doi: 10.1111/sji.12592

The Lymphocytes Stimulation Induced DNA Release, a Phenomenon Similar to NETosis

Y. C. Rocha Arrieta*, M. Rojas†,‡, G. Vasquez‡ & J. Lopez*

Abstract

The release of DNA into the extracellular milieu by neutrophil during a process called NETosis has been postulated as an additional source of autoantigens; a process believed to be important in the pathogenesis of some autoimmune disease, such as systemic lupus erythematosus (SLE). However, it is not established if the B and T cells undergo the release of DNA to the extracellular milleu, in response to different stimuli. In this study, it was observed that the treatment of B and T cells with PMA, ionomycin and the serum from patients with SLE induced the extracellular DNA presence in B and T cells. These findings suggest that the phenomenon were similar to those observed in neutrophil's Etosis; B and T cells also released their DNA into the extracellular milieu. The findings express that serum from patients with SLE and SLEDAI ≤ 8 triggers the release of extracellular DNA in neutrophils, B and T cells, that suggested the presence of soluble factors in the serum that favoured this phenomenon.

*Grupo de Inmunodeficiencias Primarias/Lab 530-531, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia; †Unidad de Citometría/Lab 420, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia; and ‡Grupo de Inmunología Celular e Inmunogenética/Lab 510-511, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia

Received 5 February 2017; Accepted in revised form 30 July 2017

Correspondence to: Y. C. Rocha Arrieta, Grupo de Inmunodeficiencias Primarias/Lab 530-531, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia. E-mail: yermis248@gmail.com

Introduction

Neutrophil extracellular traps (NETs) are structures formed by a mixture of DNA with histones, cytoplasmic and granular proteins that are released into the extracellular milieu. This allows the neutrophil to trap different microorganisms as effector mechanisms. It has been reported that eosinophils, basophils, mast cells, monocytes and macrophages also release DNA in response to different activating stimuli, as part of a process known as ETosis [1–4]. However, whether it may occur in T and B cells in response to activation is not known.

Actually, it is considered that NETs may contribute to autoantigens and neo-antigens; especially when these structures are not completely eliminated, as it is considered to occur in systemic lupus erythematosus (SLE) [5–7].

Previous *in vitro* studies have shown that the neutrophils from patients with SLE release NETs in the absence of any stimuli, favouring the exposure to autoantigens that may undergo further oxidative changes and their antigenicity, endothelial damage and IFN-α production by pDCs [8–12]. In addition to this excessive production of NETs, Hakkim *et al.* [5] proved that a group of patients with SLE had a lowered capacity to degrade NETs, while developing lupus nephritis to a higher proportion. In addition, Leffler *et al.* [6] showed that patients who did not degrade NETs had a more active disease and presented lower level of complement C4 and C3, in serum.

The NETs releases have been observed in the absence of infectious processes, suggesting that the soluble factors in patients' serum triggered it [8, 10]. Furthermore, elevated proportion of apoptotic cells, defects in their clearance and release of NETs have been reported in SLE [7, 13]. For these reasons, it was considered relevant to know if the serum induced DNA release into the extracellular milieu is related to cell death events or not. This could provide insights about the increase in the antigenic charge and lost of the immunological tolerance, which do not necessarily entail death cell.

This study evaluated the presence of DNA extracellular in neutrophils, B and T cells in response to activating stimuli, including serum from patients with SLE. It was observed that PMA, ionomycin, anti-IgM + LPS and serum from patients with SLE and SLEDAI ≤ 8 induced the presence of DNA into the extracellular milieu in neutrophils, B and T cells. In lymphocytes, it also resulted to a decline in phosphatidylserine exposure and an increase in cell membrane damage. However, in T lymphocytes, a higher $DIOC_6$ uptake through mitochondrial membrane was observed. Conversely, serum with SLEDAI > 8

SCANDINAVIAN JOURNAL OF Immunology AN INTERNATIONAL MONTHLY JOURNAL

Table 1 Demographic and clinical characteristics of patients and controls..

	Patients			
	Healthy controls	SLE with SLEDAI ≤8	SLE with SLEDAI >8	OAD
n	22	28	18	5
Ago, median (IQR)	27 (25–29)	26 (20–36)	25 (20–28)	37.5 (19–49)
Sex, F/M	11/11	25/3	15/3	4/1
Treatment [frequency (%), dose]				
Prednisolone	–	79, 12.5 mg/days (5.8–21)[a]	94, 50 mg/days (32.5–57.5)[a]	–
Chloroquine	–	64, 168 mg/days[b]	56,188 mg/days[b]	–
Methylprednisolone	–	7, 500 mg[b]	33, 564 mg[b]	–
Cyclophosphamide	–	14, 950 mg (600–1000)[a]	50, 725 mg (650–850)[a]	–
Others	–	50	39	–

The patients with SLE were classified into two groups based on the SLEDAI. One group consisted of patients who had a SLEDAI ≤8 (n = 28) and another one who had a SLEDAI >8 (n = 18). The group of patients with OAD, was composed of patients with rheumatoid arthritis (n = 1), granulomatosis with polyangiitis (n = 1), Sjögren syndrome (n = 1), dermatomyositis (n = 1) and cryoglobulinemic vasculitis (n = 1). The median and interquartile ranges are showed for age. The frequency (%) and the dose of the medications that patients with SLE received are showed. Prednisolone and chloroquine were the most used drugs in the two groups of patients.

[a] Median and interquartile range.

[b] Mean.

triggered the presence of extracellular DNA in lower proportions in the three cell populations evaluated; also B and T lymphocytes had increased cell membrane damage, established by the increased propidium iodide staining.

Materials and methods

Reagents. Ficoll-Hypaque, RPMI 1640 medium, PMA (Phorbol 12-myristate 13-acetate), ionomycin, lipopolysaccharide from Escherichia coli 0111:B4, DPI, modified Dulbecco's phosphate-buffered saline (DPBS) and cyclosporine A were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Penicillin/streptomycin, Sytox Green, Hoecsht, DNase I, RNase A, $DIOC_6$ (3.3′- dihexyloxacarbocyanine iodide), Polymorphprep and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The RosetteSep™ B cell enrichment cocktail was acquired at Stem Cell Technologies (Vancouver, Canada). The anti-CD3 (clones UCHT1 and SK7), anti-CD45 (clone HI30), anti-CD19 (cloneSJ25C1), anti-CD14 (clone M5G2) and anti-CD16 (clone B73.1) antibodies were purchased from BD Biosciences (San Diego, CA, USA).

The TACS annexin V–FITC apoptosis detection kit was acquired from R&D Systems (Minneapolis, MN), and the Goat F(ab') 2 Fragment antibody to human IgM was obtained from MP Biomedicals (Solon, OH, USA).

Patients and controls. Forty-six serum samples from patients with SLE were processed (Table 1). The patients were diagnosed at the Rheumatology Service in University Hospital San Vicente Foundation, Medellin-Colombia, according to the criteria established by the American College of Rheumatology in 1982 [14]. Two groups of patients were established according to Systemic Lupus Erythematosus Disease Activity Index (SLEDAI): patients with mild to moderate SLE (SLEDAI ≤ 8) and those with active SLE (SLEDAI > 8).

