Interferon-α Abrogates the Suppressive Effect of Apoptotic Cells on Dendritic Cells in an *In Vitro* Model of Systemic Lupus Erythematosus Pathogenesis

Lucie Abeler-Dörner, Cosima C. Rieger, Bartlomiej Berger, Heiko Weyd, Daniela Gräf, Sandra Pfrang, Ingo H. Tarner, Andreas Schwarting, Hanns-Martin Lorenz, Ulf Müller-Ladner, Peter H. Krammer, and Annegret Kuhn

ABSTRACT. ***Objective.*** An increased incidence of apoptotic cells and an increased activation of dendritic cells (DC) may be involved in the pathogenesis of systemic lupus erythematosus (SLE). We investigated the characteristics of apoptotic neutrophils and monocyte-derived DC of patients with SLE, their interaction, and the influence of autoantibodies and inflammatory cytokines on this interaction.

Methods. Kinetics of neutrophil apoptosis and DC activation were studied by flow cytometry. To analyze the interaction of apoptotic cells with phagocytes, crossover coculture experiments were performed with DC from patients with SLE and apoptotic Jurkat T cells as well as with apoptotic neutrophils from patients with SLE and the monocytic cell line U937. SLE serum and cytokines were added to this coculture, and activation and suppression of DC were quantified by levels of inflammatory cytokine secretion.

Results. Apoptotic neutrophils and DC from patients with SLE showed no inherent defects compared to healthy controls, and the suppressive nature of their interaction was not affected. Autoantibodies as well as the inflammatory cytokines interleukin 17 (IL-17) and IL-1β had no influence on the interaction in this setup. Interferon (IFN)-α, however, substantially reduced the suppressive effect of apoptotic cells on DC.

Conclusion. The data suggest that aberrant immune reactivity in SLE is not generally due to an intrinsic defect in apoptotic cells, their processing, or their interaction with DC, but likely arises from the milieu in which this interaction takes place. Our study highlights the importance of IFN-α during early stages of SLE and its potential as a therapeutic target. (First Release Sept 15 2013; J Rheumatol 2013;40:1683–96; doi:10.3899/jrheum.121299)

Key Indexing Terms:
AUTOIMMUNE DISEASES | SYSTEMIC LUPUS ERYTHEMATOSUS | APOPTOSIS
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Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease with a broad spectrum of clinical manifestations. Patients are diagnosed with SLE if 4 or more of the classification criteria of the American College of Rheumatology (ACR) are fulfilled[1,2]. The pathogenesis of the disease is still not well understood, and mostly has genetic and environmental components[3,4,5]. Although classical immunosuppressive agents have accounted for a significant improvement in survival and have slowed the progression to endstage renal failure in SLE, they lack selectivity for the underlying immune dysregulation[6]. Drug resistance and relapses after treatment, as well as side effects, are of major concern and reduce the quality of everyday life for many patients[7,8]. Therefore, understanding

From the Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, Heidelberg; Department of Rheumatology and Clinical Immunology, University of Giessen, Giessen; Internal Medicine I, Division of Rheumatology, University of Mainz, Mainz; Internal Medicine V, Division of Rheumatology, University of Heidelberg, Heidelberg; Department of Dermatology, University of Münster, Münster, Germany.

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L. Abeler-Dörner, PhD, Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, currently at Peter Gorer Department of Immunobiology, King's College, London, UK; C.C. Rieger, PhD; B. Berger; H. Weyd, PhD; D. Gräf; S. Pfrang, Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center; I.H. Tarner, MD, Department of Rheumatology and Clinical Immunology, University of Giessen; A. Schwarting, MD, Internal Medicine I, Division of Rheumatology, University of Mainz; H-M. Lorenz, MD, Internal Medicine V, Division of Rheumatology, University of Heidelberg; U. Müller-Ladner, MD, Department of Rheumatology and Clinical Immunology, University of Giessen; P.H. Krammer, MD, Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center; A. Kuhn, MD, Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, and Department of Dermatology, University of Münster.

L. Abeler-Dörner, C. Rieger, B. Berger, and H. Weyd contributed equally to this work.

Address correspondence to Dr. A. Kuhn, Department of Dermatology, University of Münster, Von-Esmarch-Strasse 58, D-48149 Münster, Germany. E-mail: kuhnan@uni-muenster.de

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the inherent factors contributing to this disease might lead to development of more specific pharmacological agents for the treatment of SLE in the future.

In SLE patients with active disease, many cells of the immune system are hyperactivated or impaired in numbers, and the serum contains proinflammatory cytokines such as interferon (IFN)-α and interleukin 17 (IL)-17 as well as autoreactive antibodies[9,10,11]. It is, however, not clear which of the observed effects are causative and which appear secondarily. One of the earliest symptoms and hallmarks of SLE is the presence of autoantibodies against dsDNA or other intracellular antigens such as Ro/SSA, La/SSB, snRNP, and Sm, which appear on the surface of apoptotic cells after exposure to UV irradiation[12]. The presence of autoantibodies against epitopes from apoptotic cells and the observation of increased numbers of apoptotic cells in the tissue of patients with SLE[13,14] have supported the hypothesis that a defect in the clearance of apoptotic cells leads to the activation of the immune system by unscavenged material[15] and that therapy directed toward augmenting clearance and decreasing concomitant inflammation may result in improved management of the disease[16,17].

Under normal circumstances, the uptake of apoptotic cells is an immunologically silent event leading to the suppression of proinflammatory cytokine secretion by the phagocyte rather than its activation[18]. Phagocytes that have taken up apoptotic material can migrate to the lymph node,[19] where the presentation of self-material in a nondanger context silences potentially autoreactive T cells, thus maintaining the tolerance toward self-antigens in the periphery[20].

In our study, we focused on the question of whether dendritic cells (DC) and apoptotic neutrophils of patients with SLE differ inherently from cells of healthy controls in their interaction with the other cell types and if this interaction is further influenced by the surrounding autoimmune milieu. In particular, we aimed to determine whether the interaction between DC and apoptotic cells in patients with SLE can initiate a breakdown of self-tolerance or whether the abnormalities reported for DC activation and uptake of apoptotic cells by phagocytes were secondary effects caused by autoantibodies and inflammatory cytokines present at the site of interaction. To this end, we established an *in vitro* coculture system in which the function of phagocytes and apoptotic cells can be studied separately. Inherent defects in neutrophil apoptosis, DC activation, or the interaction of the 2 cell types have not been observed, but we found that the presence of IFN-α significantly reduces the suppressive effect of apoptotic cells on DC. These results demonstrated that one of the mechanisms to ensure the safe disposal of apoptotic cells is substantially reduced in an SLE-relevant milieu *in vitro* and stressed the important role IFN-α has been shown to play in the pathogenesis of SLE and related autoimmune conditions[11,21].

