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CD4$^+$CD69$^+$ T cells and CD4$^+$CD25$^+$FoxP3$^+$ Treg cells imbalance in peripheral blood, spleen and peritoneal lavage from pristane-induced systemic lupus erythematosus (SLE) mice

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Abstract

Background: Adaptive immune cells, including CD4$^+$CD69$^+$ and CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (Treg) cells, are important for maintaining immunological tolerance. In human systemic lupus erythematosus (SLE), CD4$^+$CD25$^+$FoxP3$^+$ Treg cells are reduced, whereas CD69 expression is increased, resulting in a homeostatic immune imbalance that may intensify autoreactive T cell activity. To analyze the mechanisms implicated in autotolerance failure, we evaluated CD4$^+$CD69$^+$ and CD4$^+$CD25$^+$FoxP3$^+$ T cells and interleukin profiles in a pristane-induced SLE experimental model.

Methods: For lupus induction, 26 female Balb/c mice received a single intraperitoneal 0.5 ml dose of pristane, and 16 mice received the same dose of saline. Blood and spleen samples were collected from euthanized mice 90 and 120 days after pristane or saline inoculation. Mononuclear cells from peripheral blood (PBMC), peritoneal lavage (PL) and splenocytes were obtained by erythrocyte lysis and cryopreserved for further evaluation by flow cytometry using the GuavaEasyCyte TM HT. After thawing, cells were washed and stained with monoclonal antibodies against CD3, CD4, CD8, CD25, CD28, CD69, FoxP3, CD14 and Ly6C (BD Pharmingen TM). Interleukins were quantified using Multiplex® MAP. The Mann-Whitney test and the Pearson coefficient were used for statistical analysis, and $p < 0.05$ considered significant.

Results: Compared with the controls, SLE-induced animals presented increased numbers of CD4$^+$CD69$^+$ T cells in the blood on T90 and T120 ($p = 0.022$ and $p = 0.008$) and in the spleen on T120 ($p = 0.049$), but there were decreased numbers in the PL ($p = 0.049$) on T120. The percentage of Treg was lower in blood ($p < 0.005$ and $p < 0.012$) on T90 and T120, in spleen ($p = 0.043$) on T120 and in PL ($p = 0.001$) on T90. Increased numbers of CD4 + CD69+ T cells in the PL were positively associated with high IL-2 ($p = 0.486$) and IFN-γ ($p = 0.017$) levels, whereas reduced Treg cells in the blood were negatively correlated with TNFα levels ($p = 0.043$) and positively correlated with TGFβ1 ($p = 0.038$).

Conclusion: Increased numbers of CD4$^+$CD69$^+$ T cells and reduced numbers of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells with an altered interleukin profile suggests loss of autotolerance in pristane-induced lupus mice, which is similar to human lupus. Therefore, this model is useful in evaluating mechanisms of cellular activation, peripheral tolerance and homeostatic immune imbalance involved in human SLE.

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Introduction

Systemic lupus erythematosus (SLE) is a complex multifactorial disease characterized by loss of autotolerance, autoreactive T cell activation and production of inflammatory mediators and auto-antibodies. Immune tolerance is the state of unresponsiveness of the immune system to substances or tissues that have the potential to induce an immune response and comprises central and peripheral mechanisms [1]. Central tolerance is the primary response that allows immune system to discriminate self from nonself [2] [3], and it is centered in the thymus, bone marrow and spleen; however, peripheral tolerance controls self-reactive immune cells and prevents overreactive immune responses to various environmental factors [3], and it takes place in tissues and lymph nodes after lymphocyte maturation.

The balance between adaptive immune cells such as CD4+CD69+ effector T cells and CD4+CD25+FoxP3+ suppressor/regulatory T (Treg) cells is important for the maintenance of immunological tolerance [4] [5] [6]. In a normal immune response, the CD69 receptor is a protective inducible activation marker expressed on effectors T cells [7]. Because Treg cells can suppress the activation and proliferation of those effector T cells, Treg cells play a key role in the pathogenesis of inflammatory conditions [7]. If this process fails, the loss of tolerance may result in autoimmune disorders, including SLE [3]. Dysregulation of both adaptive and innate immune systems mechanisms are marked in SLE, culminating with tissue and organ damage induced by chronic inflammation and a variety of clinical manifestations [8] [9] [10].

Abnormal T cell activation and signaling problems appear to contribute to chronic disease activity in patients with SLE. Increased expression of the CD69 activation receptor [8] [11] [12] may enhance the activity of autoreactive T cells associated with disease severity [8] [6] [13] [14] [15]. Additionally, Treg cell numbers are reduced in SLE patients [16] [17] [18] and may explain the increased activity and autoreactivity of CD4 T cells [18] [19], which results in a homeostatic immune imbalance [20]. The mechanisms implicated in autotolerance failure during SLE development, maintenance and chronicity remain unknown, highlighting the importance of studies using experimental disease models [21] [22] [23]. Several genetic lupus mouse models are available, however the high cost of developing and maintaining these modified mouse lines prompts the need for studies using induced disease murine models [3], such as pristane-induced lupus mice. Pristane is a mineral oil that is injected intraperitoneally and is capable of generating clinical and laboratory abnormalities similar to those observed in human SLE, including the production of autoantibodies and inflammatory mediators and the development of arthritis [20] [24] [22, 25]. Therefore, to determine some aspects involved in immune dysregulation in murine lupus, we analyzed the expressions of CD4+CD69+ T cells and Treg cells and some interleukin profiles in pristane-induced Balb/c mice.

Materials and methods

Mice

Six-to-eight-week-old (18–22 g) female wild-type Balb/c mice were purchased from the Centro Multidisciplinar para Investigação Biológica – CEMIB/UNICAMP (Campinas, Brazil) and housed in the animal facility of the Rheumatology Division of the University of São Paulo School of Medicine (São Paulo, Brazil). All animal protocols were approved by the Institutional Animal Care and Research Advisory Committee (CAPPesq HC-USP Protocol # 009/11 - Comissão de Ética para Análise de Projetos de Pesquisa do Hospital das Clínicas da Faculdade de Medicina da USP) and were strictly conducted according to the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Mice were maintained in a conventional animal facility at $22 \pm 2\,°C$ with a 12-h light/dark cycle and fed a pelleted commercial chow ad libitum (Nuvilab CR1, São Paulo, Brazil) with free access to drinking water.

SLE induction by pristane in Balb/c mice

Twenty-six mice received a single intraperitoneal (i.p.) injection of 0.5 ml pristane (2,6,10,14 tetramethylpentadecane, Sigma Chemical Co., St. Louis, MO, USA) for SLE induction. Before injection, pristane was filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA). Sixteen control mice received i.p. injections of 0.5 ml 0.9% saline. At 90 and 120 days following SLE induction (T90, T120), 13 SLE-induced animals and 8 controls were euthanized with CO_2 to obtain peripheral blood, spleen and PL samples. The time points of 90 and 120 days were chosen for euthanasia and experimental procedures based on previous reports that described autoantibody production observed after 2 weeks [20] [22] [26] [27] [28] [29] and the development of arthritis 3 months [24] [30] [28] [31] after pristane induction, indicating the beginning of the inflammatory SLE process. In addition, we conducted a pilot experiment in which these periods were shown to be the most important for assessing the beginning and perpetuation of the lupus inflammatory processes, such as autoantibody production, arthritis development, cellular imbalance and inflammatory mediator alterations [32].

