive spontaneous abortions before the 10th week of gestation, and 2 or more positive tests (performed at least 12 wks apart) for lupus anticoagulant (performed by 3 different phospholipid-dependent coagulation tests) and/or anticardiolipin antibodies (IgG and IgM measured by ELISA) and/or anti-ß$_2$-GPI antibodies (IgG and IgM measured by ELISA) were considered to have APS. Patients were not included if less than 12 weeks or more than 5 years separated the positive aPL test and the clinical manifestation. Among patients with APS, there were no women with premature births before the 34th week of gestation because of eclampsia, severe preeclampsia, or placental insufficiency. In this article, patients with APS were stratified into 2 groups according to the absence (Group A1) or presence (Group A2) of ischemic or thrombotic events during clinical followup.

Disease controls: Recurrent abortion was defined by the loss of 2 or more consecutive pregnancies of nonviable fetuses. Negative tests for aPL [lupus anticoagulant, anticardiolipin antibodies (IgG and IgM), and anti-ß$_2$-GPI antibodies (IgG and IgM)] were documented on at least 2 occasions in these patients.

In order to exclude other causes of recurrent abortion, examinations for maternal and paternal chromosomes, uterine anomalies, endocrine pathologies, and other thrombophilias had been previously performed. To exclude possible causes of alterations in the functionally distinct lymphocyte subsets, histories of APS patients and disease controls were carefully reviewed by the same immunologist for any clinical criteria of concomitant systemic autoimmune diseases, chronic infections (hepatitis B, hepatitis C, HIV), severe acute infection requiring prescription of therapy, immunization, immunosuppressive or immunomodulatory therapies, active substance abuse, or ongoing treatment for malignancy within 6 months prior to the study. Patients with any of the above underlying clinical conditions were excluded from the study. The diagnosis of autoimmune disease was considered when the patients met complete specific criteria for systemic lupus erythematosus (SLE), rheumatoid arthritis, polyarteritis nodosa (American College of Rheumatology), Behçet disease (International Study Group for the Diagnosis of Behçet Disease), or Sjögren's syndrome (American-European Consensus Group)[15-19].

Parous controls: As pregnancy may have an effect on some lymphocyte subsets, we included women who had live-born babies but no abortions as parous controls[20-22].

To establish the relationship between lymphocyte subset abnormalities and clinical findings in women with APS, any history of APS-related complications was recorded at the time of the study. Doppler ultrasound, pulmonary ventilation-perfusion (VQ) scanning, magnetic resonance imaging (MRI), and angio-MRI had been employed to document vascular events as required. Patients with obstetric APS without thromboses were receiving antiplatelet agents, while patients with arterial or venous thromboses were receiving anticoagulants.

To avoid the acute-phase effect on the immune profile, immunological studies of patients were performed at least 6 months after the last abortion, successful pregnancy, or APS-related clinical complications that could influence the results. Immunological studies of parous female controls were performed at least 1 year after the last successful pregnancy.

Other immunological measures that were collected at the time of the study included antinuclear antibodies (ANA; measured by indirect immunofluorescence), anti-DNA antibodies (anti-DNA; measured by ELISA), and complement C3, C4 and factor B (FB) (measured by nephelometry). We defined high-titer ANA when the titer was ≥ 1/160. Low levels of complement factors C3, C4, and FB were defined according to reference values of the immunochemistry laboratory: < 91, < 18, and < 19 mg/dl, respectively.

The study protocol was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by our hospital research ethics committee. All subjects were included in the study after informed consent had been obtained.