MATERIALS AND METHODS

Patients. In our study, 19 patients (18 women and 1 man; mean age ± SD 39.1 ± 13.6 yrs) who fulfilled at least 4 of the revised ACR criteria for SLE[1] and who were positive for autoantibodies against dsDNA were included from the Department of Rheumatology and Clinical Immunology, University of Giessen; the Department of Internal Medicine I, Division of Rheumatology, University of Mainz; and the Department of Internal Medicine V, Division of Rheumatology, University of Heidelberg, Germany (Table 1). The disease activity of the patients with SLE was assessed using the European Consensus Lupus Activity Measurement[22]. Seventeen patients were treated with antimalarials and/or low (≤ 10 mg/day), medium (12.5-25.0 mg/day), or high (> 50 mg/day) doses of prednisolone and/or immunosuppressive drugs (azathioprine or mycophenolate mofetil). Nineteen sex-matched and age-matched healthy individuals served as a control population. The study was approved by the ethics committees of the universities of Giessen, Mainz, and Heidelberg, and was conducted according to the ethical guidelines at the institutions as well as the Helsinki Declaration.

Cell sample preparation. Primary human neutrophils were freshly isolated from peripheral blood by Polymorphprep (Axis Shield) according to the manufacturer's instructions and used in an *in vitro* coculture system to analyze the influence of apoptotic cells on the immune response initiated by DC in SLE. Isolated cells were seeded at 2 million cells/ml in RPMI 1640/10% fetal calf serum (FCS)/1% glutamine (Gibco BRL). A common and well-established protocol was used for the differentiation of primary human monocyte-derived DC[23]. Cells were isolated from the lymphocyte fraction of the Polymorphprep by magnetic cell sorting using anti-CD14 microbeads, according to the manufacturer's instructions (Miltenyi). Obtained monocytes were differentiated into immature DC by culturing for 7 days in 6-well plates (Falcon, BD Pharmingen) in DC medium [RPMI 1640 medium supplemented with 10% FCS, 1% glutamine, 1000 u/ml recombinant granulocyte-macrophage colony-stimulating factor (Leukine, Schering) and 500 u/ml recombinant IL-4 (Immunotools)]. Cytokines were replenished after 3 days of culture.

Flow cytometric analysis. Early apoptosis was assessed by staining neutrophils with annexin V (Immunotools), antiannexin A1 antibody (DAC5)[24], and propidium iodide in annexin V staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). DNA fragmentation was analyzed as described by Nicoletti, *et al*[25]. For surface receptor analysis, DC were preincubated in 10% normal mouse serum (Dako) and stained for expression of CD83, CD86, and HLA-DR (BD Pharmingen). To assess binding of autoantibodies, apoptotic cells incubated with serum were washed and stained with an FITC-labeled antibody directed against human immunoglobulin G (Jackson Immunoresearch).

Cellular assays. Primary human monocyte-derived DC (100,000/well) were seeded in 48-well plates (Greiner) and incubated with 200,000 to 500,000 apoptotic Jurkat T cells for 3-4 h (final assay volume 750 μl). After the preincubation, the DC were stimulated with lipopolysaccharide (LPS; *Escherichia coli*, Sigma Aldrich), and secretion of tumor necrosis factor (TNF) analyzed by ELISA as a measure of DC activation 18 h after stimulation, according to the manufacturer's protocol (BD Pharmingen). For induction of apoptosis, Jurkat T cells were irradiated with 50 mJ/cm^2 UV-C in a Stratalinker 1800 (Stratagene) 2 h before addition to the phagocytes. For assays involving the addition of cytokines, the apoptotic cells were centrifuged for 10 min at 300 × g before addition to the assay. Cytokines were added to the DC at a final concentration of 1000 u/ml immediately prior to the addition of apoptotic cells. For coculture assays involving addition of cytokines, the content of serum in DC medium was reduced to 1% human AB serum.

Phorbol myristate acetate (PMA; 10 mg/ml) was used for 4 days to differentiate U937 cells. The adherent differentiated U937 cells were detached with Trypsin/EDTA (Gibco BRL) and 100,000 cells were seeded in 48-well plates (Greiner) and incubated at 37°C/5% CO_2 for 2 h. Medium was removed from adherent cells and 500,000 apoptotic Jurkat T cells

Table 1. Characteristics of patients with systemic lupus erythematosus (SLE) at the time of the study.

Patient No.	Diagnosis	Age, yrs	Sex	Serological Features	Treatment	ECLAM
1	SLE	33	F	ANA (no titer indicated), dsDNA	Antimalarials	5
2	SLE	21	F	ANA (1:1280), dsDNA	IS, medium steroids	2
3	SLE	39	F	ANA (1:320), dsDNA	—	1
4	SLE	18	F	ANA (1:10240), dsDNA, Ro/SSA, La/SSB	IS, medium steroids	3
5	SLE	74	F	ANA (1:20480), dsDNA, Ro/SSA	IS, low steroids	3
6	SLE	58	F	ANA (1:2560), dsDNA	IS, low steroids	1
7	SLE	62	F	ANA (1:40000), RNP	Antimalarials, low steroids	3
8	SLE	42	F	ANA (1:5120), dsDNA, Ro/SSA	IS, low steroids	2
9	SLE	39	F	ANA (1:2560), dsDNA, ACA, La/SSB	IS, antimalarials, low steroids	3.5
10	SLE	35	F	ANA (1:1280), dsDNA	Antimalarials, low steroids	2.5
11	SLE	43	M	ANA (no titer indicated), dsDNA, RNP	IS, medium steroids	1
12	SLE	27	F	ANA (1:5120), dsDNA, Ro/SSA, La/SSB	IS, antimalarials	0
13	SLE	37	F	ANA (1:2560), dsDNA	—	1
14	SLE	42	F	ANA (1:640), dsDNA	IS, low steroids	3
15	SLE	45	F	ANA (1:320), dsDNA	IS	2
16	SLE	32	F	ANA (no titer indicated), dsDNA	Antimalarials, low steroids	0.5
17	SLE	30	F	ANA (1:10240), dsDNA	High steroids	2
18	SLE	24	F	ANA (1:1280), dsDNA	Antimalarials, low steroids	1.5
19	SLE	42	F	ANA (1:20000), dsDNA	Antimalarials, low steroids	0

ACA: anticardiolipin antibodies; ANA: antinuclear antibodies (HEp-2 cells); IS: immunosuppressive agents; ECLAM: European Consensus Lupus Activity Measure.