The therapeutic regimen included prednisolone, chloroquine (that was the most used among all groups of patients), azathioprine, methotrexate, mycophenolate mofetil, methylprednisolone and cyclophosphamide (Table 1).

As a control, the serum from five patients, with other autoimmune diseases (OAD) that had similar treatment with SLE patients, was included. Also, neutrophils, B and T cells, were isolated from 22 healthy controls (HCs). Serum samples from HCs were employed to prepare pool of human serum (NHS).

The exclusion criteria for the selection of patients were infections, pregnancy, diabetes or neoplastic processes. The HCs have no history of autoimmune diseases, diabetes or cancer. All patients and HCs signed an informed consent form. According to the Declaration of Helsinki, the Medical Research Ethics Committee, from the School of Medicine University of Antioquia, approved this form.

Isolation of lymphocytes and neutrophils. Peripheral blood samples were obtained from healthy controls (Table 1) and collected in EDTA tubes. B cells were isolated through negative selection, using a RosetteSep™ B cell enrichment kits according to the manufacturer's instructions. The purity of B cells was assessed by flow cytometry, using anti-CD45, anti-CD19, anti-CD3, anti-CD14, and anti-CD16 antibodies on a FACSCanto (BD Biosciences, San Jose, CA, USA). The purity of B cells was 81% on average.

T cells were separated by sorting them from PBMC in a Moflo XDP (Beckman Coulter, Brea, CA, USA), using anti-CD45 and anti-CD3 antibodies. The purity and efficiency were 99% and ≥95% on average, respectively. To sum up, neutrophils were isolated, using Polymorphprep according to manufacturer's instructions. Neutrophils were left to adhere for 30 min at 37 °C and 5% CO_2, before the stimulation.

Stimulation of neutrophils, B and T cells, using different activating agents. The neutrophils, B and T cells were obtained from HCs. The B cells (2×10^5) were stimulated with PMA (0.2 μg/ml or 0.5 μg/ml), ionomycin (5 or 10 μg/ml) or anti-IgM (12.5 μg/ml) + LPS (20 μg/ml). On the other hand, the T cells (2×10^5) were stimulated with ionomycin (5 μg/ml) for 18 h.

The neutrophils, B and T cells, were stimulated during 4 h (neutrophils) or 18 h (lymphocytes), to a final concentration of 10% of serum from patients with SLEDAI ≤ 8 or >8, 10% from the NHS and 10% for serum from patients with OAD. The release of DNA was assessed using Sytox Green in the fluorescence microscopy and semi-quantified in the spectrofluorometer. The presence of extracellular DNA was detected by fluorescence microscopy (Olympus CKX41 and Carl Zeiss Axio Vert A1), using Sytox Green (0.25 μM), Hoecsht (3 μg/ml) or propidium iodide (1 μg/ml) for DNA staining, and semi-quantified by a spectrofluorometer (SpectraMax Gemini, Molecular Device) using 96-well cell culture plates with dark walls (Greiner Bio-one, Frickenhausen, Germany). A scan of 9 points per well was performed at an excitation wavelength of 485 nm, emission of 535 nm. The DNA was detected with Sytox Green (2.5 μM). The results were reported as relative fluorescence units (RFU).

For data normalization, RFUs detected in the serum samples were subtracted from the RFUs detected in treated cells. The experiments were performed in triplicate. Only neutrophils were fixed with 4% paraformaldehyde for 20 min, at room temperature, and it was observed with fluorescence microscopy, after incubation time.

The resulting data in fluorescence microscopy and spectrofluorometry were analysed using the single-blind technique; in which the demographic and clinical backgrounds of SLE patients that provided the serum samples were unknown to the analyst.

Assessment of events associated with cell death. Events associated to cell death were assessed in B and T cells to determine whether these were related with the release of DNA into the extracellular milieu. The cells were treated for 18 h, using serum from patients with SLEDAI ≤ 8 or >8. Accordingly, 2×10^5 cells were cultured in 200 μl of RPMI (final volume) supplemented with 2% penicillin/streptomycin in 12 × 75-mm polystyrene tubes (Falcon, Tewksbury, MA, USA). After the incubation time, phosphatidylserine exposure and alteration of plasma membrane permeability were evaluated, using the apoptosis kit according to the manufacturer's instructions. Also, 2×10^5 of the T or B cells was incubated briefly in the dark for 20 min, using 50 μl of Annexin V incubation reagent and propidium iodide (PI, 2 μl). After the incubation time, measurements were made with a FACSCanto. The percentage of Annexin V^+ cells corresponded to the sum of Annexin V^+PI^- and Annexin V^+PI^+ cells.

Alteration in mitochondrial function was assessed with $DIOC_6$ (230 nM) and PI (0.4 μg/mL), using a mixture of $DIOC_6$, PI and DPBS. Staining was performed in the dark within 20 min. Lastly, the measurements were made with a FACS Canto cytometer. The mean fluorescence intensity (MFI) for $DIOC_6$ was determined in the cell population, $DIOC_6^+$ PI^-.

Statistical analysis. GraphPad Prism, version 6, (GraphPad Software, Inc, San Diego, CA, USA) was used for the statistical analysis. Comparisons among the groups were made with a nonparametric, one-tailed, Wilcoxon test, ran in paired samples. The value of $P < 0.05$ was considered significant.

Results

Demographic and clinical characteristics of serum sources

The demographic characteristics of patients and healthy controls used as serum sources are shown in Table 1. The age range among the patients and healthy controls ($P > 0.05$) was comparable. For patient with SLEDAI ≤ 8, the median age was 26 years (interquartile range (IQR) 20–36); patients with SLEDAI > 8, 25 years (IQR: 20–28); and healthy controls, 27 years (IQR: 25–29). For patients with OAD, the median age was 37.5 years (IQR 19–49). In all groups of patients (SLE and OAD), the major subjects were women, in a proportion of 7:1.

Activating Stimuli and Serum from patients with SLE induced the release of DNA into the extracellular milieu in neutrophils and lymphocytes

The presence of DNA extracellular in B and T cells was found, after 18 h stimulation with PMA (0.2 μg/ml or 5 μg/ml) or ionomycin (5 μg/ml or 10 μg/ml) (Fig. 1). The presence of extracellular DNA was observed too with the more physiological stimuli such as anti-Ig M (12.5 μg/ml) + LPS (20 μg/ml) used to stimulate B cells (Fig. 1). The presence of extracellular DNA was verified through treatment with DNase I and staining with other DNA intercalating agents (data not shown); confirming the presence of DNA in the structures extracellularly observed with Sytox Green.

Evaluation was carried out to determine whether the serum of SLE patients was able to induce the release of DNA by neutrophils, B and T cells, from healthy individuals.