SLE induced in Balb/c mice

A) Clinical characteristics: lipogranulomas, splenomegaly and arthritis in front and back paws were evaluated by visualization. The presence and severity of arthritis was graded visually using the scoring system described by Patten (2004) [31].

B) Histopathological features: the spleens of all SLE-induced and control mice were removed, fixed in paraformaldehyde (4%), and embedded in paraffin; 5-µm spleen sections were then stained with H&E. Histopathological features were examined by an independent and experienced pathologist blinded to the study protocol using Panoramic Viewer software (3DHistech, Budapest, Hungary).

C) Plasma and peritoneal lavage evaluation: antinuclear Abs (ANAs) were detected by indirect immunofluorescence using Hep2 cell slides (NOVA Lite™ IFA) with 1:40 diluted plasma and peritoneal lavage, followed by staining with FITC antibody anti-.

IgG-conjugated at a dilution of 1:50 (Southern Biotechnology, Birmingham, AL). Anti-dsDNA, anti-Sm and anti-RNP Abs were measured by ELISA (1:250 dillution of plasma and peritoneal lavage) according to the manufacturer's instructions (ALPHA DIAGNOSTICS and eBioscience). Levels of interleukin (IL)-1, IL-2, IL-10, interferon I (IFN-I) and transforming growth factor (TGF) $_{\beta 1}$, $TGF_{\beta 2}$ and $TGF_{\beta 3}$ were quantified using Multiplex® MAP (multi-analyte panels) according to the manufacturer's instructions (Luminex® Technology, Millipore, Minneapolis, NM, USA) and using Analyst Milliplex software (Millipore).

D) Total and differential leucocyte counts: total peripheral leukocytes in the blood, spleen and peritoneal lavage (PL) were counted in a Neubauer chamber using blue methylene (1:1), and the differential counts of polymorphonuclear cells (PMNs), monocytes and lymphocytes were realized by smears of blood, spleen and PL.

Obtaining peripheral blood mononuclear cells (PBMCs) and blood plasma

Peripheral blood (200 µl) was collected from the caudal veins of all animals 90 days after induction of SLE. The samples were centrifuged (1200 rpm for 10 min at 4 °C) for plasma separation and frozen (– 80 °C) until further laboratory analyses. Red blood cells (RBC) were then lysed in whole blood by incubating the samples in FACS™ Lysing Solution (Becton Dickinson). The cell solution was centrifuged and washed several times and resuspended in RPMI (*Roswell Park Memorial Institute*) medium 1640. PBMCs obtained from the pellet were cryopreserved with DMSO (dimethyl sulfoxide) and fetal bovine serum (FBS) (1:4) and frozen in liquid nitrogen for at most 2 weeks until flow cytometry experiments.

Obtaining peritoneal lavage mononuclear cells (PLMCs) and peritoneal lavage supernatants

Cell suspensions were collected from the peritoneal cavities of all euthanized mice after local asepsis with 70% ethanol, inoculation with 2 ml RPMI medium 1640 and abdominal incision. PLMCs obtained were centrifuged to separate supernatants, washed with RPMI medium 1640,

and cryopreserved in DMSO and FBS until flow cytometry experiments.

Extracting the spleen and obtaining splenocytes

Spleens were removed, weighed for evaluation of splenomegaly, chopped with a sterile scalpel and crushed in a mortar with a sterile pestle using a fine mesh metal sieve in a petri plate with RPMI medium 1640. The cell suspension was transferred to a Falcon tube and allowed to stand for approximately 2 min to allow precipitation of larger tissue blocks. The supernatant was transferred to another Falcon tube and centrifuged (800 g for 10 min at 4 °C). The pellet containing splenocytes were washed twice with RPMI medium 1640 and cryopreserved with DMSO and FBS for at most 2 weeks until further evaluation by flow cytometry.

Flow cytometry

To perform the flow cytometry experiments, PBMCs, splenocytes and PLMCs were unfrozen, resuspended with RPMI medium 1640 at room temperature, washed and centrifuged (1500 rpm for 10 min at 4 °C) twice and resuspended with PBS and FBS. All samples were stained for one hour in the dark at 4–8 °C with a variety of anti-mouse monoclonal Abs (BD Biosciences): CD3; CD4; CD8; CD25; CD28; CD69; FoxP3; CD14; and-Ly6C. Cells were permeabilized with permeabilization buffer (Biolegend) at a dilution of 1:10 to stain for intracellular Foxp3.

After incubation with each of above monoclonal Abs, the samples were centrifuged and washed with PBS and then fixed in 4% paraformaldehyde. Flow cytometry analysis was conducted using a Guava EasyCyteTM HT (Millipore), and analyses was conducted with InCyte software (Millipore).

Statistical analysis

Data are expressed as the mean ± SD of percentages of positive cells to monoclonal antibodies tested. The chi-square test was used in qualitative analyses, and the Mann-Whitney test was used to analyze quantitative differences between both SLE-induced and control groups. The Pearson coefficient was used to evaluate correlation. *P* values ≤0.05 were considered statistically significant.

Results

SLE-induced experimental model

a) **Clinical characteristics (Fig. 1).** All 26 SLE-induced mice presented lupus clinical alterations compared to controls (A, D and F) on both T90 and T120 evaluations. SLE-induced animals were heavier than controls on T90 (28 g ± 2 vs. 26 g ± 2, *p* < 0.001) and on T120 (29 g ± 3 vs. 27 g ± 2, p < 0.001). Lipogranulomas (B and C) and splenomegaly (E) developed in the peritoneal cavity, and greater

Fig. 1 Peritoneal cavity of a control animal (**a**) and of a SLE induced animal (**b**) with lipogranulomas on liver and spleen surface (arrows). Lipogranuloma's detail in (**c**). Spleen of control (**d**) and SLE induced mice (**e**) showing splenomegaly. Later members of normal (**f**) and of SLE induced animal (**g**) showing joints affect by arthritis.

spleen weights were demonstrated in the SLE-induced mice compared to controls (0.25 g ± 0.07 vs. 0.12 g ± 0.03, $p < 0.001$ on T90 and 0.11 g ± 0.01 vs. 0.30 g ± 0.04, p < 0.001 on T120). Arthritis was observed in 7 of 13 mice on T90 (53%, score of 0.9 ± 0.9) and in all 13 mice on T120 (100%, score of 1.8 ± 0.6), whereas no control mice had this manifestation.

b) Histological characteristics. Fig. 2 shows spleens from control group animals with preserved capsules of dense connective tissue and splenic parenchyma consisting of red and white pulp with preserved architecture (A and B). On the other hand, spleens from SLE-induced animals showed rarefaction of the white pulp and loss of lymph node architecture (C and D, arrow), randomly distributed deposits of greasy material (Fig. 1 D, tip of arrow), and increased inflammatory cells, primarily neutrophils, suggesting a greater inflammatory process in this organ. These alterations were intensified on T120 (Fig. 1 D, tip of arrow).