(irradiated as above) or primary early apoptotic d1 neutrophils were added (final assay volume 500 μl). For assays including serum, serum from patients with SLE and healthy controls was dialyzed against a 100 kDa cutoff to isolate the containing antibodies and remove serum proteins and cytokines. Apoptotic Jurkat T cells (irradiated as above) were incubated with the dialyzed serum for 20 min and then added to U937 cells. After 4 h of preincubation with the apoptotic cells, the U937 cells were stimulated with LPS, and TNF secretion was measured as described.

Statistical analysis. Data are presented as the median or mean. Statistical significance was determined using test 2-tailed Mann-Whitney U test for unpaired samples or tobit regression analysis. A p value < 0.05 was regarded as statistically significant. Analyses were performed using the software GraphPad Prism 4 (GraphPad Software Inc.) or Stata 11.1 (StataCorp).

RESULTS

Neutrophils from patients with SLE display apoptosis kinetics comparable to neutrophils from controls. To investigate whether the kinetics of apoptotic cell death are impaired in cells of all or a subgroup of patients with SLE, we used freshly isolated primary human neutrophils as a well-established model system for apoptosis kinetics[24]. As hallmarks of apoptosis, we monitored translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane, the appearance of DNA fragments of subgenomic size, and the loss of membrane integrity. In addition, we monitored the surface exposure of annexin A1, a marker of early apoptotic cells, which partly mediates the suppressive effect of apoptotic cells on DC[24]. Neutrophils from patients with SLE and controls started to expose PS on Day 1 after isolation and reached a maximum after 2 to 3 days. This mirrors the biology of apoptosis, because PS exposure is an early event that is likely to have reached full effect by Day 1 (Figure 1A; median SLE vs controls, mean fluorescence intensity d0: 480 vs 897, d1: 3125 vs 2413, d2: 2589 vs 3003, d3: 2276 vs 3884). The median value of PS expression on Day 0 up to Day 3 of culture was not significantly different between patients with SLE and controls. The observed pattern was confirmed by evaluating the percentage of subgenomic DNA produced by apoptotic DNA cleavage (Figure 1B; median SLE vs controls, percent DNA fragmentation d0: 7.8 vs 7.8, d1: 51.3 vs 22.3, d2: 72.3 vs 74.0, d3: 85.1 vs 78.4). The continuous rise of subgenomic DNA content up to Day 3 reflects the known kinetics of apoptosis, because DNA degradation starts after PS exposure and continues. Loss of membrane integrity was observed in a minority of cells on Day 1 after isolation and was seen in the majority of cells on Day 3 (Figure 1C;