When neutrophils from healthy individuals were treated with serum from lupus patients with SLEDAI ≤ 8 for a period of 4 h, the presence of extracellular DNA was evidenced through the increase in RFUs ($P < 0.005$), by spectrofluorometry and visualization using fluorescence microscopy (Fig. 2). In serum with SLEDAI > 8, there was not any increase in the RFUs or detection with the

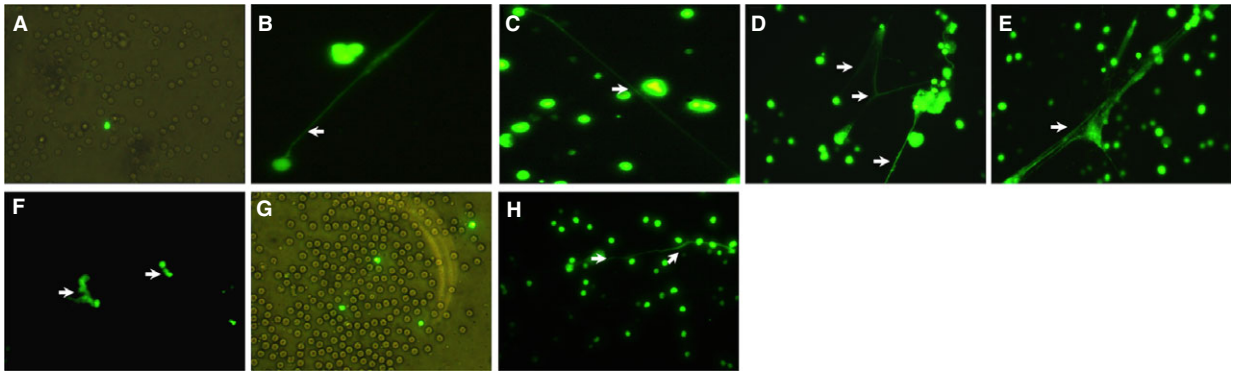

Figure 1 B and T cells release DNA into the extracellular milieu. Representative bright field and fluorescence field images of B and T cells obtained from healthy individuals. B cells (2×10^5) unstimulated (A) or stimulated with PMA 0.2 μg/ml (B) or 5 μg/ml (C) or ionomycin 5 μg/ml (D) or 10 μg/ml (E) and anti-lg M (12.5 μg/ml) + LPS (20 μg/ml) (F). T cells (2×10^5) unstimulated (G) or stimulated with ionomycin (5 μg/ml) (H). Original magnification 40×. White arrows indicate the structures compatible with extracellular DNA, which was stained with Sytox Green. Experiments were performed in triplicate.

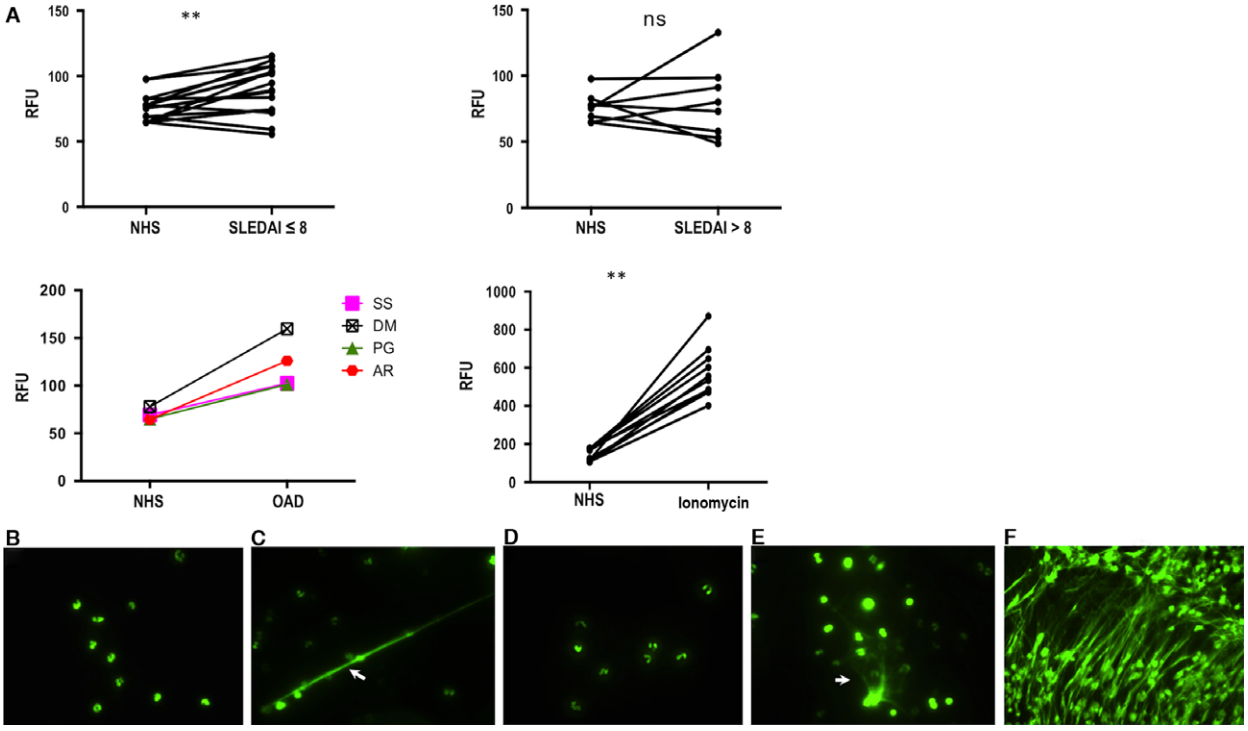

Figure 2 Serum from patients with SLE induces presence of NETs. (A) Neutrophils were stimulated with serum from patients with SLEDAI ≤ 8 (n = 16) or SLEDAI > 8 (n = 8), with serum from patients with OAD (n = 4) or ionomycin (5 μg/ml) during 4 h. Experiments were performed in triplicate and expressed as RFUs. Representative fluorescence images are shown for neutrophils treated with NHS (B), serum with SLEDAI ≤ 8 (C), serum with SLEDAI > 8 (D), serum from patient with OAD (E) or ionomycin (5 μg/ml) (F). Original magnification 63×. White arrows indicate the structures compatible with extracellular DNA. Wilcoxon test $^{**}P < 0.005$. $^{*}P < 0.05$. ns: not significant.

microscope (Fig. 2). The treatment of these cells with serum from patients with OAD induced RFUs increase, and the presence of structures similar to those of NETs was observed in the serum of patients with SLEDAI ≤ 8 (Fig. 2).

The effects of sera from patients with SLE in B and T cells were also evaluated. The serum from patients with SLEDAI ≤ 8 significantly increased RFUs ($P < 0.005$) and induced the presence of extracellular DNA in B cells, in a similar way to what was observed in neutrophils (Fig. 3). Despite the serum of patients with SLEDAI > 8 increased the RFUs ($P < 0.05$) compared to NHS, extracellular DNA was not detected by fluorescence microscopy in most of them (Fig. 3).