Lymphocyte phenotype analysis. Venous blood for immunophenotyping was collected in EDTA. Three-color immunophenotyping of peripheral blood lymphocytes was performed by flow cytometry within 2 h of blood collection. The monoclonal antibodies used were directly conjugated with the fluorochrome fluorescein isothiocyanate (FITC; FL1), phycoerythrin (PE; FL2), or peridinin chlorophyll protein (PerCP; FL3). We enumerated T cell subsets using FITC/PE/PerCP combinations of CD27/IgD/CD19; CD40/CD5/CD19; CD45RA/CCR7/CD4; HLADR/CD38/CD4; HLA-DR/CD25/CD4; CD28/HLADR/CD8; CD56/CD-16/CD3; and isotypic controls. Lymphocyte staining was carried out using a whole-blood lysis technique, according to the recommended methodology and quality control procedures. Stained samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) with Cellquest Pro software (Becton Dickinson). Data were collected for 5000 events in a lymphocyte gate defined by both forward and side scatter characteristics. Background staining was assessed with the appropriate isotype-and fluorochrome-matched control monoclonal antibodies directed against an irrelevant target and was subtracted from all the results. Total CD3, CD4, CD8, CD19, and CD3-CD16+CD56+ (NK) subsets are expressed as a percentage of total lymphocytes. Percentages of the functionally distinct naive and memory, activated, and regulatory T cells and of naive and memory B cell subpopulations are expressed as a percentage of total CD4, CD8, or CD19 lymphocytes, respectively. To determine the percentage of CD4 or CD8 T cells coexpressing one or both of the additional markers under analysis, gates were set that included only PerCP-positive cells with the same intensity of fluorescence as the CD4 and CD8 cells. We used a combination of anti-CD27 and anti-IgD monoclonal antibodies for quantitative analysis of naive and memory B lymphocytes. Percentages of lymphocyte subsets (from flow cytometry) and total lymphocyte count (determined by the Hematology Department) were used to obtain absolute counts of distinct lymphocyte subsets.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). The normality of the distribution of variables in each test group was tested by the Kolmogorov-Smirnov goodness-of-fit test. Comparisons between 2 sample populations were made with Student's t-test; p values less than 0.05 between 2 groups were considered significant. Overall differences between

groups were assessed by ANOVA. Those significantly different were identified using the post-hoc least significant difference test. Pearson's and Spearman correlation coefficients calculated the association between variables. Logistic regression analysis was used to assess the association between subsets and the prevalence of distinct clinical complications.

RESULTS

Comparison between APS groups and controls. Patient and control characteristics are shown in Table 1. Mean time of clinical followup from diagnosis of APS to the performance of the immunological study was $6.9 \pm$ SD 4.9 years, range 1–17 years. Immunological characteristics of patients with APS and disease controls are presented in Table 2. None of the APS women with positive ANA or positive anti-DNA fulfilled SLE classification criteria.

The percentages and absolute numbers of total peripheral blood lymphocytic subpopulations of APS groups and controls are shown in Table 3. Total CD3+, CD4+, and CD8+ lymphocyte subsets were not different between both APS groups and controls.

Percentages of the functionally distinct T and B cell subsets are shown in Figures 1–3. Group A1 women had significantly higher mean percentages of CD4+CD-45RA–CCR7+ central memory cells, lower percentages of CD4+CD45-RA–CCR7– effector memory cells, and higher percentages of activated CD4+CD25+ lymphocytes (49% ± 10% vs 44% ± 8%; p = 0.046) than those observed in Group D (Figure 1). The mean percentage of NK cells in these APS women was lower in comparison with Group B (Table 3). Lower levels of CD4+CD45RA–CCR7– effector memory

cells (111 ± 72 vs 160 ± 67 cells/μl; p = 0.007) and of CD3–CD56+CD16+ NK cells (Table 3) as compared with Group D were confirmed when employing absolute numbers.

In Group A2, immunophenotypic abnormalities were more evident affecting distinct lymphocyte compartments. Within the CD19+ B lymphocytic population, there was a significant increase in the percentage of naive (CD19+CD-27–IgD+) cells compared to Group A1 patients and compared to all control groups. Lower percentages of memory class-switched (CD19+CD27+IgD–) as well as memory non-class-switched (CD19+CD27+IgD+) cells were also observed in APS women who had thrombosis as compared to Group A1 patients and compared to all control groups (Figure 2). A higher naive/memory class-switched ratio was observed in these patients compared with all control groups (data not shown). Higher CD19+ naive B cell absolute counts were confirmed in Group A2 women (158 ± 99 cells/μl) as compared with Group A1 patients (88 ± 63 cells/μl; p = 0.004), Group B (81 ± 52 cells/μl; p = 0.001), Group C (89 ± 72 cells/μl; p = 0.003), and Group D (158 ± 99 cells/μl; p = 0.025).

Among T cell subsets, percentages of activated CD4+HLADR+ and CD4+CD25+HLA–DR+ lymphocytes were increased compared to all controls (Figure 3). Significantly higher absolute numbers of both subsets [80 ± 52 vs 56 ± 28 cells/μl (p = 0.048); and 43 ± 25 vs 32 ± 14 cells/μl (p = 0.047)] were observed in Group A2 patients as compared with nonparous and parous controls, respectively.