Plasma and peritoneal lavage characteristics of the SLE-induced experimental model

a) Antinuclear (ANA), anti-dsDNA, anti-Sm and anti-RNP autoantibodies. Prior to SLE induction, ANA, anti-dsDNA, anti-RNP and anti-Sm autoantibodies were negative in plasma and PL from all 42 animals. Following induction, control mice remained negative for these autoantibodies, whereas 13 SLE-induced mice presented at least one autoantibody: 9 ANA (69%), 5 anti-dsDNA (38%), 4 anti-Sm (31%) and 4 (31%) anti-RNP in plasma and 6 (46%), 9 (69%), 3 (23%) and 4 (31%) were positive for the same antibodies in the PL on T90; 11 (85%), 7 (54%), 3 (23%) and 5 (38%) mice had ANA, anti-dsDNA, anti-Sm and anti-RNP antibodies in plasma and 6 (46%), 8 (62%), 7 (54%) and 2 (15%) were positive for the same antibodies in the PL on T120. Interestingly, all 4 autoantibody negative SLE-induced mice on T90 also had no signs of arthritis but showed large percentages of $CD4^+CD69^+$ T cells, mainly in the blood and PL. In
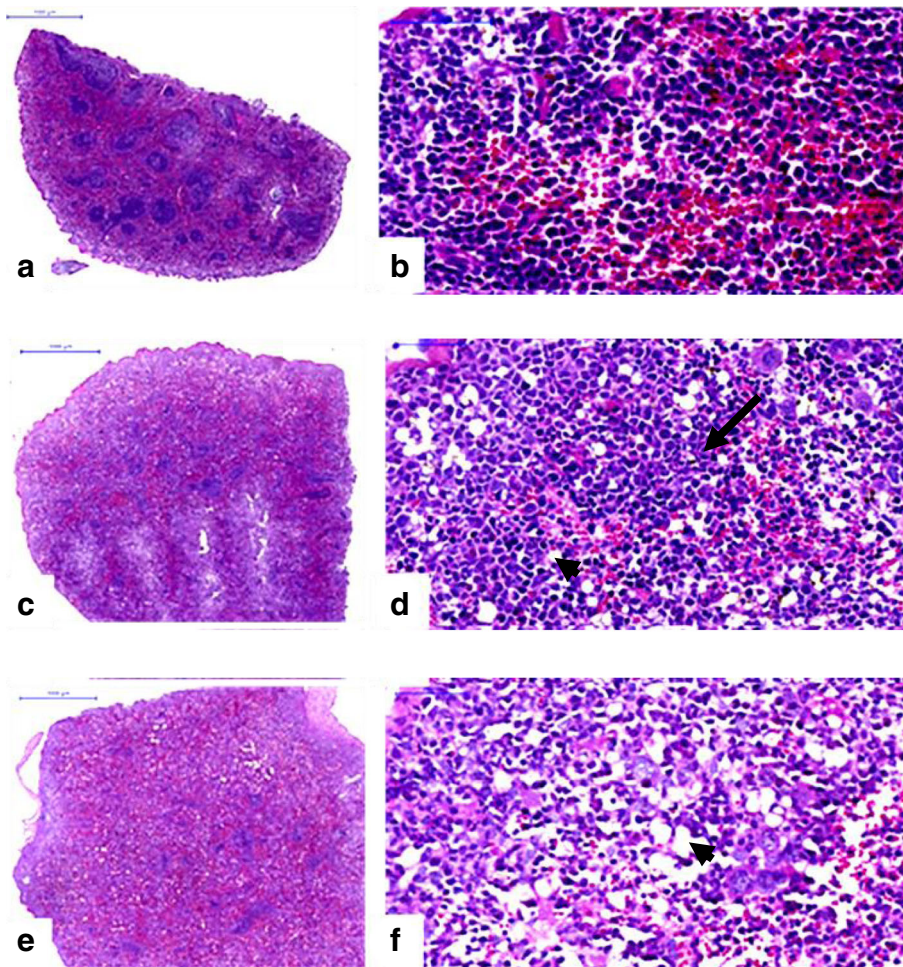
Fig. 2 Panoramic histological cut of spleen from control group animal and SLE induced animal. **a**) Spleen of control animal showing preserved capsule of dense connective tissue and septa dividing the interior tissue of the organ in lobules interconnected. **b**) splenic parenchyma consisting of red pulp rich in sinusoids capillaries and splenic tissue cords constituted by macrophages, plasma cells, reticular cells and blood cells within the standards of normality. It is observed still rare megakaryocytes cells. The white pulp is constituted by lymphatic nodes characterized by lymphocyte cells arranged around the arterial branches with preserved architecture. **c**) Spleen of a SLE induced animal revealing rarefaction of white pulp characterized by loss of architecture of lymphatic nodes (**d**, arrow) which is characterized by replacement of lymphatic nodes by deposits of greasy material distributed diffusely (tips of arrows). These alterations are intensified on T120 (**e** and **f**, tips of arrows) Original magnification X2 and X40.

contrast, on T120, although 2 SLE-induced animals were ANA negative, they had signs of arthritis and increased percentage of CD4$^+$CD69$^+$ T cells in blood and spleen, but not in the PL.

b) Interleukins (Table 1). The production of plasma TNFα (12.3 ± 3.9 vs. 9.1 ± 1.4, $p < 0.048$, T90 10.2 ± 6.2 vs. 6.0 ± 2.2, $p < 0.001$, T120); TGF$_{\beta 1}$ (2780.0 ± 1050.0 vs. 1255.0 ± 821.4, $p < 0.010$, T90); and TGF$_{\beta 2}$ (79.3 ± 28.6 vs. 38.7 ± 31.9, $p < 0.027$, T90, and 169.6 ± 68.0 vs. 125.8 ± 64.9, $p = 0.031$, T120) was higher in SLE-induced animals compared to controls. In contrast, SLE-induced and control animals had similar IL-2, IL-10 and IFN-γ levels. In the PL, a similar interleukin profile was observed on T90. However, on T120, SLE-induced animals had higher TGF$_{\beta 1}$ (525.0 ± 110.3 vs. 318.1 ± 223.6, $p = 0.039$) and

lower IL-2 and IL-10 secretions (0.4 ± 0.3 vs. 2.1 ± 1.8, $p = 0.007$ and 0.9 ± 0.6 vs. 7.8 ± 10.0, $p = 0.007$, respectively) compared to controls.

Cellular characteristics of the SLE-induced experimental model

a) Quantification of polymorphonuclear, monocytes and lymphocytes in blood, spleen and peritoneal lavage. The peritoneal blood from SLE-induced mice had higher PMN numbers on T90 and T120 (14.7 ± 15.2 vs. 4.3 ± 3.2, $p = 0.049$; 7.5 ± 3.6 vs. 2.8 ± 1.8, $p = 0.002$) and fewer lymphocytes on T120 (13.5 ± 7.6 vs. 33.53 ± 14.2, $p = 0.003$) compared to controls, whereas the number of monocytes and total leucocytes were similar in both groups. In the spleen, SLE-induced animals had more

Table 1 Data represent plasma and PL levels (mean ± standard deviation) of interleukins in SLE induced and control mice on both periods T90 and T120