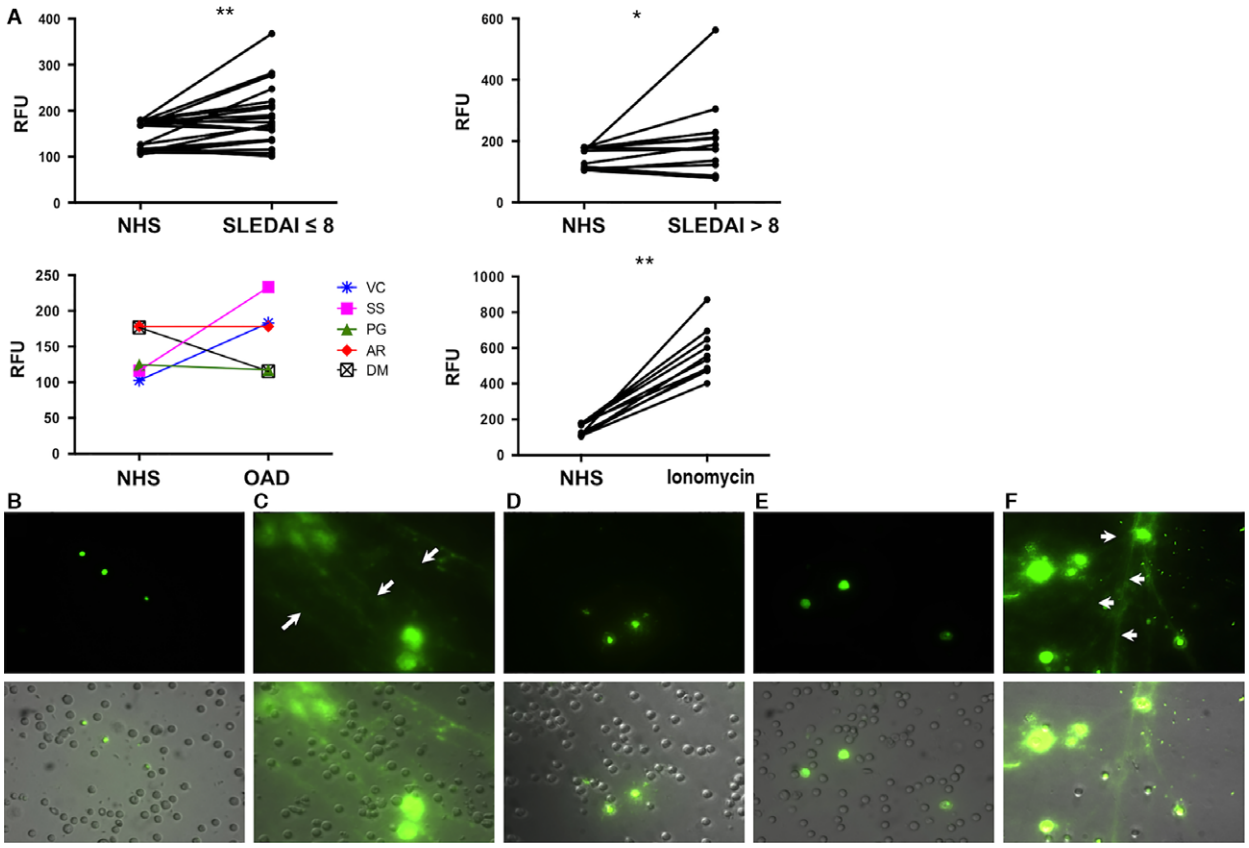

Figure 3 The serum from patients with SLE induces presence of extracellular DNA in B cells. (A) B cells were stimulated with serum from patients with SLEDAI ≤ 8 (n = 20) or SLEDAI > 8 (n = 13), with serum from patients with OAD (n = 5) or ionomycin (10 μg/ml) during 18 h. Experiments were performed in triplicate. Representative bright field and fluorescence field images for B cells treated with: NHS (B), serum with SLEDAI ≤ 8 (C), serum with SLEDAI>8 (D), serum from patient with OAD(E) or ionomycin (10 μg/ml) (F). Original magnification 63×. White arrows indicate the structures compatible with extracellular DNA. Wilcoxon test $^{**}P < 0.005$. $^{*}P < 0.05$. ns: not significant.

Neither the presence of extracellular DNA nor the increase in RFUs was observable, when peripheral blood B cells were treated with the serum from patients with OAD (RA: rheumatoid arthritis, GPA: granulomatosis with polyangitis and DM: dermatomyositis). However, the presence of extracellular DNA and increase in RFUs was observed, when the mentioned cells were treated with serum from patients with Sjögren syndrome (SS) and cryoglobulinaemia, as well as from one subject with cryoglobulinaemic vasculitis (CV) (Fig. 3 and data not shown).

When T cells were stimulated with the serum from patients with SLE and SLEDAI ≤ 8, the DNA extracellular was also observed; whereas, the phenomena were not evident in most of the cells treated with serum from patients with SLEDAI > 8 (Fig. 4). The treatment with serum from patients with OAD (RA, GPA, DM and SS) induced the release of the DNA into the extracellular milieu, in similar amounts to those observed with NHS. Nevertheless, a greater presence of extracellular DNA per evaluated field was observed, when cells were treated with the serum from the patient with CV (Fig. 4 and data not show).

Together, all these findings suggest that soluble factors present in serum of lupus patients with SLEDAI ≤ 8 induced the presence of structures similar to NETs in the three types of cells evaluated. However, patients with SLEDAI > 8 do not favour its occurrence. Also, the results showed that patients with large amounts of immune complexes, such as those with SS or CV, induced the phenomenon in B cells. Only the serum from patient with CV managed to do so in T lymphocytes; contrary to obtained results in the case of neutrophils, in which the serum of all patients with OAD induced release of DNA.

Serum from patients with SLE induces increased DIOC6 uptake without phosphatidylserine exposure, in B and T lymphocytes

To confirm that the release of DNA observed in the previous assays was not due to a type of death cell, we evaluated several events associated with cell death in sorted B and T cells, in response to serum from lupus patients stimulated during 18 h with serum from patients with SLE and SLEDAI ≤ 8 or >8.