Table 1. Clinical characteristics of patients with APS and control groups.

| Characteristics | APS Patients | | Control Groups | | |
	A1, n = 26	A2, n = 10	B, n = 36	C, n = 36	D, n = 36
Age, yrs, mean (range)	38 (28–48)	39 (30–48)	37 (30–43)	37 (22–48)	36 (27–50)
Abortions, mean (range)	2.88 (1–8)	2.60 (1–9)	2.89 (2–7)	—	—
Abortions, < 10 wks, mean (range)	1.64 (0–8)	1.10 (0–5)	1.89 (0–4)	—	—
Abortions, > 10 wks, mean (range)	1.16 (0–7)	1.50 (0–4)	0.92 (0–3)	—	—
Live-born babies, mean (range)	0.23 (0–2)*	0.40 (0–4)*	0.44 (0–3)	1.64 (1–3)	—
Complications, n (%)					
Ischemic stroke	0 (0)	6 (60)	—	—	—
Deep venous thrombosis	0 (0)	3 (30)	—	—	—
Retinal thrombosis	0 (0)	1 (10)	—	—	—
Pulmonary embolism	0 (0)	1 (10)	—	—	—
Epilepsy	1 (3.8)	3 (30)**	—	—	—
Migraine	1 (3.8)	2 (20)	—	—	—
Livedo reticularis	1 (3.8)	3 (30)**	—	—	—
Abruptio placentae	1 (3.8)	0 (0)	—	—	—
Preeclampsia	1 (3.8)	1 (10)	—	—	—

A1: obstetric APS without thrombosis; A2: obstetric APS with thrombosis; B: women with spontaneous recurrent abortions without antiphospholipid antibodies; C: healthy parous controls; D: health nonparous controls.
* Analysis of variance post hoc test, p < 0.001 vs parous C. ** Pearson chi-square, p < 0.05 vs A1.

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Higher percentages of activated CD8+DR+ lymphocytes were observed in Group A2 as compared to Group A1 women and parous and nonparous controls (Figure 3). Similar differences were observed when using absolute counts of CD8+DR+ T cells [177 ± 67 vs 119 ± 77 cells/μl ($p = 0.045$), vs 125 ± 63 cells/μl ($p = 0.047$) and vs 115 ± 89 cells/μl ($p = 0.028$), respectively].

The mean percentage of CD28–CD8+ regulatory lymphocytes was higher in these women with previous thrombosis in comparison with nonparous controls (Figure 3). Activated (HLA-DR+) CD28–CD8+ cell percentages were significantly higher if compared with Group A1 patients and with all control groups (Figure 3). Higher absolute counts of this subset were also detected in APS women with thrombosis (26 ± 9 cells/μl) as compared with Group A1 patients (16 ± 9 cells/μl; $p = 0.027$), Group B (17 ± 9 cells/μl; $p = 0.029$), Group C (17 ± 13 cells/μl; $p = 0.023$), and Group D (14 ± 12 cells/μl; $p = 0.003$).

The percentages and absolute numbers of CD3–CD-56+CD16+ NK cells in the total lymphocyte population were significantly decreased in these patients compared with all control groups (Table 3).

Mean percentages (Figure 3) and absolute counts (data not shown) of regulatory CD25-high CD4+ lymphocytes in both APS subgroups of patients were not different from controls.

Correlation among immunological variables. In correlation analysis including all patients with APS, the percentage of naive B cells directly correlated with the level of IgG anticardiolipin and with IgG anti-ß₂-GPI antibodies [correlation

Table 2. Immunological characteristics of patients with APS and disease controls.

Characteristics	APS Patients A1, n = 26	A2, n = 10	Disease Controls B, n = 36
More than one aPL, n (%)	21 (81)	7 (70)	0 (0)
Anticardiolipin alone, n (%)	1 (4)	0 (0)	0 (0)
Anti-ß₂-glycoprotein I alone, n (%)	4 (15)	2 (20)	0 (0)
Lupus anticoagulant alone, n (%)	0 (0)	1 (10)	0 (0)
C3, mg/dl, mean (SD)	99 (20)	98 (25)	105 (27)
C4, mg/dl, mean (SD)	19 (7)*	17 (5)*	24 (7)
Factor B, mg/dl, mean (SD)	30 (7)	35 (8)**	31 (6)
Positive ANA, n (%)	3 (11.5)	1 (10)	4 (11)
Positive anti-DNA, n (%)	2 (7.7)	2 (20)	3 (8.3)

A1: obstetric APS without thrombosis; A2: obstetric APS with thrombosis; B: women with spontaneous recurrent abortions without antiphospholipid antibodies. Antiphospholipid antibodies include anticardiolipin (IgG or IgM), anti-ß₂-glycoprotein I (IgG or IgM), or lupus anticoagulant. Positive ANA: antinuclear antibody titer > 1/160; positive anti-DNA: ELISA anti-DNA antibodies > 15 U/ml. * Analysis of variance post hoc test, $p < 0.05$ vs B. ** $p < 0.05$ vs A1.