Interleukins	Periods (days)	Blood			PL		
		Control n = 8	SLE-induced n = 13	*p*	Control n = 8	SLE-induced n = 13	*p*
IL-1	T90	74.6 ± 136.6	45.4 ± 20.7	0.289	5.7 ± 9.9	71.9 ± 45.6	0.114
	T120	223.4 ± 154.0	236.1 ± 239.5	1.000	8.9 ± 8.8	2.3 ± 2.2	0.450
IL-2	T90	3.3 ± 1.2	2.9 ± 1.4	0.616	0.9 ± 0.4	2.5 ± 0.9	0.057
	T120	7.5 ± 9.2	3.2 ± 1.9	0.817	2.1 ± 1.8	0.4 ± 0.3	**0.007**
TNFa	T90	9.1 ± 1.4	12.3 ± 3.9	**< 0.048**	0.8 ± 1.2	18.4 ± 11.8	0.057
	T120	6.0 ± 2.2	10.2 ± 6.2	**< 0.001**	3.1 ± 6.2	2.4 ± 3.2	0.314
TGF$_{\beta 1}$	T90	1255.0 ± 821.4	2780.0 ± 1050.0	**< 0.010**	759.3 ± 1186.0	516.3 ± 337.8	1.000
	T120	4514.0 ± 2653.0	5498.0 ± 2184.0	0.415	318.1 ± 223.6	525.0 ± 110.3	**0.039**
TGF$_{\beta 2}$	T90	38.7 ± 31.9	79.3 ± 28.6	**< 0.027**	376.3 ± 205.4	204.3 ± 102.1	0.228
	T120	125.8 ± 64.9	169.6 ± 68.0	**0.031**	294.4 ± 171.1	258.2 ± 98.1	0.451
TGFβ3	T90	6.4 ± 2.0	7.1 ± 1.5	0.404	16.3 ± 3.8	13.0 ± 6.2	0.400
	T120	6.9 ± 0.7	8.0 ± 2.4	0.400	16.7 ± 5.2	13.7 ± 5.9	0.291
IL-10	T90	10.7 ± 5.3	17.6 ± 25.9	0.923	3.7 ± 5.7	17.3 ± 10.6	0.114
	T120	8.3 ± 4.1	11.2 ± 6.2	0.379	7.8 ± 10.0	0.9 ± 0.6	**0.007**
IFNg	T90	3.7 ± 1.0	3.0 ± 1.1	0.245	1.1 ± 1.1	3.4 ± 2.6	0.229
	T120	85.8 ± 163.5	3.3 ± 3.3	0.239	1.3 ± 1.1	0.3 ± 0.1	0.119

p < 0.05 are set in bold
IL = interleukin; *IFN* = interferon; *TNF* = tumor necrosis factor; *TGF* = transforming growth factor

total leucocytes (76.7 ± 36.4 vs. 43.3 ± 20.5, $p = 0.048$, T90), more PMNs (8.3 ± 8.0 vs. 1.9 ± 2.6, $p = 0.009$; 11.6 ± 6.4 vs. 1.0 ± 0.8, $p < 0.001$) and more monocytes (126.2 ± 448.8 vs. 0.3 ± 0.5, $p = 0.036$; 4.2 ± 3.0 vs. 0.3 ± 0.8, $p = 0.001$) in both periods, T90 and T120, respectively, than control animals, and the numbers of lymphocytes were similar in both groups. In turn, SLE-induced animals had more PMNs (8.6 ± 7.2 vs. 0.8 ± 1.4, $p = 0.003$; 3.2 ± 2.4 vs. 0.5 ± 0.4, $p = 0.001$) and more monocytes (1.5 ± 1.5 vs. 0.4 ± 0.4, $p = 0.019$, T90) in the PL compared to controls.

Flow cytometry
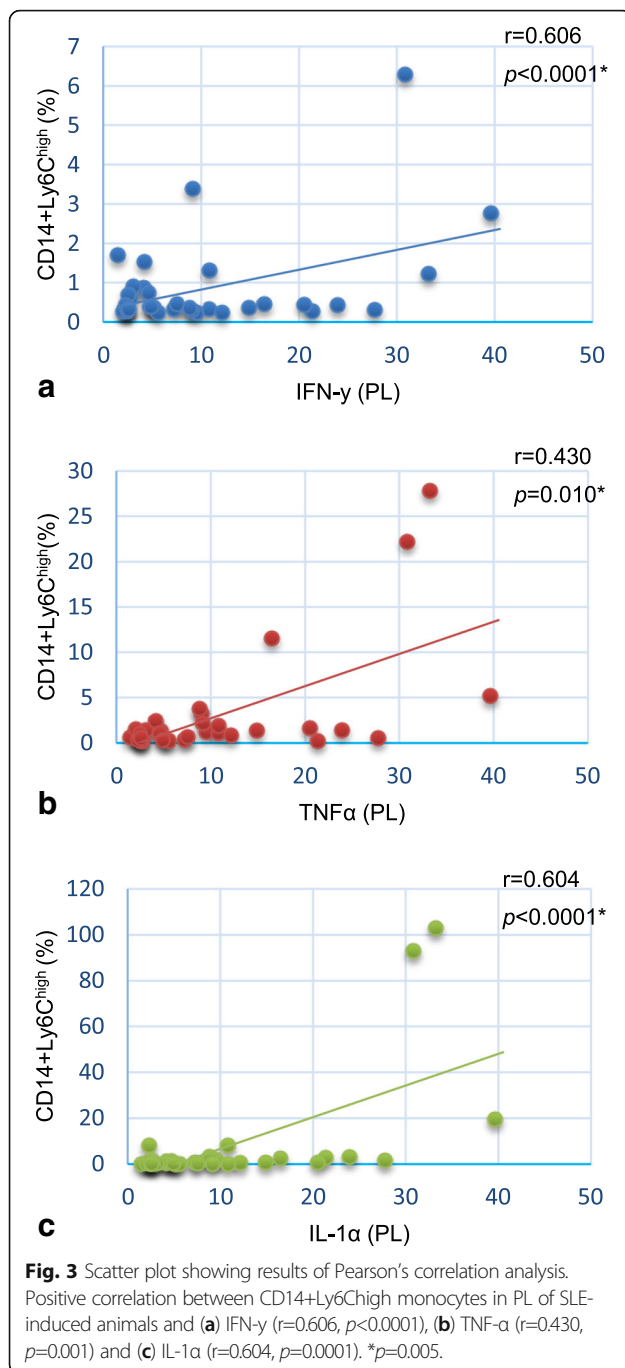**a) Monocytes CD14$^+$Ly6C^{high}.** The expression of CD14$^+$Ly6C^{high} on monocytes from SLE-induced mice was significantly higher in both periods, T90 and T120, respectively, in the blood (7.8 ± 3.4 vs. 4.0 ± 1.9, $p = 0.031$; 12.1 ± 3.0 vs. 8.1 ± 1.7, $p = 0.001$) and in the PL (26.7 ± 12.2 vs. 9.0 ± 1.5, $p = 0.031$; 5,4 ± 2.3 vs. 13.1 ± 6.5, $p < 0.005$) compared to controls, but it was similar in the spleen (8.0 ± 4.3 vs. 8.8 ± 2.7, $p = 0.203$; 7.8 ± 8.0 vs. 17.7 ± 12.6, $p = 0.068$). There was a positive correlation between PL monocytes CD14$^+$Ly6C^{high} and the proinflammatory cytokines IFN-γ ($p < 0.0001$), TNFα ($p = 0.010$) and IL-1 ($p < 0.0001$) as shown in Fig. 3.