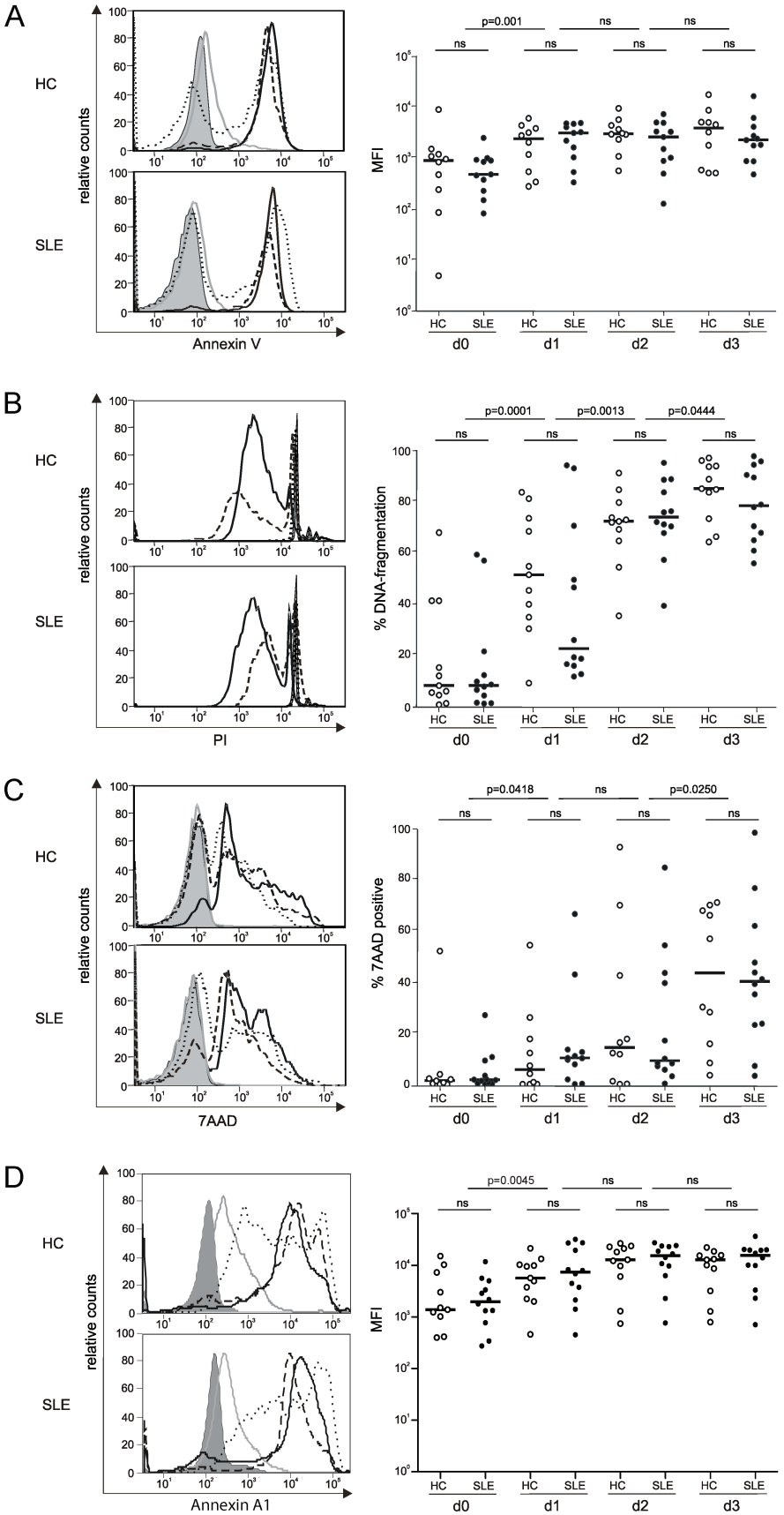

Figure 1. Apoptotic neutrophils from patients with systemic lupus erythematosus (SLE) display apoptosis kinetics comparable to neutrophils from healthy controls (HC). Left panels are representative experiments; right panels are cumulative data. A. Primary neutrophils from patients with SLE and controls were stained with FITC-labeled annexin V on 4 consecutive days after isolation and were analyzed by flow cytometry to monitor the onset of apoptosis. B. Nuclei of primary neutrophils were stained for DNA fragmentation by Nicoletti stain and analyzed by flow cytometry to quantify the appearance of subgenomic DNA characteristic for apoptosis. C. Primary neutrophils from patients with SLE and controls were stained with 7-AAD on 4 consecutive days after isolation and analyzed by flow cytometry to determine loss of membrane integrity as a late event of apoptosis. D. Primary neutrophils from patients with SLE and controls were stained with an FITC-labeled antibody against human annexin A1, a suppressive molecule on the surface of apoptotic cells, on 4 consecutive days after isolation and analyzed by flow cytometry. Filled gray lines: background; solid gray lines: d0; dotted lines: d1; dashed: d2; solid black: d3.

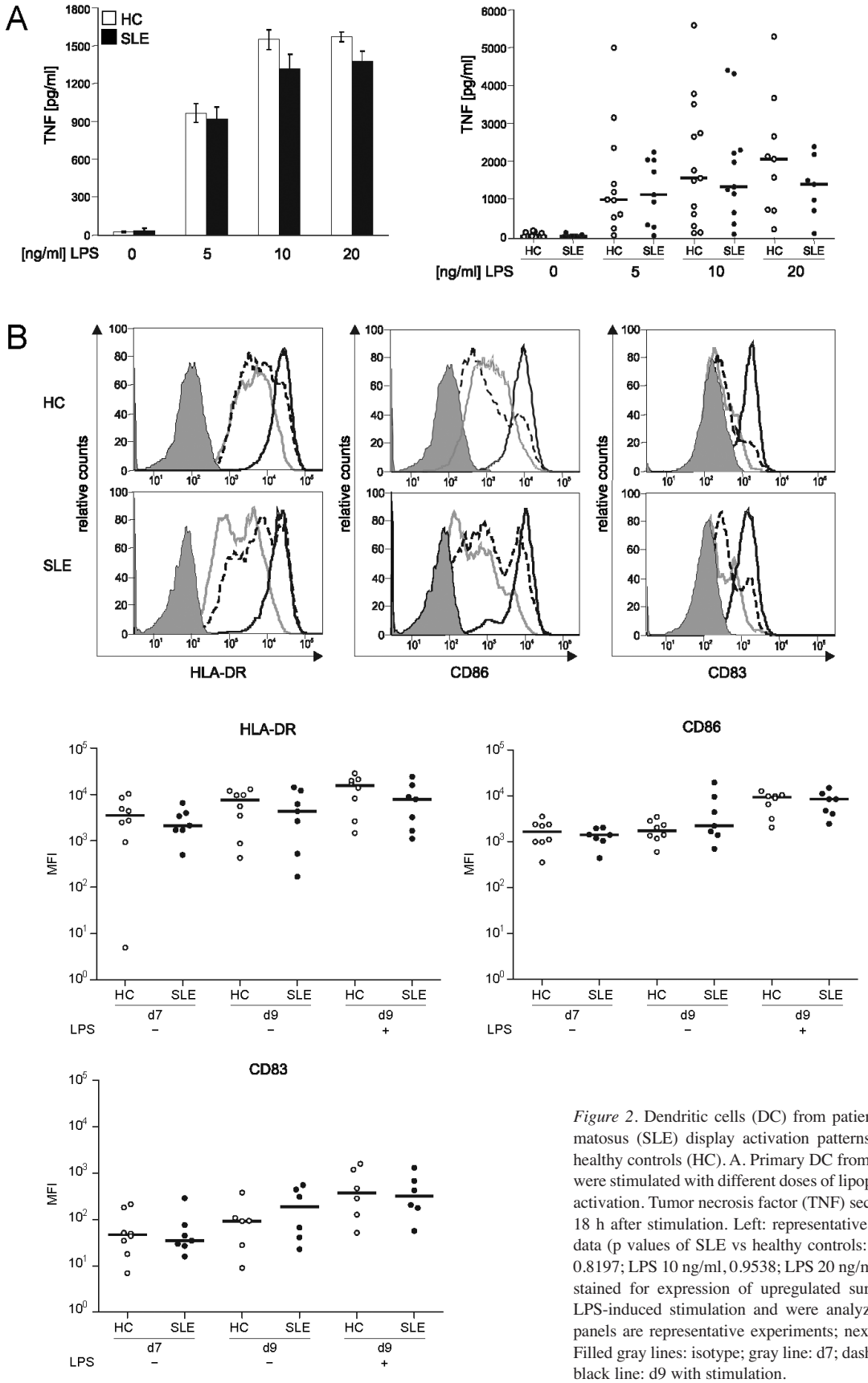

Figure 2. Dendritic cells (DC) from patients with systemic lupus erythematosus (SLE) display activation patterns similar to those of DC from healthy controls (HC). A. Primary DC from patients with SLE and controls were stimulated with different doses of lipopolysaccharide (LPS) to monitor activation. Tumor necrosis factor (TNF) secretion was measured by ELISA 18 h after stimulation. Left: representative experiments; right: cumulative data (p values of SLE vs healthy controls: no LPS, 0.3750; LPS 5 ng/ml, 0.8197; LPS 10 ng/ml, 0.9538; LPS 20 ng/ml, 0.3510). B. Primary DC were stained for expression of upregulated surface markers before and after LPS-induced stimulation and were analyzed by flow cytometry. First 3 panels are representative experiments; next 3 panels are cumulative data. Filled gray lines: isotype; gray line: d7; dashed line: d9 without stimulation; black line: d9 with stimulation.

median SLE vs controls, percent 7-AAD positive cells d0: 1.7 vs 1.7, d1: 7.3 vs 10.3, d2: 14.4 vs 16.0, d3: 43.7 vs 41.3). The exposure of annexin A1 mirrored the exposure of PS, because annexin A1 translocates from the inside to the outside of early apoptotic cells, where it binds to PS. There was no significant difference in annexin A1 exposure between apoptotic neutrophils from patients with SLE and controls. Slightly delayed kinetics of apoptosis in neutrophils from patients with SLE were observed throughout the experiment; however, because of the high donor variability observed in patients as well as in controls, this difference was not significant.