Table 3. T, B, and NK total lymphocyte subsets of patients with APS and control groups, Immunophenotypic definition of NK cells: CD3–CD56+CD16+.

Lymphocyte Subsets	APS Patients A1, n = 26	A2, n = 10	Control Groups B, n = 36	C, n = 36	D, n = 36
	Mean ± SD (95% CI for mean)				
CD3+ T cells, %	72 ± 6 (69–74)	73 ± 8 (68–79)	71 ± 6 (69–73)	72 ± 8 (69–75)	71 ± 8 (68–73)
CD3+ T cells, cells/μl	1340 ± 458 (1147–1534)	1439 ± 634 (986–1893)	1428 ± 334 (1314–1542)	1344 ± 220 (1267–1418)	1433 ± 193 (1368–1499)
CD4+ T cells, %	43 ± 7 (40–46)	44 ± 6 (40–46)	43 ± 6 (41–46)	43 ± 8 (41–46)	43 ± 6 (41–45)
CD4+ T cells, cells/μl	796 ± 274 (681–912)	876 ± 455 (527–1225)	862 ± 242 (778–944)	798 ± 175 (738–857)	873 ± 151 (822–924)
CD8+ T cells, %	22 ± 8 (19–25)	26 ± 8 (20–31)	23 ± 6 (21–25)	24 ± 8 (21–26)	23 ± 7 (20–25)
CD8+ T cells, cells/μl	426 ± 213 (338–514)	491 ± 212 (340–642)	463 ± 155 (410–515)	433 ± 155 (381–485)	457 ± 149 (406–507)
CD19+ B cells, %	9 ± 4 (7–11)	11 ± 6* (7–15)	8 ± 3 (7–8)	8 ± 4 (7–10)	9 ± 3 (8–10)
CD19+ B cells, cells/μl	165 ± 97 (123–207)	209 ± 113 (128–290)	155 ± 76 (129–181)	159 ± 95 (127–191)	181 ± 65 (159–203)
NK cells, %	11 ± 5* (9–13)	8 ± 6** (4–13)	14 ± 5 (12–16)	13 ± 6 (11–15)	13 ± 6 (11–15)
NK cells, cells/μl	201 ± 100† (159–243)	154 ± 118** (70–238)	278 ± 125 (235–321)	245 ± 116 (205–285)	273 ± 136 (227–320)

A1: obstetric APS without thrombosis; A2: obstetric APS with thrombosis; B: women with spontaneous recurrent abortions without antiphospholipid antibodies; C: healthy parous controls; D: health nonparous controls. * Analysis of variance post hoc test, $p < 0.05$ vs B. ** $p < 0.05$ vs all control groups. † $p < 0.05$ vs B and D.
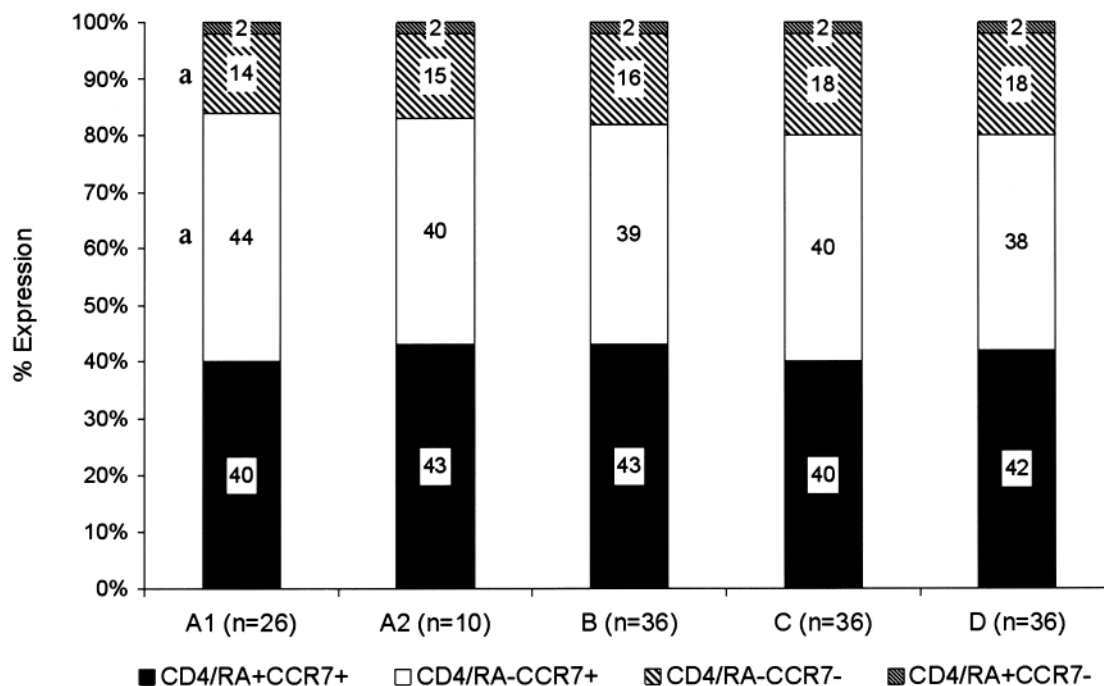
Figure 1. Naive and memory CD4 subsets of patients with antiphospholipid syndrome (APS) and control groups. Subsets are expressed as mean percentage of total CD4 T cells. Phenotypic definitions: CD45RA+CCR7+ are naive, CD45RA–CCR7+ central memory, CD45RA–CCR7– memory effector, CD45RA+CCR7– final effector CD4 T cells. A1: obstetric APS without thrombosis; A2: obstetric APS with thrombosis; B: women with spontaneous recurrent abortions without antiphospholipid antibodies (aPL); C: healthy parous controls; D: healthy nonparous controls. a: ANOVA post hoc test, $p < 0.05$ vs D.