b) CD4$^+$ T cells and CD8$^+$ T cells. The expression of CD4$^+$ T cells was similar in the blood (80.7 ± 4.6 vs. 83.9 ± 1.6, $p = 0.933$), PL (89.6 ± 2.3 vs. 85.0 ± 2.5, $p = 0.324$) and

spleen (89.6 ± 2.3 vs. 91.0 ± 2.7, $p = 0.511$) between SLE-induced and control animals. The same trend was observed with respect to CD8$^+$ T cells, which had similar expression levels in both groups: 12.7 ± 3.9 vs. 11.2 ± 3.8 ($p = 0.412$) in blood, 1.8 ± 1.5 vs. 1.6 ± 1.0 ($p = 0.373$) in PL and 2.9 ± 1.1 vs. 3.7 ± 2.0 ($p = 0.968$) in spleen.

c) CD69$^+$ expression on CD4$^+$ T cells (Fig. 4). At T90, SLE-induced animals had higher expression of CD4$^+$CD69$^+$ T cells in the blood (16.4 ± 8.3 vs. 7.0 ± 2.6 $p < 0.022$) compared to controls, whereas in both the spleen and PL, the expression levels of these cells were similar (22.6 ± 10.9 vs. 15.1 ± 4.3, $p > 0.05$ and 18.0 ± 11.4 vs. 18.3 ± 5.0, $p > 0.05$, respectively). However, 120 days after SLE induction, the expression of CD4$^+$CD69$^+$ T cells was higher in the blood (18.3 ± 6.9 vs. 10.1 ± 2.7, $p = 0.008$) and spleen (23.8 ± 6.3 vs. 19.0 ± 4.9, $p = 0.049$) but lower in the PL (8.2 ± 3.9 vs. 15.0 ± 5.0, $p = 0.001$) compared to controls. Increased percentages of CD4$^+$CD69$^+$ T cells in the PL were positively associated with higher levels of IL-2 ($p = 0.486$) and IFN-γ ($p = 0.017$).

d) Coexpression of CD28$^+$ and CD69$^+$ on CD4$^+$ T cells. At T90, SLE-induced animals had increased coexpression of CD28$^+$ and CD69$^+$ on CD4$^+$ T cells in blood (9.7 ± 4.5 vs. 3.5 ± 3.8, $p < 0.05$) compared to control animals, although expression was similar in the PL (13.2 ± 6.6 vs. 12.6 ± 3.1, $p > 0.999$) and in the spleen (9.0 ± 6.3 vs. 4.1 ± 0.8, $p = 0.405$). At T120, CD28$^+$ and CD69$^+$ coexpression remained enhanced in the blood

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Fig. 3 Scatter plot showing results of Pearson's correlation analysis. Positive correlation between CD14+Ly6Chigh monocytes in PL of SLE-induced animals and (**a**) IFN-y (r=0.606, p<0.0001), (**b**) TNF-α (r=0.430, p=0.001) and (**c**) IL-1α (r=0.604, p=0.0001). *p=0.005.

(9.2 ± 5.9 vs. 3.8 ± 1.9, $p = 0.020$), whereas in the PL, it was significantly reduced (6.9 ± 2.9 vs. 11.9 ± 5.6, $p = 0.031$).

e) **Expression of CD4⁺CD25⁺FoxP3⁺ Treg cells.** Figs. 5 demonstrates that the expression of CD4⁺CD25⁺FoxP3⁺ Treg cells was lower in SLE-induced mice compared to controls in blood at T90 and T120, respectively (2.6 ± 1.8 vs. 5.1 ± 2.4, $p < 0.005$, 3.5 ± 1.2 vs. 5.7 ± 1.8, $p = 0.012$), but in the PL, this same trend occurred only at T90 (3.2 ± 3.0 vs. 7.0 ± 4.3,

$p = 0.001$). At T120, these cells were also increased in the spleen (2.6 ± 1.5 vs. 4.6 ± 0.7, $p = 0.018$). In addition, in the blood, Treg cells were negatively correlated with TNFα production ($p = 0.043$) and positively correlated with TGF$_{\beta 1}$ levels ($p = 0.038$).

Discussion

This study shows for the first time, that in pristane-induced SLE Balb/c mice, there is an imbalance of CD4⁺CD69⁺ T cell and CD4⁺CD25⁺FoxP3⁺ Treg expressions in blood, spleen and PL. This alteration might be involved in the breakdown of immune autotolerance contributing to lupus development and chronicity.

All of our SLE-induced mice developed lupus clinical characteristics with great amounts of lipogranulomas in the peritoneal cavity, especially in the diaphragm and the surfaces of the liver and spleen; they also developed splenomegaly and arthritis similar to previous studies in this model performed by Utbonaviciute et al. (2013), Bossaller et al. (2013) and Leiss et al. (2013) [33] [33] [34] [35] [33] [30] [36]. Spleen histomorphology changes, such as hyperplasia of the red pulp and reduction of the white pulp, increased infiltration of inflammatory cells (especially neutrophils), and random oil deposits were observed in all SLE-induced animals as described in the studies by Leiss et al. (2013) [30] and Zhuand et al. (2014) [37]. Likewise, in human SLE [38], atrophic changes with lower volume and numbers of splenic corpuscles (lymphatic nodes or follicles) were also evidenced. These histological findings suggest that pristane-induced murine SLE model is more similar to human SLE than other commonly used models of this disease such as B/WF1 mice, in which only slight inflammatory cell infiltration [39] is observed.