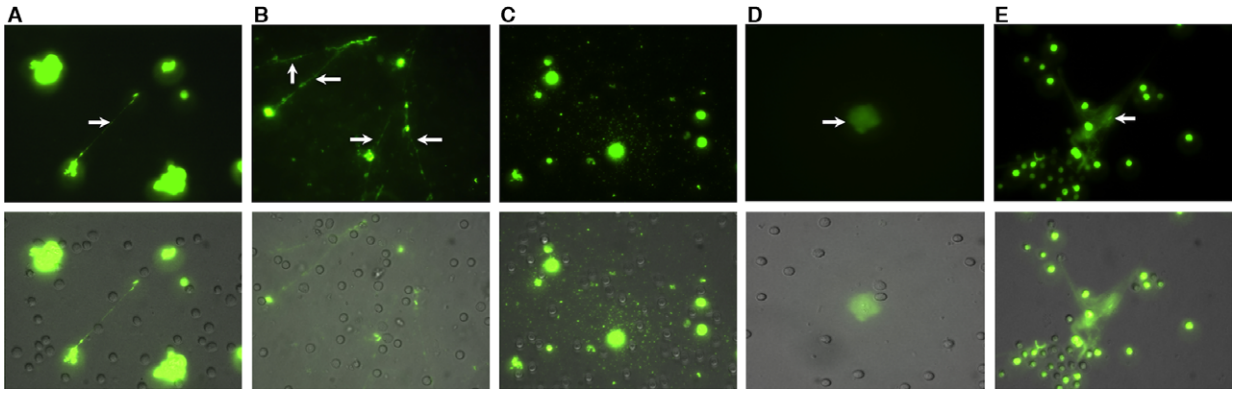

Figure 4 The serum from patients with SLE induces presence of extracellular DNA in T cells. Representative bright field and fluorescence field images of T cells. These cells were treated with NHS (A), serum with SLEDAI ≤8 (B), serum with SLEDAI >8 (C), serum from patient with OAD (D) or ionomycin (5 μg/ml) (E) are showed. Original magnification 63×. White arrows indicate the structures compatible with extracellular DNA.

It was found that the treatment of B and T cells with NHS, induced phosphatidylserine exposure (Annexin V^+ PI^- and V^+ PI^+ cells) in a 13.1% (IQR: 5.1–13.7%) and 3.4% (IQR: 2.4–4.4%), respectively, but when the cells were treated with serum from patients with SLEDAI ≤ 8, the percentage of cells with phosphatidylserine exposure was 6.9% (IQR: 4–10%) for B cells and 2.1% (IQR: 1.4–3.7%) for T cells. Such decrease was statistically significant in both cases ($P < 0.05$, Figs. 5 and 6). Likewise, serum from patients with SLEDAI ≤ 8 increased propidium iodide-positive cells (Annexin V^- PI^+ cells) 2.1% (IQR: 0.9–4.6%) for B cells compared to treatment with NHS, which resulted in 1% (IQR: 0.7–1.1%) and 1.6% (IQR: 0.7–2%) for T cells, compared to treatment with NHS which was of 0.5% (IQR: 0.4–0.7%) ($P < 0.005$, Figs. 5 and 6).

Furthermore, the treatment with serum for patients with SLEDAI ≤ 8 in T cells caused a higher uptake of $DIOC_6$ by the mitochondrial membrane, with a median for the MFI of 16364 (IQR: 13830–21765 MFI). When compared to the treatment with NHS, the median for the MFI was 10306 (IQR: 9,583–14,677 MFI) ($P < 0.05$, Fig. 6).

The treatment with serum from patients with SLEDAI > 8 only increased in B and T cells incorporation of propidium iodide ($P < 0.05$) 2.1% (IQR: 1.7–2.5%) and 0.4% (IQR: 0.4–2%) respectively, compared to the treatment with NHS. Other events associated with cell death such as phosphatidylserine exposure and alteration in mitochondrial membrane potential did not result in a statistically significant effect, when compared to the treatments with NHS (Figs. 5 and 6).

Such findings suggest that serum from lupus patients with SLEDAI ≤ 8 can modulate events associated with cell activation and/or cell death, which have not necessarily explained the release of DNA into the extracellular milieu observed.

Discussion

ETosis has been reported in basophils, eosinophils, mast cells, monocytes and macrophages, but not in lymphocytes [2, 4, 15]. However, this study reports the release of DNA into the extracellular milieu by peripheral blood B and T lymphocytes, in response to the stimulation with PMA, ionomycin and serum from patients with autoimmune disease.

The treatment with anti-IgM + LPS, which resembles a physiological stimulation of B cells, also induced the release of DNA into the extracellular milieu, being a clear indication of the phenomenon occurrence *in vivo.*

This study did not evaluate proteins associated with DNA; however, it is important that subsequent studies evaluate the composition of the DNA released into the extracellular milieu by B and T cells. The detection of proteins such as histones or citrullination of the same will permit exclusion of other forms of cell death, where there is DNA release.

Otherwise, the semiquantification or quantification of the release of DNA, made by spectrofluorometry, must be carefully analysed; excluding other types of cell death that allow the release of the DNA. For this reason, the integrity of the DNA released by peripheral blood B cells was evaluated, using agarose gel electrophoresis (data not shown). In this case, it was observed that the extracellular DNA detected after stimulation with ionomycin had a high molecular weight, which had not suffered random or internucleosomal fragmentation as occurs in necrosis or apoptosis, respectively [16]. This DNA presented a similar pattern (high molecular weight and no fragmentation) to that observed previously in the literature for NETs [15, 17].

The spontaneous NETosis described in SLE suggests that soluble factors (autoantibodies, immune complex, cytokines and complement factors) could be inducing the phenomenon. This work reports that the serum from patients with SLEDAI ≤ 8 induced the release of DNA, by