coefficient 0.36 (p = 0.03) and 0.49 (p = 0.003), respectively]. Absolute numbers of naive B cells correlated with the level of IgG anti-β_2-GPI antibodies [correlation coefficient 0.40 (p = 0.02)]. The percentage of activated CD4+CD-38+DR+ lymphocytes positively correlated with the titer of IgG anticardiolipin antibodies [correlation coefficient 0.36 (p = 0.04)].

Among Group A1 patients there was an inverse correlation between central memory CD4 cell percentages and serum C4 levels [correlation coefficient –0.397 (p = 0.04)].

Within Group A2 women, there was a strong inverse correlation of naive B cell and of activated CD4+CD25+ cell percentages with the serum concentrations of C4 and C3 [correlation coefficients –0.895 (p = 0.001) and –0.857 (p = 0.007), respectively].

Relationship of lymphocyte subset abnormalities with development of thrombosis. APS-related clinical complications in women with APS are shown in Table 1. A total of 13 patients with APS (36.1%) had at least 1 episode of 1 or more of these complications. Ten women with APS (16.7%) had a history of thrombotic or ischemic events. Obstetric manifestations of APS occurred before thrombotic complications in all women. Mean time from the last abortion to thrombotic complication was 3 ± 2.6 years (range 1–8 yrs) in these women. Mean time from thrombosis to the performance of

the immunological study was 3.9 ± 3 years (range 1–8 yrs). The prevalence of risk factors for thrombosis (overweight, use of oral contraceptives, arterial hypertension, diabetes, dyslipidemia, or smoking) was the same in Groups A1 and A2 (data not shown).

In logistic regression analysis, increased percentages of CD8+DR+ T cells (per each increase of 10%, relative risk (RR) 2.43, 95% CI 1.09–5.44, p = 0.02) and of naive B cells (RR 3.05, 95% CI 1.30–7.11, p = 0.009) were associated with development of thrombotic complications during followup. There was no correlation between both lymphocyte subsets (correlation coefficient 0.108, p = 0.537).

Among the distinct aPL, IgG anti-β_2-GPI antibodies were associated with the risk of development of thrombosis (per each increase in 10 unit/ml, RR 2.38, 95% CI 1.11–5.09, p = 0.02). Distinct clinical variables were not associated with development of thrombosis, including age (p = 0.21), time of followup after diagnosis of APS (p = 0.19), presence of humoral or autoimmune abnormalities (hypocomplementemia or positive antinuclear antibodies, respectively, p = 0.73), or risk factors for thrombosis (p = 0.82).