Previous reports have detected ANAs in the pristane SLE model following the first two weeks after induction [40] [30] [29] [36] [35]. We also found ANAs in plasma and/or in PL from most of our SLE-induced mice. Interestingly, at T90, 4 SLE-induced (30%) mice were ANA negative and had no signs of arthritis but had higher percentages of CD4⁺CD69⁺ T cells in the blood and PL, suggesting the beginning of the inflammatory process. Remarkably, at T120, only 2 SLE-induced animals (15%) were ANA negative and had arthritis and increased percentages of CD4⁺CD69⁺ T cells in the blood and spleen although not in PL, suggesting a possible migration of these activated inflammatory cells from the LP to other sites such as the blood and spleen. These data may indicate that by T90, the inflammatory process is in the very early stages when sufficient apoptotic cells are not yet present to trigger ANA production. Hence, we have demonstrated that, according to the postinduction period evaluated, different cellular and clinical alterations were observed during disease development and progression.
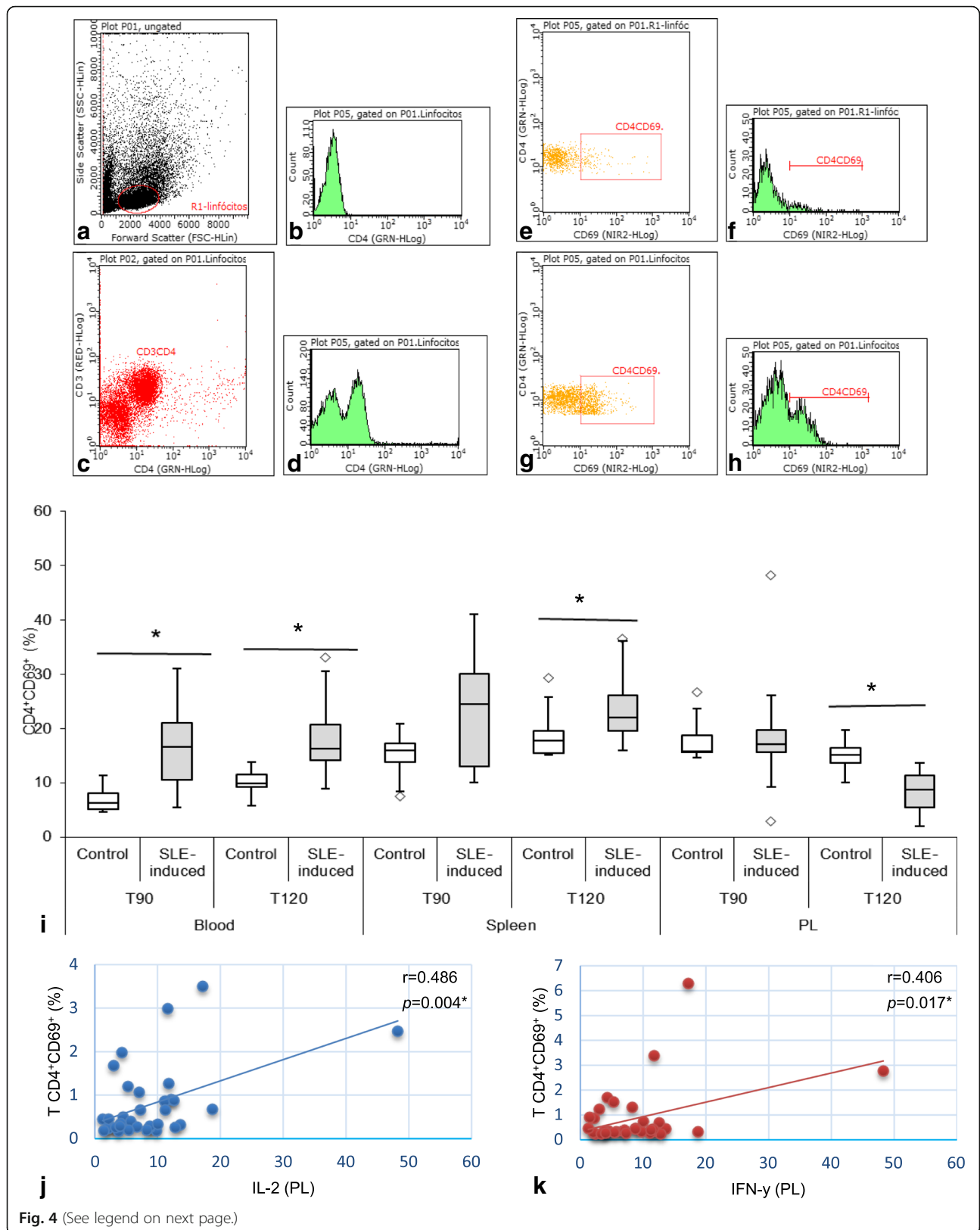
Fig. 4 (See legend on next page.)

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Fig. 4 Dot-plot and histograms of lymphocytes separated by size and granularity (**a** and **b**) and the subpopulation of CD4 T cells (**c** and **d**) which express CD69, highlighted in the central quadrant, from a control (**e** and **f**) and a SLE induced animal (**g** e **h**). **i**: Expression of CD69 in CD4+ T cells in blood, spleen and PL of SLE induced animals (dark bars) and controls (light bars) on D90 and D120. Results expressed as mean ± SD. Mann-Whitney Test, *p*=0.005. Scatter plot showing results of Pearson's correlation analysis. Positive correlation between CD4+CD69+ T cells in PL of SLE-induced animals and (**j**) IL-2 (r=0.606, *p*<0.0001) and (**k**) IFN-γ (r=0.604, *p*=0.0001). *p*=0.005.

Interestingly, the interleukin profile was different between the two groups of induced and controlled animals. In fact, we have shown increased TNFα, $TGF_{\beta1}$ and $TGF_{\beta2}$ levels in plasma from SLE-induced animals while IL-1, IL-2, IL-10, $TGF_{\beta3}$ and IFN-γ levels were similar in both induced and control animals. In contrast, in PL, IL-2 e IL-10 levels were reduced and $TGF_{\beta1}$ was increased 120 days after induction, while IL-1, TNFα, $TGF_{\beta1}$, $TGF_{\beta2}$ e IFN-γ were similar on both groups. Therefore, SLE-induced animals had increased production of pro inflammatory plasmatic TNFα. In human SLE, TNFα is increased in plasma [41] and influences the regulation of INF-γ production [42] involved in inflammation and in apoptosis [43]. Despite increased TNFα in our SLE-induced mice, INF-γ production was not altered, similar to the study by Mizutani et al. (2015) [22]. However, Xu et al. (2015) noted increased INF-γ only 6 months after pristane SLE induction that returned to basal levels after this period [42]. In humans and B/WF1 mice, IL-2 is reduced [44] and IL-10 is usually increased [45] [46] [47]. Lower production of IL-2 in human SLE suggests diminished T helper cell function and an imbalance in Th1 and Th2 cells [48], whereas IL-10 may be related to B cell proliferation and apoptosis [49] and is linked to disease severity [50]. Interestingly, similar levels of plasma IL-2 and IL-10 were detected in our SLE-induced animals compared to controls; in contrast, in the PL, both cytokines were reduced on T120 suggesting that the inflammatory process is not maintained in the peritoneal cavity. $TGF_{\beta1}$ and $TGF_{\beta3}$ have important roles in controlling cytotoxic T cell proliferation and differentiation [51]. In human SLE, they down regulate chronic lymphocyte hyper responsiveness, despite decreased production [52]. In our SLE-induced animals, the production of plasma $TGF_{\beta1}$ and $TGF_{\beta3}$ was significantly higher, suggesting an attempt to regulate immune homeostasis during the inflammatory process; in fact, TGF_{β} can inhibit the proliferation of naive but not activated T cells [53] and therefore promotes high plasmocytic activity [51].

White cell count was higher in the spleens from SLE-induced animals at T90, suggesting more pronounced inflammatory activity during this period. CPMN number was higher in the blood, spleen and PL of SLE-induced mice at T90 and T120, and monocyte numbers were increased in the spleen (T90 and T120) and PL (T90). On the other hand, lymphocyte numbers were higher only in the blood (T120) and similar in the spleen and PL between both groups. The presence of pristane in the peritoneal cavity months after inoculation could cause lymphocyte and dendritic cell apoptosis, as evaluated by Calvani et al. (2005) [26], and explain the increase in CPMN and monocytes, which are professional phagocytes.

Increased numbers of activated $CD14^+Ly6C^{high}$ monocytes were observed in the blood and PL from SLE-induced mice on T90 and T120, suggesting a possible role of these cells in the beginning and perpetuation of the inflammatory process observed in this model. In a similar way, Lee et al. (2008b) [54] and Bossaller et al. (2013) [33] also observed increases in these cells two weeks after induction accompanied by high expression of INF-I [54]. Furthermore, in the PL, there was a positive correlation between high numbers of $CD14^+Ly6-C^{high}$ monocytes and the production of IFN-γ, TNFα and IL-1. In fact, monocytes play a fundamental role in the production of proinflammatory cytokines present in the peritoneal cavity and plasma from SLE-induced mice [54]. IFN-γ and TNFα [28] contribute to the great influx of CD4 T cells towards the peritoneal cavity in the first months after induction [55], whereas IL-1 has been considered a biomarker of disease activity or organ involvement in humans [56] [57].

The percentages of CD4 and CD8 T cells from SLE-induced and control animals were similar in all sites evaluated. In human lupus, a variety of changes in CD4 and CD8 numbers and proportions have been described [6] [7] [15] [11] [12], but the role of these cells in the development and maintenance of disease activity is still controversial.