DC from patients with SLE displayed activation similar to DC from controls. DC are unique mediators of both tolerance and immunity, and aberrant DC function and activation can promote autoimmune responses through a number of mechanisms[26,27]. To study whether DC of patients with SLE are more easily activated than DC from controls, we investigated their capacity to be activated by Toll-like receptor (TLR) stimulation. We analyzed the secretion of several cytokines by DC upon stimulation with different TLR stimuli (Appendix 1A) and decided to use LPS stimulation with a readout of TNF for our patient samples because the signal is strong and reliable. In addition, TNF rises dose-dependently over a wide range of LPS concentrations and correlates well with secretion of other inflammatory cytokines, such as IL-12 and IL-6 (Figure 2A). In this setup, monocyte-derived DC from patients with SLE and controls exhibited a comparable dose-dependent secretion of TNF (Figure 2A; median SLE vs controls, LPS 5 ng/ml: 979 vs 1105; 10 ng/ml: 1318 vs 1548; 20 ng/ml: 1383 vs 2051). The levels of MHC II, CD86, and CD83 after LPS stimulation were also comparable between DC from patients with SLE and those of controls (Figure 2B). Overall, DC from patients with SLE were activated by LPS to the same extent as DC from controls.

DC from patients with SLE are inhibited by apoptotic cells to an extent comparable to DC from controls. Dying cells *in vivo* are rapidly taken up by professional phagocytes and surrounding tissue cells. The context of this uptake determines whether an immune response is or is not initiated[28,29]. A proinflammatory context is often exploited for tumor therapy[30,31]; however, during steady-state conditions *in vivo,* apoptotic cells have been shown to suppress the activation of DC[18,19]. We have analyzed this suppression in an *in vitro* system, taking care to generate apoptotic cells with suppressive properties. Under these conditions, DC preincubated with apoptotic cells showed a reduced activation upon subsequent stimulation with different TLR ligands[18] (Appendix 1A). To analyze the responsiveness of DC for suppressive signals from apoptotic cells, we cocultured monocyte-derived DC from patients with SLE and controls with apoptotic Jurkat T cells and compared their reactivity toward LPS (Figure 3A). When added to the phagocytes, the apoptotic Jurkat T cells were in an early stage of apoptosis, showing PS exposure but no loss of membrane integrity (Appendix 1B).

Coculture with apoptotic Jurkat T cells suppressed the LPS-induced TNF secretion of DC derived from patients with SLE and controls to about 40% (Figure 3A; median in patients with SLE vs controls, LPS 5 ng/ml: 56 vs 70; 10 ng/ml: 62 vs 71; 20 ng/ml: 91 vs 96). The efficacy of the suppression was especially prominent when low concentrations of LPS were used; however, there was no significant difference between DC from patients with SLE and controls in these settings.

Apoptotic neutrophils from patients with SLE inhibit monocytic U937 cells to an extent comparable to apoptotic neutrophils from controls. An inherent defect in suppressive mediators of apoptotic cells in SLE might lead to a reduced tolerance of DC and finally to autoimmunity. To study whether apoptotic cells from patients with SLE have an inherent defect in their ability to suppress phagocytes, we cocultured apoptotic neutrophils from patients with SLE and controls with monocytic U937 cells. These cells can be differentiated by PMA into cells resembling human phagocytes in terms of surface receptor expression and TNF secretion[32,33] (Appendix 2A). Like immature DC, differentiated U937 cells phagocytized apoptotic cells, dose-dependently secreted TNF in response to LPS stimulation (Appendix 2B), and showed a reduced activation when cocultured with apoptotic Jurkat T cells (Appendix 2C). To assess the suppressive capacity of apoptotic cells from patients with SLE, apoptotic neutrophils from patients and controls were cocultured with U937, and cocultures were subsequently stimulated with LPS. Apoptotic neutrophils from patients with SLE and controls suppressed the LPS-induced TNF secretion of U937 cells on average by 42% and 46%, respectively (Figure 3B). The degree of suppression was comparable to that of apoptotic Jurkat T cells (44%), which served as a control. A significant difference in the suppressive capacity of neutrophils from patients with SLE and controls could not be observed.

Autoantibodies from SLE serum bind to apoptotic cells but do not influence suppression of TNF secretion in the absence of complement. The serum of patients with SLE contains autoantibodies, nucleosomes from dying cells, and autoantibody-DNA complexes, which have been shown to promote the differentiation and activation of DC in this disease[11,34,35]. To test whether autoantibodies present in the serum of patients with SLE might influence the suppressive capacity of apoptotic cells, we added antibodies from sera of patients with SLE to our *in vitro* system. Patient serum was dialyzed using a 100 kDa cutoff membrane, thus removing other potentially active compounds such as cytokines and complement. Flow cytometric analysis revealed that antibodies from the sera of patients with SLE bound signifi-