In multiple logistic regression analysis, increased levels of CD8+DR+ (p = 0.025) and of naive B cells (p = 0.015) were independent of IgG anti-β_2-GPI titer for identification of groups with a higher frequency of thrombosis.
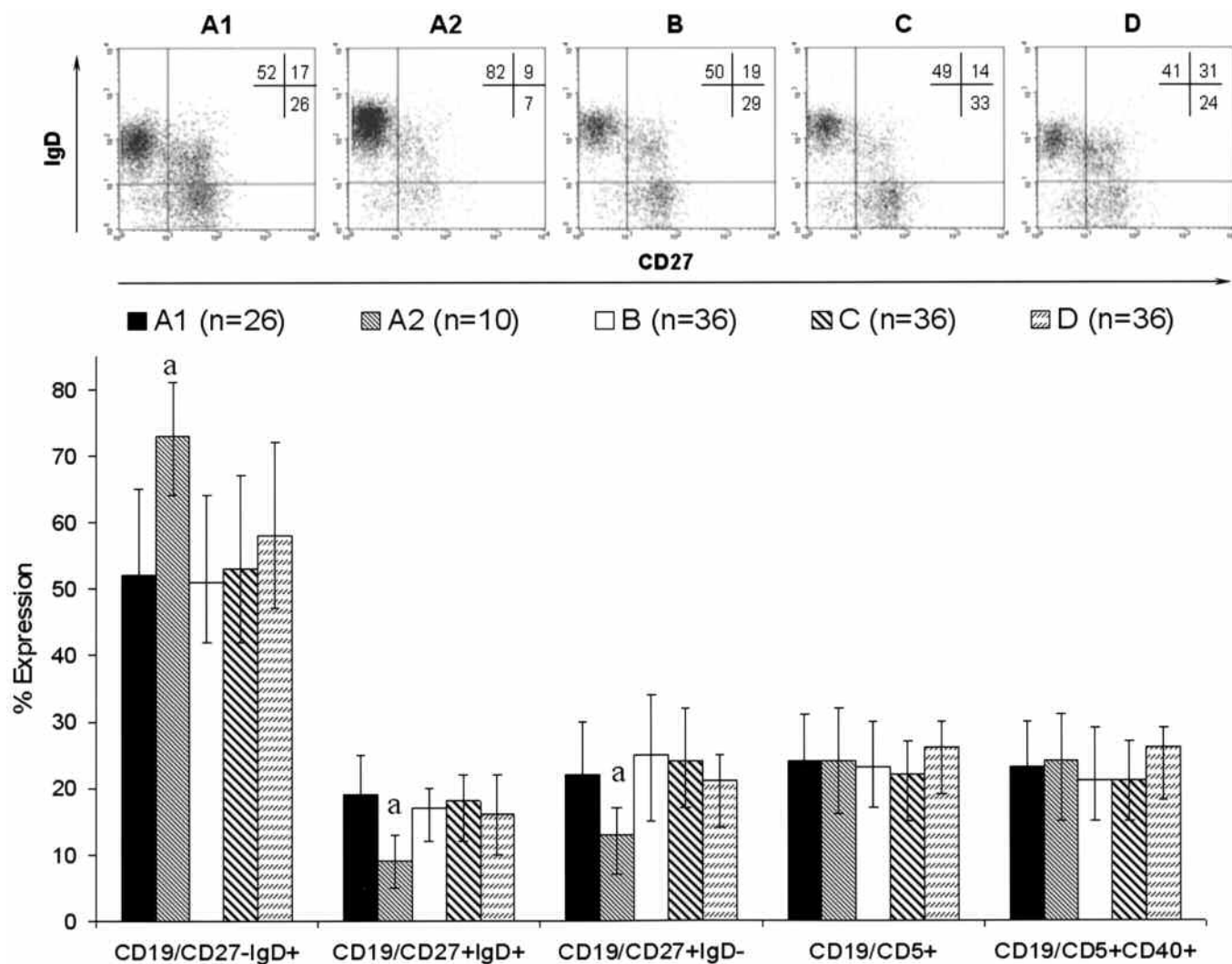
Figure 2. B lymphocyte subsets of patients with APS and control groups (group definitions as given in Figure 1). Dot plots show CD19-gated naive/memory B cells from representative subjects. Relative frequencies of each quadrant are indicated. Bar graphs show mean values and 25th-75th percentile for different B lymphocyte subsets. a: ANOVA post hoc test, $p < 0.05$ vs all control groups and vs A1.