SLE-induced mice had increased activated $CD4^+CD69^+$ T cells similar to human SLE [17] [18] [7], suggesting peripheral auto tolerance breakdown [58] [59] [60] [7]; this alteration may be even greater in patients with active disease [58] [60] [7]. We have also shown increased activated CD4 T cells expressing CD69 in the spleen only 120 days after induction possibly due to migration of immune cells from LP to other regions and consequent increases in inflammatory infiltrates in the spleen. In contrast, in B/WF1 mice, $CD4^+CD69^+$ T cells are increased in the spleen [13] [61] [20] [22] [62] [63] and lymph nodes but not in the peripheral blood, indicating continuous activation of CD4 T cells in lymphoid organs [6] [13] [20] [22]. In MRL animals as well, $CD4^+CD69^+$ T cell expression in the blood and peripheral lymphoid organs are similar to those of normal Balb/c [64]. Therefore, the behavior of $CD4^+CD69^+$ T cell expression in our pristane induced
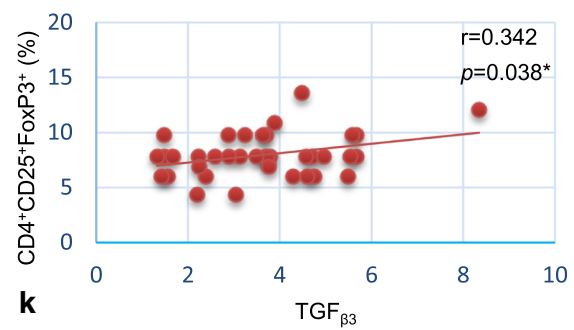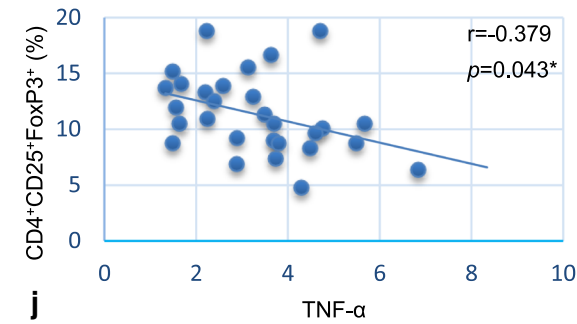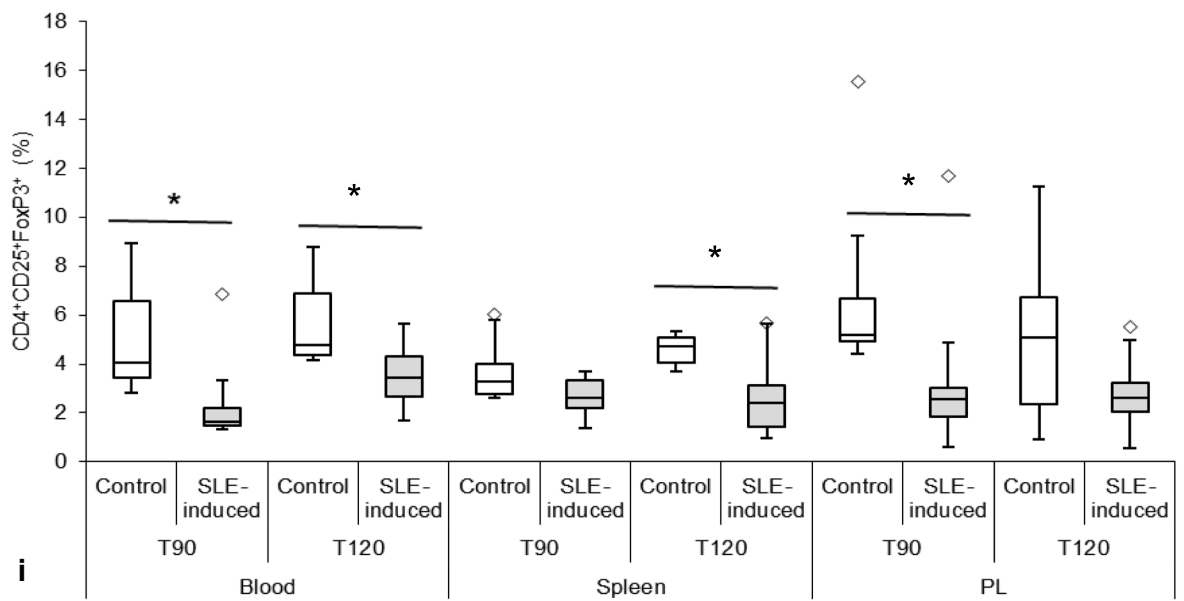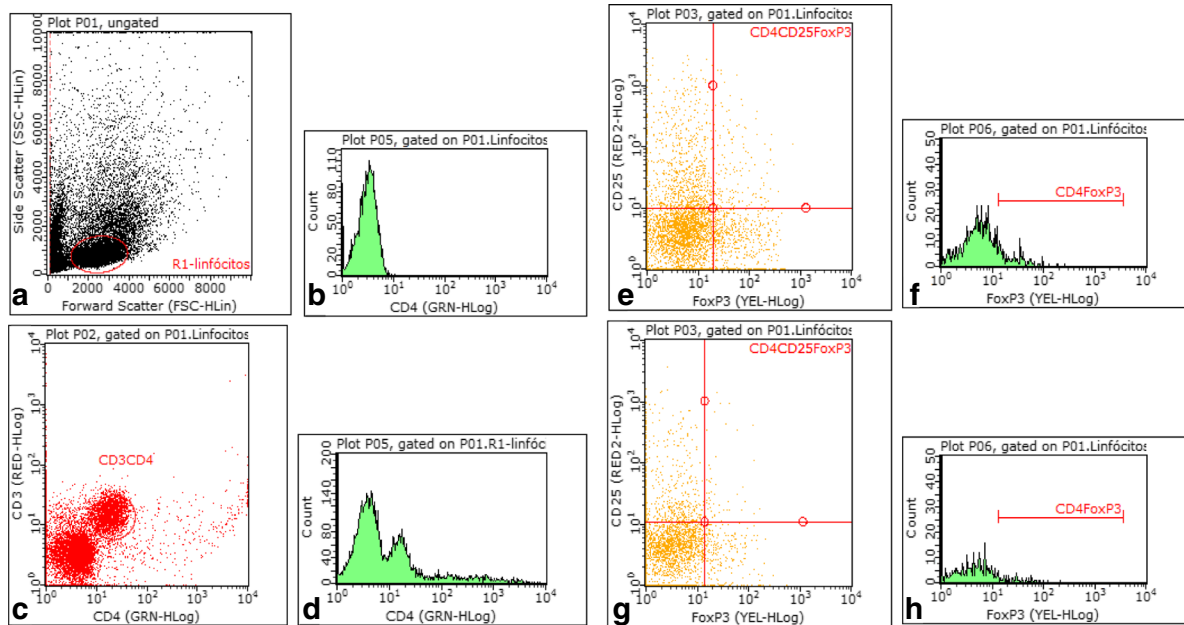
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Fig. 5 Dot-plot and histograms of lymphocytes separated by size and granularity (**a** and **b**) and the subpopulation of CD4 T cells (**c** and **d**) which expresses CD25 and FoxP3, highlighted in the larger quadrant, of a control (**e** and **f**) and a SLE induced animal (**g** and **h**). **i**: Expression of Treg CD4+CD25+FoxP3+ cells in blood (**a**). spleen (**b**) and PL (**c**) from SLE induced animals (dark bars) and controls (light bars) on T90 and T120. The results are expressed by mean ± SD. Mann-Whitney Test. *$p<0.005$. Scatter plot showing results of Pearson's correlation analysis. Positive correlation between Treg cells in blood of SLE-induced animals and (**j**) TNF-α (r=0.379, $p<0.043$) and (**k**) TGFβ3 (r=0.342, $p=0.038$). *$p=0.005$.