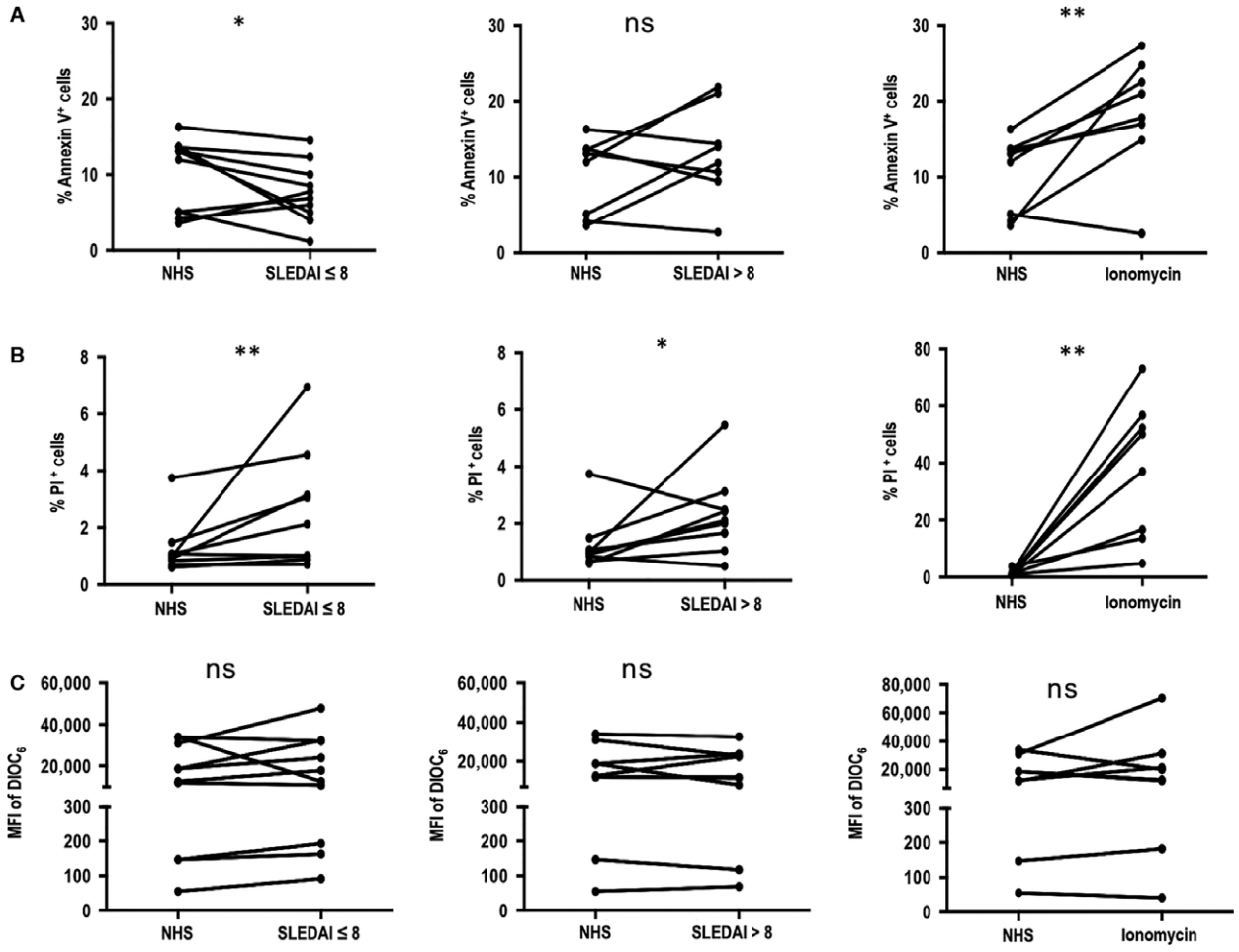

Figure 5 Serum from patients with SLE induced in B cells decreases in phosphatidylserine exposure and increase of propidium iodide positive cells. B cells were stimulated with serum from patients with SLE with SLEDAI ≤8 (n = 10), SLEDAI >8 (n = 9) or ionomycin (5 μg/ml) during 18 h. (A) Percentage of cells positive for annexin V (annexin V^+ IP^- + annexin V^+ IP^+). (B) Percentage of cells positive for IP (annexin V^- IP^+). (C) MFI of DiOC6 (DiOC6 $^+$ IP^-). Wilcoxon test $^{**}P < 0.005$. $^{*}P < 0.05$. ns: not significant.

peripheral blood neutrophils, B and T cells from healthy controls. Even though the soluble factors responsible for such phenomenon were not evaluated, previous studies have shown that antibodies (anti-LL37, anti-HNP and anti-RNP), cytokines (IFN-α, IFN-γ and TNF-α), immune complexes and complement proteins enable the release of NETs. Such evidence could provide an explanation to the results in this study, at least in neutrophils [12, 18–20].

With regard to B cells, there is evidence considering that immune complexes can induce FcγRIIB activation, which is registered as a signal of cellular stress in the absence of BCR-specific antigen binding [21, 22]. Immune complexes could also mediate BCR cross-linking and induce downstream signalling pathways, which allow release of DNA. In such sense, it may prove convenient to assess whether a similar mechanism affected the B cells from healthy controls, in which FcγRIIB or BCR activation could have been important in the release of DNA into the extracellular milieu.

The presence of extracellular DNA was detected in T cells in response to NHS treatment or with the serum from patients with OAD (RA, GPA, SS and DM), but greater release was noted in the case of the serum from lupus patients with SLEDAI ≤ 8 or patients with CV. This suggests that soluble factors in the serum pool of healthy controls and, to higher levels, in patients with SLE and OAD were responsible for the release of DNA by T cells. However, the phenomenon was not observable in T cells treated with FBS.

When the serum from OAD was evaluated, NETs were released from neutrophils stimulated with the serum from patients with RA, GPA and DM in agreement with previous reports in the literature. [23–25]. The release of extracellular DNA caused by the serum from a patient with SS was observed in neutrophils too. The patients with these diseases also presented autoantibodies against ribonucleoproteínas, increased levels of IFN type I and circulating immune complexes [26]; therefore, the possibility of their role in this results should not be discarded.