DISCUSSION

Our study adds information regarding peripheral blood T and B lymphocyte homeostasis in obstetric APS. In contrast with previous studies, we set apart patients with obstetric APS who had thrombosis to avoid the possible effect of previous thrombosis and therapy. Immunophenotypic abnormalities among APS women with abortions but no history of thrombosis included increased levels of central memory (CD45RA–CCR7+) CD4+ lymphocytes and of activated CD4+CD25+ cells. Cells within the CD45RA–CCR7+ subset are more likely to express interleukin 2 (IL-2) than interferon-γ (IFN-γ), in keeping with a previous report in patients with APS, in which an increase in IL-2-producing CD4+ T cells was found[23]. IL-2 has been implicated in the pathogenesis of recurrent abortion[24]. Interestingly, central memory CD4 cells inversely correlated with C4 concentration.

The potential role for complement as an effector of pregnancy loss has been suggested[25,26].

In obstetric patients with APS who developed thrombosis, we have observed functionally distinct immunophenotypic abnormalities. For the first time, we have examined the distribution of naive and memory B cells in APS. Increased percentages and absolute counts of naive B cells were observed in these women with APS. Interestingly, higher levels of naive B cells were strongly associated with lower concentrations of serum C4. Within all women with APS, naive B cell percentages correlated with IgG anticardiolipin and with IgG anti-ß2-GPI antibody titers. Naive B cells have been suggested to be involved not only in antigen presentation processes but also in autoantibody production[12]. A disturbance in B cell trafficking or an increased activation of B cells, as well as an alteration in peripheral B cell differenti-
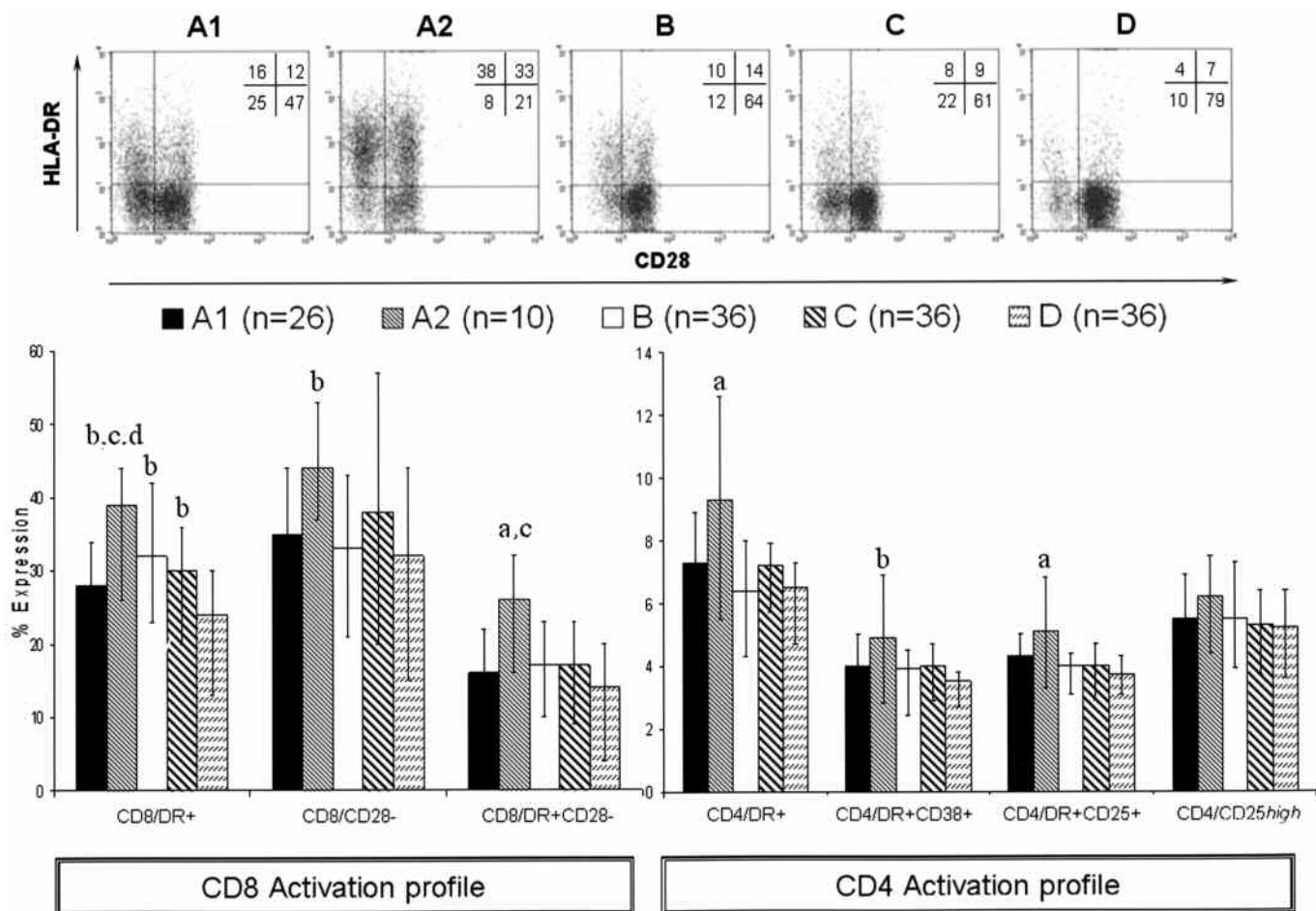
Figure 3. Activated and regulatory T cell subsets of patients with APS and control groups (group definitions as given in Figure 1). Dot plots show CD8-gated cells from representative subjects. Relative frequencies of each quadrant are indicated. Bar graphs show mean values and 25th-75th percentile for different CD8 and CD4 subsets. a: ANOVA post hoc test, $p < 0.05$ vs all control groups; b: $p < 0.05$ vs D; c: $p < 0.05$ vs A1; d: $p < 0.05$ vs C.