mice is similar to human disease in contrast to B/WF1 and MRL mice models, indicating that our model may be a better experimental murine model for the study of inflammatory process involved in lupus. Remarkably, in the PL from our mouse model, the increase in CD4+CD69+ T cells was directly correlated with high IL-2 and IFN-γ levels. This reinforces the concept that IL-2, which is primarily produced by T cells, can exert stimulatory effects on immune responses by expanding effector T cells populations [65] and thus promoting positive feedback in this model. Interestingly, CD4+CD69+ T cells [13] and IFN-γ levels were strongly associated with disease activity [13] [66]; in fact, IFN-γ was recently described as a potential biomarker in human SLE [66]. In contrast, there was no correlation between CD4+CD69+ T cells and IL-10 levels, and actually, a pathogenic role for IL-10 in human SLE remains controversial. Even though IL-10 is mainly produced by Treg cells and acts as a regulator of the immune response, paradoxically, it also improves B cells proliferation and Ig class switching, thus increasing antibody secretion [67].

We further demonstrated that SLE-induced animals had increased CD4 T cells coexpressing CD69 and CD28, suggesting amplified proliferation of activated CD4 T cells. Yang et al. (2008) proposed that CD69 is necessary but not sufficient for activation of CD4 T cells [68]. The stimulation of CD28, continuously expressed in CD4 T cells [69], in the presence of CD69 intensively increases the proliferation of CD4 T cells [26], as well as the production and secretion of IL-2 [70]. The reduction in CD4 + CD25 + FoxP3+ Treg cells in peripheral blood from our pristane SLE-induced animals suggests the loss of peripheral autotolerance and a homeostatic immune imbalance [71] [34] [10]. This alteration was also observed in the spleen (T120) and PL (T90), suggesting that Treg cells play an important role in the development of autoimmunity in this model. Moreover, the initial reduction of Treg cells in LP and in spleen suggests that the inflammatory process may starts in the peritoneum and is followed by Treg cell migration to inflammatory sites such as joins, kidneys and lungs [24] [32] [72]. Therefore, the current study cannot completely rule out possible causes of the differences in cell numbers found in PL and spleen which may result from a variety of possibilities such as cell migration, destruction, impaired production, etc. In fact, we have previously shown that pristane induced lupus animals exhibited increased

mesangial cell proliferation in glomerulus, increased IgG levels and proteinuria [24], reproducing lupus nephritis. Moreover, Kluger (2016) observed multipotent Treg cells expressing FoxP3 with proinflammatory properties in the peritoneum of SLE-induced mice 3 weeks after induction, indicating the participation of these cells at the beginning of lipogranuloma formation [35]. Similarly, in lupus patients, the number of CD4+CD25+FoxP3+ peripheral Treg cells is also reduced, showing dysregulation of peripheral tolerance [73] [19] [74] [40] [60] [10]. In addition, some studies have revealed that the reduction in these cells may be inversely correlated with disease activity, although correlation with SLEDAI was not significant [18]. In the B/WF1 murine model, the percentage of CD4+CD25+FoxP3+ Treg cells in peripheral blood, spleen and lymph nodes [73] [68] [20] [39] [62] is reduced with time compared to Balb/c [68] [27]. In MRL mice, the expression of Treg cells in the blood and peripheral lymphoid organs is similar to that in Balb/c [64] and its suppressive capacity is normal. Interestingly, in our model, the percentage of Treg cells in the blood was negatively correlated with TNFα and positively correlated with TGFβ1; no correlation between Treg cells and IL-2 and IL-10 was observed. Thus, higher titers of TNFα [57] may play an important role in SLE development and could be responsible for an increased proinflammatory response especially in active disease [57] [75]. TGFβ1 promotes the development of peripheral Treg cells, and curiously, IL-2, produced by activated T cells [72], may regulate Treg proliferation that contributes to homeostasis and maintenance of Treg suppressive capacity [65], which suggests a negative feedback. While IL-2 is generally considered to promote T-cell proliferation and enhance effector T-cell function, recent studies have demonstrated that treatments that utilize low-dose IL-2 unexpectedly induce immune tolerance and promote Treg development [76]. However, that capture of IL-2 was dispensable for the control of CD4+ T cells but was important for limiting the activation of CD8+ T cells [72]. This may explain our data, however, overall, our findings may not have power enough to rule out such hypothesis.

Additional experiments including immunohistochemical, immunophenotyping and functional assays in order to evaluate Treg cells suppressor activity and migration of are currently being conducted for better understanding about the mouse model capabilities and deficiencies.

Conclusion

In conclusion, our study has shown for the first time, higher expression of CD4$^+$CD69$^+$ T cells and reductions in CD4$^+$CD25$^+$FoxP3$^+$ Treg cells with altered interleukin profiles in pristane-induced SLE mice peripheral blood, spleen and PL, suggesting loss of autotolerance and a homeostatic immune imbalance similar to human SLE. Therefore, this easily reproducible experimental low cost model may help to generate new knowledge concerning cellular immune defects related to human lupus as well as the study of future therapies capable of reestablishing the immune homeostatic balance.

Abbreviations

Ab: Antibody; ANA: Anti-nuclear antibody; BALB/c: a mouse strain; CD: "Cluster of differentiation"; DMSO: dimethyl sulfoxide; ELISA: enzyme-linked immunosorbent assay; FACS: fluorescence-activated cell sorter; FBS: fetal bovine serum; h: hour; H&E: hematoxylin and eosin; i.p: intraperitoneal; IFN: interferon (e.g., IFN-γ); IL: interleukin (e.g., IL-2); mAb: monoclonal Ab; min: minute; PBMC: peripheral blood mononuclear cell; PL: peritoneal lavage; PLMC: peritoneal lavage mononuclear cell; PMN: polymorphonuclear cell; rpm: revolutions per minute; RPMI: (usually RPMI 1640); SD: standard deviation; SLE: Systemic Lupus Erythematosus; TGF: transforming growth factor; TNF: tumor necrosis factor; wk: week; μg: microgram; μl: microliter

Authors' contributions

TV P conceived the presented idea, designed and carried out the experiments, interpreting the results and writing the manuscript with input from all authors, who pro provided critical feedback and helped shape the research. SC carried out the experiments and aided in interpreting the results, working and commenting on the manuscript. DA C Botte aided in interpreting the results, working and commenting on the manuscript. SC, TM L and NU carried out the experiments. ERP, pathologist involved in the interpretation of histological slide results. FGS and SBV de M were involved in planning and supervising the work. CM. Rodrigues proofread the manuscript. CG-S contributed to the interpretations of the results and supervised this work.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Approved by the Institutional Animal Care and Research Advisory Committee (CAPPesq HC-USP Protocol # 009/11 - Comissão de Ética para Análise de Projetos de Pesquisa do Hospital das Clínicas da Faculdade de Medicina da USP).
Consent to participate not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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