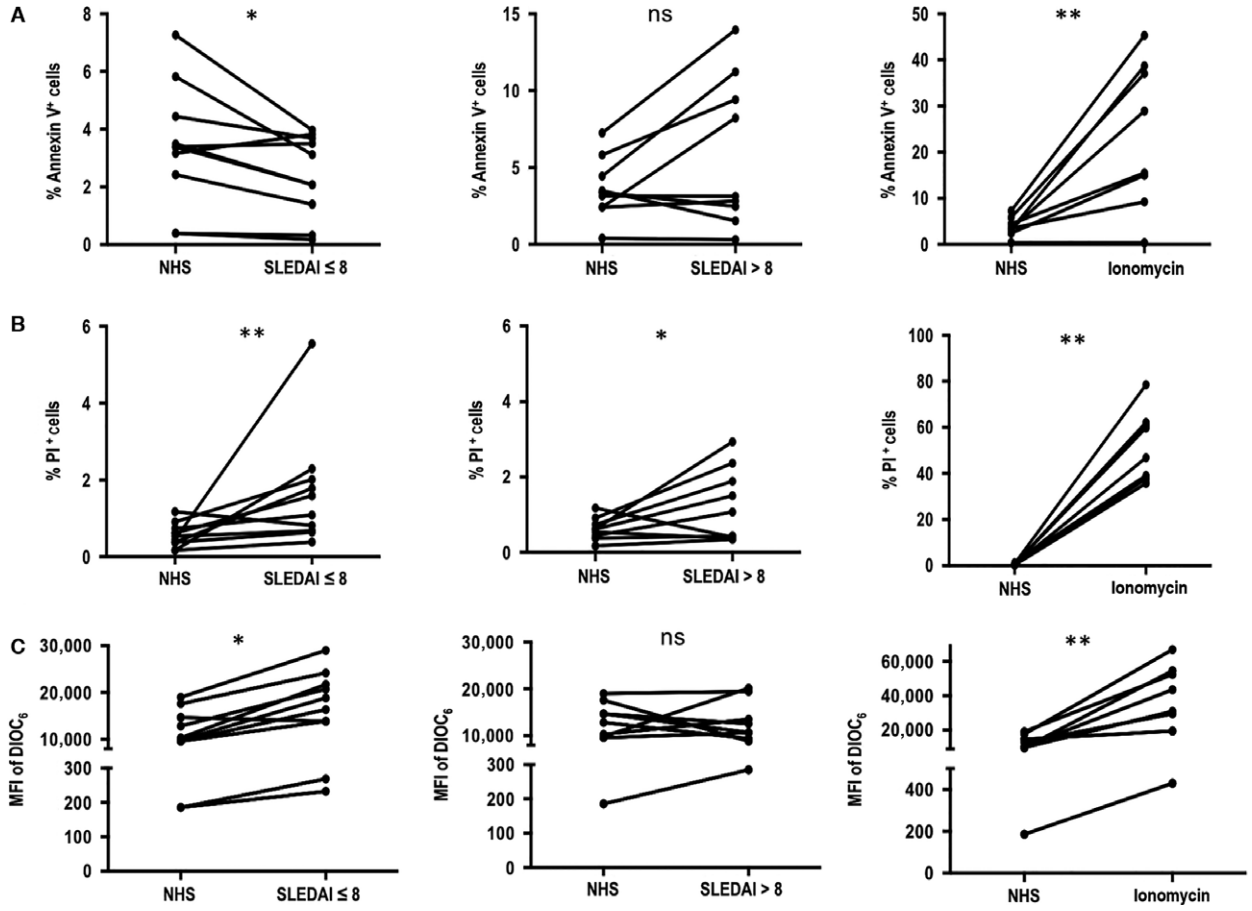

Figure 6 Serum from patients with SLE induced in T cells decrease in phosphatidylserine exposure, increase of propidium iodide positive cells and increase of mitochondrial membrane potential. T cells were stimulated with serum from patients with SLE with SLEDAI ≤8 (n = 10) or SLEDAI >8 (n = 9) or ionomycin (5 μg/ml) during 18 h. (A) Percentage of cells positive for annexin V (annexin V^+ IP^- + annexin V^+ IP^+). (B) Percentage of cells positive for IP (annexin V^- IP^+). (C) MFI of DiOC6 (DiOC6 $^+$ IP^-). Wilcoxon test $^{**}P < 0.005$.$^*P < 0.05$. ns: not significant.

In B and T cells, the release of extracellular DNA was observed with the stimulation with serum from a patient with primary SS and cryoglobulinaemia, and another with CV, similar to occurred with serum from patients with SLEDAI ≤ 8. This suggests that the presence of immune complexes containing antibodies against DNA or nuclear antigens may be one of those responsible for the phenomenon in B cells.

In contrast to the finding in B cells, only the serum from the patient with CV induced the release of DNA into the extracellular milieu, using T cells. This suggests that besides the immune complexes, other soluble mediators are important for the occurrence of the phenomenon in T cells, or that the release of DNA is not influenced solely by immune complexes.

These findings also led us to suggest that autoantibodies, immune complexes and pro-inflammatory cytokines present in other autoimmune diseases could induce release extracellular of DNA, which perpetuates autoantibody formation cycles, inflammatory response and tissue damage.

Previous works have shown that immune complexes are able to induce NETs production dependent of Fcγ receptors. For this reason, it was expected that serum from patients with SLE and SLEDAI > 8 overwhelmingly favoured the phenomenon, at least in neutrophils, due to its abundance of circulating immune complexes [24, 27, 28]. However, isolated immune complexes from patients were not employed in this study.

Therefore, it should not be overlooked that the presence of other soluble mediators or high doses of immunosuppressive agents that patients with SLE receive may somehow affect the release of DNA [29–32]. Thus, it would be convenient to evaluate whether the differences observed in the release of DNA into the extracellular milieu, among groups with SLEDAI ≤ 8 or >8, can be explained due to the drug treatment. This means that with the highest dose of pharmacological agents, the expression of soluble factors responsible for NETs release from the immune cells is considerably reduced [33].

The most commonly used immunosuppressants for the treatment of SLE are corticosteroids, such as prednisolone.

In a study by Fyfe *et al.* [29], it was shown that prednisolone affects the rearrangement of the cytoskeleton in porcine endothelial cells. It cannot be ruled out that the leucocytes could have a similar effect and alter the rearrangement of microtubules and actin cytoskeleton, which have been described as important for the release of NETs [34]. In addition, glucocorticoids may decrease or inhibit calcium release. Besides, it has been shown that an efficient induction of NETosis requires mobilization of calcium from the intracellular and extracellular reservoir, highlighting the role of calcium in the release of NETs. Drugs that alter the calcium mobilization in neutrophils, B and T cells could have important implications for regulating Etosis [30–32].

Previous reports in the literature show that chloroquine is able to inhibit autophagy, because it induces an increase in intralysosomal pH and decreases the degradation of proteins. Autophagy is an event that allows the chromatin unfolding for subsequent NETs release [35].

In this sense, it would be convenient to evaluate whether the high doses of drugs that the patients received and the differential expression of soluble factors cells derivatives could explain the differences observed in the release of DNA into the extracellular milieu, between the SLEDAI ≤ 8 and >8 groups.

Previous studies have shown that serum from patients with SLE induced apoptosis in PBMC of healthy individuals, independent of whether the disease is active or not [36, 37]. Nonetheless, in this study, the serum from patients with SLEDAI ≤ 8 induced a decrease in the apoptosis of B and T cells, in comparison with those treated with NHS.

It is then possible to suppose that one reason for the reduction in apoptosis cell percentage in B and T cells induced by the serum from patients with SLEDAI ≤ 8 could be the presence of survival factors in these patients' serum, as well as BAFF, IL-4, IL-15 and IL-17 [38–41].

Additionally, in T cells, an increased uptake of $DIOC_6$ induced by serum from patients with SLEDAI ≤ 8 was demonstrated; this suggests an increase in mitochondrial membrane potential (MMP). Mitochondrial hyperpolarization has been described in very early stages of apoptosis preceding phosphatidylserine exposure, which is indicative of cell activation and/or oxidative stress [42, 43]. On the contrary, decreased phosphatidylserine exposure was observed. Therefore, the serum from patients with SLE may be activating another type of death or cellular mechanism in T cells.

If findings on the induction of DNA release into the extracellular milieu are taken into account, it could be suggested that the soluble factors in lupus patients with SLEDAI ≤ 8 reduced apoptosis, caused mitochondrial hyperpolarization and induced the release of DNA into the extracellular milieu. However, it is not possible to say this DNA release was the reason for the increase in the cell membrane damages observed. This is because, the extracellular release of DNA did not always coincide with membrane damage and it was also a common event to both serum groups. The findings suggest that the release of DNA into the extracellular milieu can be vital, because it was not related, in any way, to the cell death markers evaluated in this study. However, it cannot be excluded that similar to what happens in neutrophils, a lytic release of DNA into the extracellular milieu leading to cell death can occur.

Based on the results of this study, it can be proposed that the release of DNA into the extracellular milieu by neutrophils B and T cells, which was induced by the serum from patients with SLEDAI ≤ 8, can provide an explanation for the persistence of the disease because: extracellular DNA can be a constant source of autoantigens for autoreactive B cells, treatment of pDCs, monocytes and macrophages using extracellular DNA obtained from lymphocytes cultures that can induce production of IFN-α and pro-inflammatory cytokines (IL-1β, IL-18, IFN-γ, TNF-α) *in vivo*, which would translate into SLE exacerbation [11, 44]. Also, extracellular DNA released by lymphocytes could bind to C1q, directly or through the autoantibodies already attached to it, which would generate complement activation and consumption aggravating the inflammatory response [6]; and finally, in the case of NETs, it has also been proven that they can induce a pre-activation in T lymphocytes, by reducing its activation threshold and increasing the antigen-specific response even under suboptimal doses [45]. Whether a similar case occurs in the presence of extracellular DNA released by lymphocytes will be determined. The T lymphocytes pre-activation would intensify the immune response in SLE.