ation leading to a bias toward plasma cell differentiation and thus fewer memory B cells, could also provide an explanation for the higher percentages and absolute counts of naive B cells observed in these patients.

Obstetric patients with APS with thrombosis disclosed higher percentages of distinct activated CD4+ and CD8+ subsets. Higher expression of activation markers on CD4+ and CD8+ T-lymphocytes has been documented in APS[10,11]. Data of chronic *in vivo* activation in patients with APS might support previous data suggesting that autoreactive CD4+ T cells in APS are continuously stimulated by exposure to serum ß$_2$-GPI, or to ß$_2$-GPI on the membrane of apoptotic cells[7-9]. Functional studies are needed to demonstrate whether increased *in vivo* CD4+ and CD8+ T cell activation in patients with APS has a role in the pathogenesis of the disease or is secondary to clinical complications. We have started functional studies to assess the immunophenotype (naive, memory, activation, regulatory) of autoreactive CD4+ T cells to ß$_2$-GPI in patients with APS. The sample size was a limitation of the analysis of immunophenotypic

abnormalities in the subgroup of women with obstetric APS who had thrombosis. The potential role of B cell homeostasis and T cell activation in the pathogenesis and clinical evolution of APS warrants further evaluation in a larger number of patients and preferably by using multiple time-point determinations.

Lower levels of regulatory CD25-high CD4 T cells have been described in distinct autoimmune diseases[27]. The potential role of these cells has not been previously explored in APS. Typing CD25-high expression by CD4 as regulatory T cells, we have encountered no changes in both APS groups in comparison with controls. Usage of other markers such as CD127 or FoxP3 to better define regulatory CD4 T cells and functional studies might be necessary to assess if these immunological mediators have a role in the pathogenesis of APS.

We have observed higher percentages of regulatory CD8+CD28– T cells in obstetric patients with APS who had thrombosis. Circulating CD8+CD28– T cells are increased not only in various infectious diseases but also in patients

with autoimmune diseases like ankylosing spondylitis[28]. However, the distribution of CD8+CD28– cells has been found to be significantly lower in other autoimmune diseases such as SLE[29]. In view of the different functions of CD8+CD28– T cells, it is difficult to propose a potential role for these cells in APS. This warrants further investigation.

Contradictory findings for NK cells in patients with APS have been described. Some authors report increased levels, while others observed the opposite compared with healthy controls[10,30]. In our study, lower percentages and absolute counts of NK cells were observed in women with APS without thrombosis compared with disease controls, and significantly lower NK cell percentages and absolute counts were observed in patients with APS who had thrombosis compared with all control groups.

The identification of lymphocyte subsets as potential risk factors for clinical complications in APS is of utmost interest. The majority of obstetric patients with APS do not have clinical complications, while a proportion of patients develop aPL-related complications during followup. Previous studies have identified that the percentage of CD8+DR+ cells was a biological measure significantly associated with clinical activity of SLE, even more powerful than classical serological markers[31,32]. It has been reported that chronic activation of T lymphocytes, especially CD8+ T lymphocytes, may be involved in the pathogenesis of ischemic events[33]. The current authors hypothesized that a chronic activation of the immune system might play a role in the pathogenesis of thrombosis in APS[34]. The predictive value of increased activated CD8+DR+ T cells and of naive B cells for the development of clinical complications such as thrombosis in women with APS should be evaluated in future prospective studies with larger numbers of patients.

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