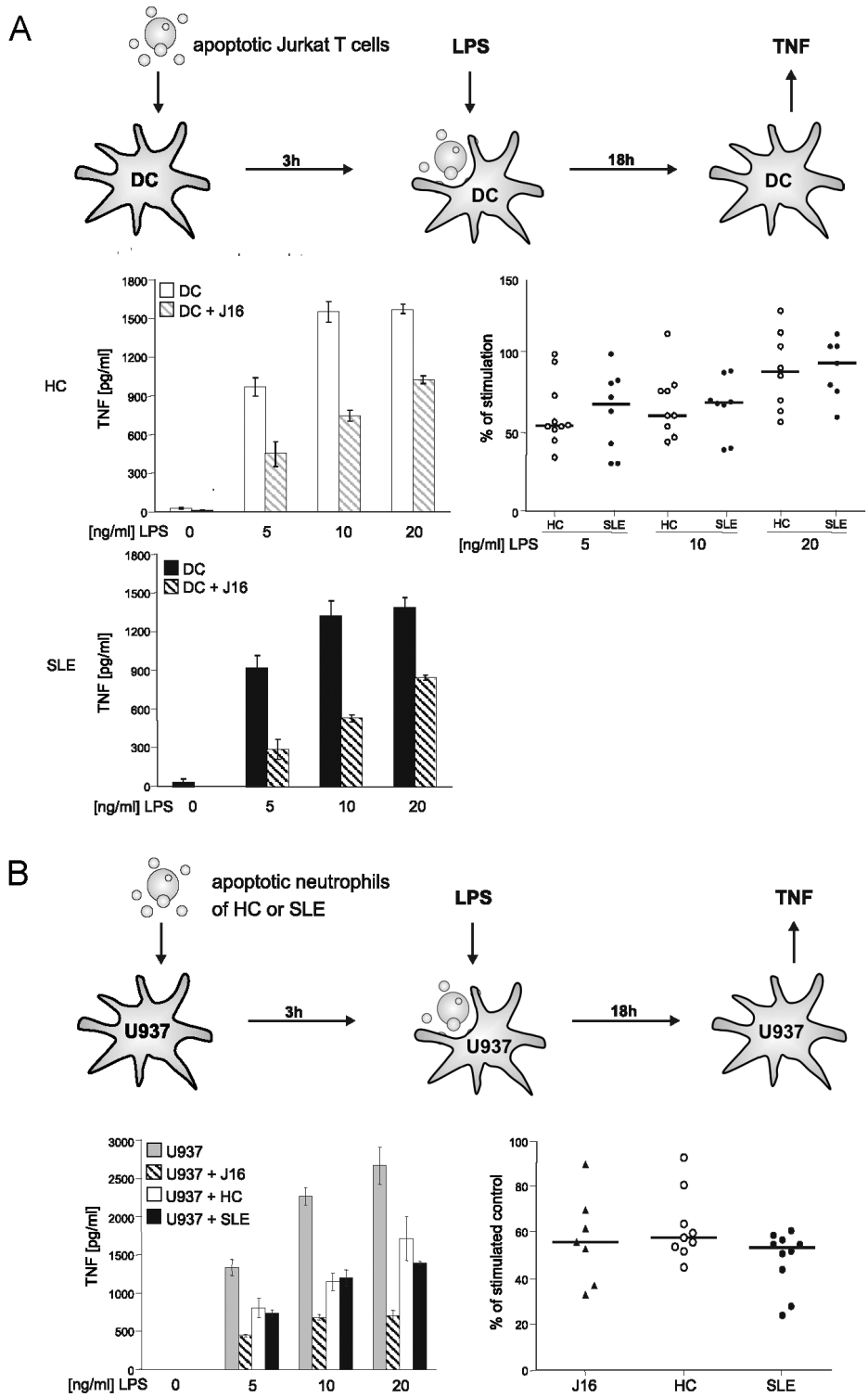

Figure 3. A. Dendritic cells (DC) from patients with systemic lupus erythematosus (SLE) are inhibited by apoptotic cells to an extent comparable to DC from healthy controls (HC). DC from patients with SLE or controls were cocultured with apoptotic Jurkat T cells for 3 h prior to lipopolysaccharide (LPS)-induced stimulation to analyze their capacity to be suppressed by apoptotic cells. Tumor necrosis factor (TNF) secretion was measured by ELISA 18 h after stimulation. Left panel: representative experiment; right panel: cumulative data. P values LPS 5 ng/ml: 0.9654; 10 ng/ml: 1.0000; 20 ng/ml: 0.9551. B. Apoptotic neutrophils from patients with SLE inhibit monocytic U937 cells to an extent comparable to apoptotic neutrophils from controls. U937 cells were preincubated with apoptotic neutrophils for 3 h prior to LPS-induced stimulation. Left panel: representative experiment; right panel: cumulative data. TNF secretion was measured by ELISA 18 h after stimulation. P value SLE vs controls: 0.0789. J16: apoptotic Jurkat T cells.

cantly stronger to apoptotic cells than did antibodies from the serum of controls (Figure 4A), reflecting increased serum levels of autoantibodies observed in SLE. The antibodies did not, however, influence the suppressive capacity of apoptotic cells in this setup. When apoptotic cells coated with antibodies of 5 different patients with SLE and 5 different controls were added to U937 cells, suppression of TNF secretion was not altered (Figure 4B). Antibodies added to U937 cells directly, in the absence of apoptotic cells, also had no influence on the secretion of TNF (Figure 4B), nor did antibodies added in the absence of LPS (data not shown). These results suggest that autoantibodies present in patient serum do not affect the suppressive interactions between isolated apoptotic cells and DC in our system in the absence of complement.