This study describes the presence of extracellular DNA in neutrophils, B and T cells induced by serum from patients with autoimmune disease, especially SLE, which could have a pathogenic potential and may help to explain the disease chronicity. Whether immune complexes, cytokines and/or complement factors are responsible for this phenomenon and whether the treatment that patients receive can influence the process should be subjected to further studies.

References

1 Brinkmann V, Reichard U, Goosmann C *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532–5.
2 Guimaraes-Costa AB, Nascimento MT, Wardini AB, Pinto-da-Silva LH, Saraiva EM. ETosis: a microbicidal mechanism beyond cell death. *J Parasitol Res* 2012;2012:929743.
3 Bosch X. Systemic lupus erythematosus and the neutrophil. *N Eng J Med* 2011;365:758–60.
4 Schorn C, Janko C, Latzko M, Chaurio R, Schett G, Herrmann M. Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front Immunol* 2012;3:277.
5 Hakkim A, Furnrohr BG, Amann K *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA* 2010;107:9813–8.

6 Leffler J, Martin M, Gullstrand B *et al.* Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol* 2012;188:3522–31.
7 Pieterse E, van der Vlag J. Breaking immunological tolerance in systemic lupus erythematosus. *Front Immunol* 2014;5:164.
8 Villanueva E, Yalavarthi S, Berthier CC *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 2011;187:538–52.
9 Denny MF, Yalavarthi S, Zhao W *et al.* A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol* 2010;184:3284–97.
10 Knight JS, Kaplan MJ. Lupus neutrophils: 'NET' gain in understanding lupus pathogenesis. *Curr Opin Rheumatol* 2012;24:441–50.
11 Garcia-Romo GS, Caielli S, Vega B *et al.* Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra20.
12 Lande R, Ganguly D, Facchinetti V *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra19.
13 Shao WH, Cohen PL. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. *Arthritis Res Ther* 2011;13:202.
14 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271–7.
15 Fuchs TA, Abed U, Goosmann C *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176:231–41.
16 Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun* 2005;73:1907–16.
17 Farrera C, Fadeel B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* 2013;191:2647–56.
18 Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* 2009;16:1438–44.
19 Cheng OZ, Palaniyar N. NET balancing: a problem in inflammatory lung diseases. *Front Immunol* 2013;4:1.
20 Dorner T. SLE in 2011: deciphering the role of NETs and networks in SLE. *Nat Rev Rheumatol* 2012;8:68–70.
21 Pearse RN, Kawabe T, Bolland S, Guinamard R, Kurosaki T, Ravetch JV. SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis. *Immunity* 1999;10:753–60.
22 Tzeng SJ, Bolland S, Inabe K, Kurosaki T, Pierce SK. The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway. *J Biol Chem* 2005;280:35247–54.
23 Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A *et al.* NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013;5:178ra40.
24 Kessenbrock K, Krumbholz M, Schonermarck U *et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009;15:623–5.
25 Zhang S, Shu X, Tian X, Chen F, Lu X, Wang G. Enhanced formation and impaired degradation of neutrophil extracellular traps in dermatomyositis and polymyositis: a potential contributor to interstitial lung disease complications. *Clin Exp Immunol* 2014;177:134–41.
26 Yao Y, Liu Z, Jallal B, Shen N, Ronnblom L. Type I interferons in Sjogren's syndrome. *Autoimmun Rev* 2013;12:558–66.
27 Chen K, Nishi H, Travers R *et al.* Endocytosis of soluble immune complexes leads to their clearance by FcgammaRIIIB but induces neutrophil extracellular traps via FcgammaRIIA in vivo. *Blood* 2012;120:4421–31.
28 Behnen M, Leschczyk C, Moller S *et al.* Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J Immunol* 2014;193:1954–65.
29 Fyfe AI, Rosenthal A, Gotlieb AI. Immunosuppressive agents and endothelial repair. Prednisolone delays migration and cytoskeletal rearrangement in wounded porcine aortic monolayers. *Arterioscler Thromb Vasc Biol* 1995;15:1166–71.
30 Passaquin AC, Lhote P, Ruegg UT. Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells. *Br J Pharmacol* 1998;124:1751–9.
31 Harr MW, McColl KS, Zhong F, Molitoris JK, Distelhorst CW. Glucocorticoids downregulate Fyn and inhibit IP(3)-mediated calcium signaling to promote autophagy in T lymphocytes. *Autophagy* 2010;6:912–21.
32 Gupta AK, Giaglis S, Hasler P, Hahn S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One* 2014;9:e97088.
33 Thomas SS, Clements PJ, Furst DE. Immunosuppressives: cyclosporine, cyclophosphamide, azathioprine, mycophenolate mofetil. In: Hochberg M, Silman A, Smolen J, Weinblatt ME, Weisman MH, eds. *Rheumatology*. Philadelphia, PA: Elsevier, 2015: 459–67. ISBN: 978-0-323-09138-1
34 Neeli I, Dwivedi N, Khan S, Radic M. Regulation of extracellular chromatin release from neutrophils. *J Innate Immun* 2009;1:194–201.
35 Amaravadi RK, Yu D, Lum JJ *et al.* Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Investig* 2007;117:326–36.
36 Klint C, Truedsson L, Andreasson A, Johansson I, Sturfelt G. Toxic effects of SLE serum on normal monocytes in vitro: cell death induced by apoptosis related to complement dysfunction. *Lupus* 2000;9:278–87.
37 Bengtsson AA, Sturfelt G, Gullstrand B, Truedsson L. Induction of apoptosis in monocytes and lymphocytes by serum from patients with systemic lupus erythematosus - an additional mechanism to increased autoantigen load? *Clin Exp Immunol* 2004;135:535–43.
38 Davidson A. The rationale for BAFF inhibition in systemic lupus erythematosus. *Curr Rheumatol Rep* 2012;14:295–302.
39 Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus* 2000;9:589–93.
40 Graninger WB, Steiner CW, Graninger MT, Aringer M, Smolen JS. Cytokine regulation of apoptosis and Bcl-2 expression in lymphocytes of patients with systemic lupus erythematosus. *Cell Death Differ* 2000;7:966–72.
41 Aringer M, Stummvoll GH, Steiner G *et al.* Serum interleukin-15 is elevated in systemic lupus erythematosus. *Rheumatology* 2001;40:876–81.
42 Gottlieb E, Armour SM, Harris MH, Thompson CB. Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ* 2003;10:709–17.
43 Matarrese P, Gambardella L, Cassone A, Vella S, Cauda R, Malorni W. Mitochondrial membrane hyperpolarization hijacks activated T lymphocytes toward the apoptotic-prone phenotype: homeostatic mechanisms of HIV protease inhibitors. *J Immunol* 2003;170:6006–15.
44 Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. *J Immunol* 2013;190:1217–26.
45 Tillack K, Breiden P, Martin R, Sospedra M. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol* 2012;188:3150–9.