Recombinant IFN-α abrogates the suppressive effect of

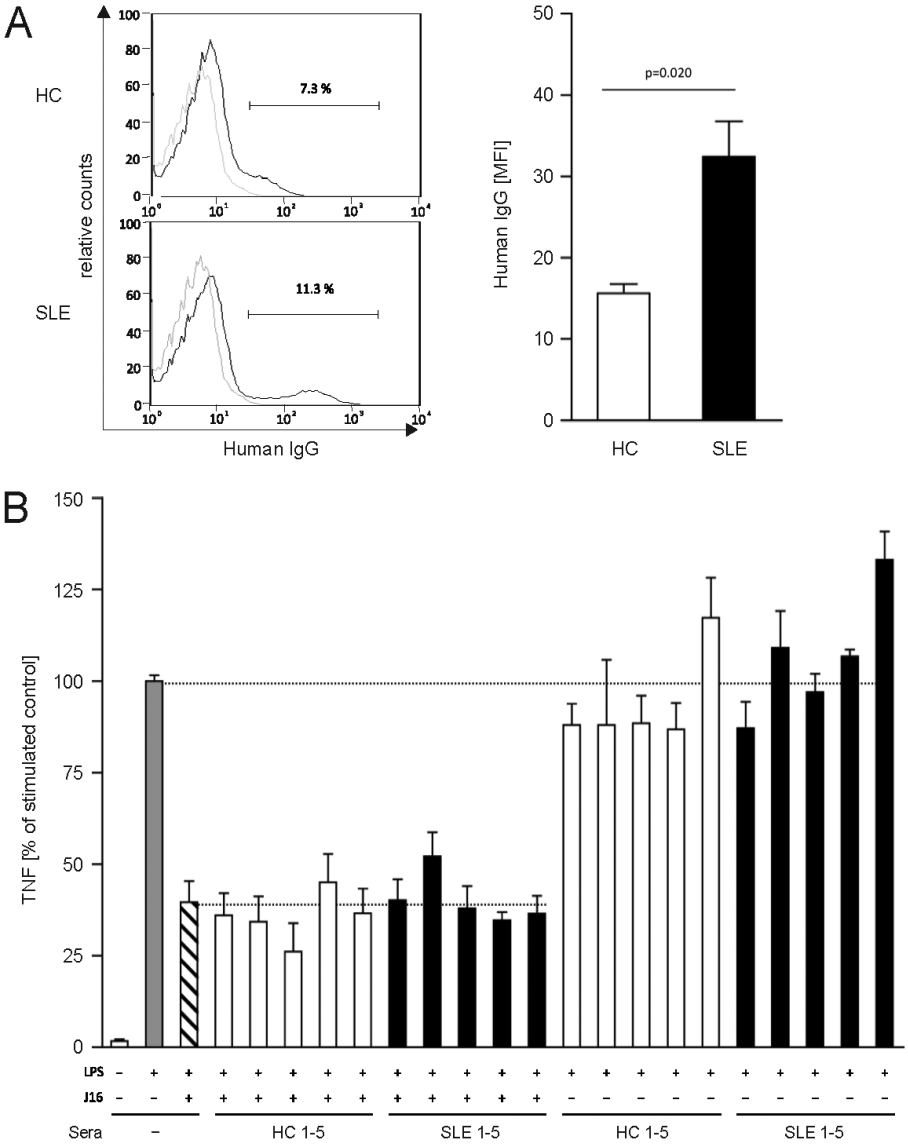

Figure 4. Autoantibodies from systemic lupus erythematosus (SLE) serum bind to apoptotic cells but do not influence suppression of tumor necrosis factor (TNF) secretion. A. Apoptotic Jurkat T (J16) cells were incubated with dialyzed serum of patients with SLE and healthy controls (HC). Gray line: J16 without serum; black line: J16 with serum from patients with SLE or controls. Left panel shows representative experiments; right panel shows cumulative data. MFI: mean fluorescence intensity. B. U937 cells were preincubated with (J16) cells coated with antibodies from dialyzed serum of controls and patients with SLE. Dialyzed serum without apoptotic cells was used as a control. Three hours later, the U937 cells were stimulated with lipopolysaccharide (LPS). TNF secretion was measured by ELISA 18 h after stimulation. Error bars represent SE of the mean of data combined from 6 independent experiments (p value apoptotic cells coated with sera from patients with SLE vs sera from controls: 0.311).

apoptotic cells on DC. Apart from autoantibodies and their complexes, high concentrations of cytokines such as IFN-α, IL-17, and members of the IL-1 family might play a role in the pathogenesis of SLE[10,11,36,37]. To analyze whether these cytokines influence the interaction of DC and apoptotic cells in our coculture system, we added apoptotic Jurkat T cells to primary human DC in the presence of recombinant IFN-α, IL-17, or IL-1β, respectively, before stimulation with LPS (Figure 5A). Remarkably, IFN-α greatly reduced the suppressive effect of apoptotic cells on the secretion of TNF and IL-6. While apoptotic Jurkat T cells alone suppressed TNF secretion to 41% of stimulated control DC, in the presence of IFN-α the TNF secretion increased to 72% of stimulated control DC. Similar results were obtained for the secretion of IL-6. Here, addition of IFN-α increased IL-6 secretion from 52% to 84% of stimulated control DC. The suppression of IL-12 p40 secretion was only slightly influenced by IFN-α. In contrast, IL-17 and IL-1β had no significant effect on the suppression of DC by apoptotic cells. A summary of the experiments is depicted in Figure 5B. These results demonstrate that IFN-α can interfere with one of the mechanisms maintaining self-tolerance to autoantigens.

DISCUSSION

Our study focused on the suppressive activity of apoptotic cells on DC, because a defect in this interaction has been suggested to be causative for the loss of peripheral tolerance in SLE[16]. For this purpose, we established a new *in vitro* coculture system that allowed us to analyze contribution of both cell types and the surrounding milieu to this process.

We found that apoptotic neutrophils from patients with SLE display kinetics of apoptosis comparable to neutrophils from controls; apoptosis in SLE neutrophils was slightly but not significantly delayed. Two previous studies found a larger effect but relied on the analysis of fewer cells over a shorter period of time[38,39]. Other reports suggested that the increased numbers of apoptotic lymphocytes observed in SLE might be due to a delayed or reduced uptake by phagocytic cells[40], a factor that was not investigated. Further, we found no difference in the exposure of the early apoptotic marker annexin A1 on the surface of neutrophils of patients with SLE and healthy controls. Annexin A1 has been shown to play a role in the suppression of DC by apoptotic cells[24]. In addition, autoantibodies against annexin A1 have been demonstrated to be present in patients with cutaneous lupus erythematosus[41], but their role in the disease has not yet been elucidated. Taken together, we did not see an obvious defect in apoptosis in cells derived from patients with SLE *in vitro*.

DC phagocytize apoptotic cells, migrate to the lymph nodes, and present apoptotic material to T cells, inducing nonresponsiveness[19,20]. We therefore used primary human monocyte-derived DC to investigate the influence of apoptotic cells on the immune response. We did not detect any significant differences in surface molecule expression between DC derived from patients with SLE and controls. There is controversy in the literature; Köller, *et al*[42], for example, found reduced expression of MHC II on mature DC from patients with SLE, whereas Ding, *et al*[43], detected an enhanced expression of MHC II, CD86, CD80, and CD1a and a reduced expression of CD83. The differences in these studies might be due to different culture media, serum supplements, and the number and variety of samples. In our study, we observed that DC from patients with SLE secrete amounts of TNF comparable to DC from controls when matured with low doses of LPS. This is in line with the results from Köller, *et al*[42], who reported the same surface expression of the LPS receptors TLR4 and TLR2 on DC of patients with SLE and controls. Our data suggest that at least *in vitro*, DC from inactive patients display a similar pattern of activation as DC from controls.

DC from patients with SLE and controls can be equally well activated by low doses of LPS, allowing us to analyze the role of apoptotic cells and phagocytes from patients and controls in coculture experiments. To separately analyze the functionality of the different cell types involved, we performed crossover experiments using DC and apoptotic cells from patients with SLE and controls, respectively.

In the first part of the crossover experiment, we focused on the phagocytes, and analyzed whether DC from patients with SLE and controls showed a comparable suppression of activation when cocultured with apoptotic cells before stimulation. As expected, DC from controls showed reduced cytokine secretion when cocultured with apoptotic Jurkat T cells. DC from patients with SLE showed a comparable reduction of TNF secretion induced by apoptotic cells. In the second part of the crossover experiment, we focused on the role of the apoptotic cells and determined to what extent apoptotic neutrophils from patients with SLE and controls can reduce LPS-induced TNF secretion of the monocytic cell line U937. Replacing primary DC with U937 cells enabled us to assess apoptotic cells of patients and controls in a donor-independent and serum-independent experimental setting. Apoptotic neutrophils from patients with SLE suppressed LPS-induced TNF secretion of U937 cells to the same extent as neutrophils from controls. These results suggest that *in vitro*, DC and apoptotic cells derived from patients with SLE are capable of having a suppressive interaction with the other cell type. The findings are consistent with the observation that the exposure of annexin A1, a molecule that partly mediates the suppressive effect of apoptotic cells on DC[24], is not altered in apoptotic neutrophils of patients with SLE. Taken together, these data suggest that apoptotic neutrophils and DC of patients with SLE do not generally have inherent defects that lead to an aberrant behavior when they are removed from their autoimmune milieu. We therefore were interested in analyzing the influence of factors likely to be present in

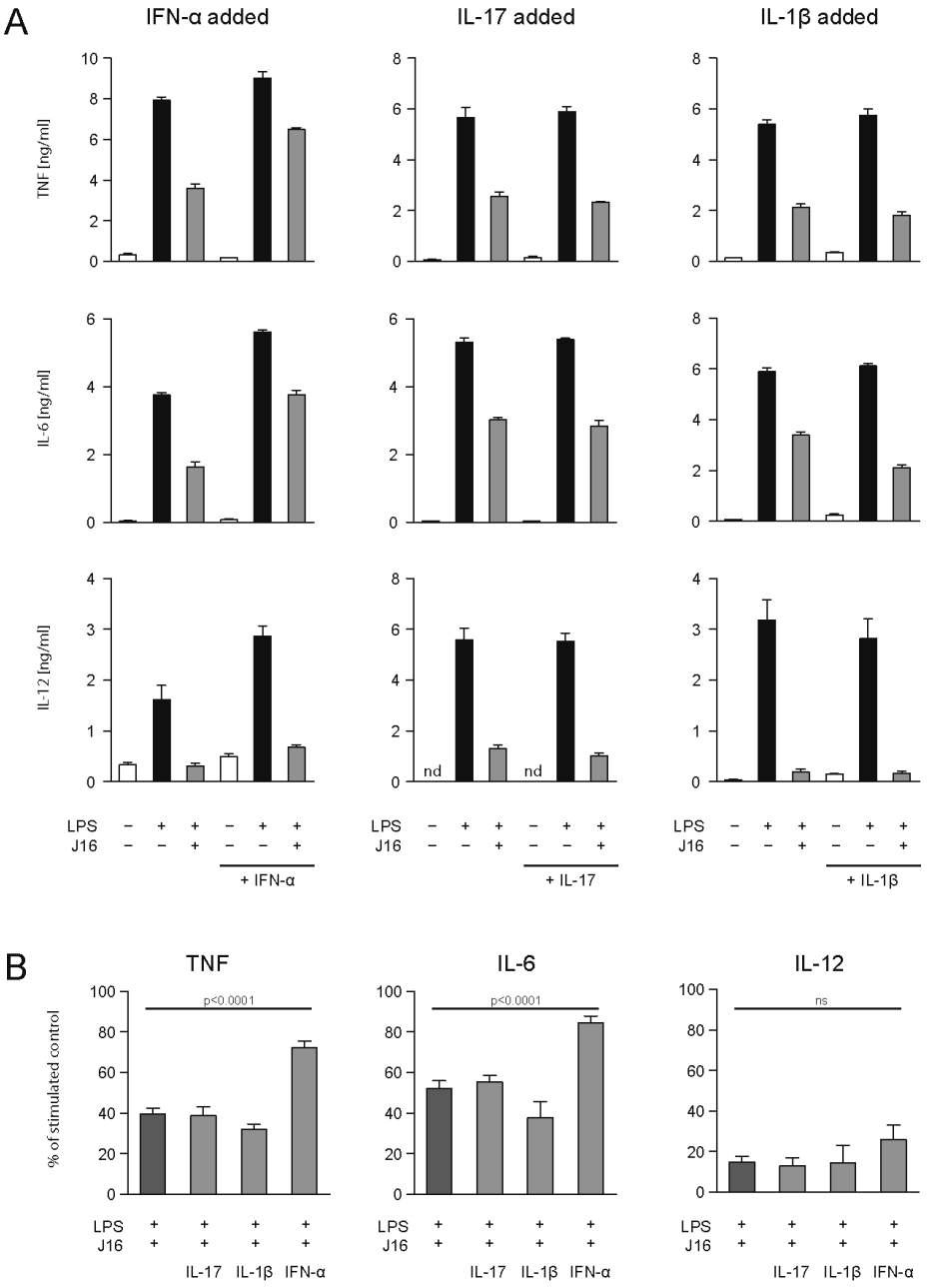

Figure 5. Interferon-α (IFN-α) abrogates the suppressive effect of apoptotic cells. A. Primary human dendritic cells (DC) were incubated with apoptotic Jurkat T cells in the presence of recombinant IFN-α, interleukin 17 (IL-17), or IL-1β (1000 u/ml) 4 h prior to stimulation with lipopolysaccharide (LPS). Recombinant IFN-α, IL-17, or IL-1β was added immediately before addition of apoptotic cells. Secretion of tumor necrosis factor (TNF), IL-6, and IL-12 (p40) was measured by ELISA 18 h after stimulation. ND: not detectable. B. Cumulative data; error bars represent SE of the mean of data combined from 3 (IL-17, IL-1β) to 5 (IFN-α) independent experiments, each performed in triplicate or duplicate wells per condition. P values for TNF-secretion apoptotic cells ± addition of cytokines: IFN-α, < 0.0001; IL-17, 0.93; IL-1β, 0.25. P values for IL-6–secretion apoptotic cells ± addition of cytokines: IFN-α, < 0.0001; IL-17, 0.70; IL-1β, 0.10. P values for IL-12 (p40) secretion apoptotic cells ± addition of cytokines: IFN-α, 0.10; IL-17, 0.77; IL-1β, 0.95.

active autoimmune disease, such as autoantibodies and inflammatory cytokines.

Autoantibodies are an early hallmark of SLE pathogenesis and have been shown to bind to apoptotic cells[12,16]. In our *in vitro* coculture system, antibodies from sera of patients with SLE and controls did not influence the

capacity of apoptotic Jurkat T cells to suppress U937 cells in the absence of complement. This is in line with a study showing that DC of patients with SLE were affected only when apoptotic cells were coated with the late apoptotic marker iC3b[44]. Another study observed differences in cytokine secretion from whole blood cells incubated with necrotic particles coated with SLE autoantibodies[45]. Both studies observe differences only under conditions of secondary necrosis, which is likely to occur *in vivo* when the phagocytic capacity of scavenging cells is insufficient to dispose of dying cells during the early phases of apoptosis. In our *in vitro* system, DC and U937 cells encounter early apoptotic cells at ratios that allow efficient phagocytosis before the cells progress to secondary necrosis. Our results show that in our *in vitro* system mimicking steady-state conditions of apoptotic cell removal, phagocyte activation as measured by TNF secretion was not influenced by the presence of autoantibodies. As we dialyzed the serum, we did not investigate the effect of complement on the interaction between DC, apoptotic cells, and autoantibodies. In particular, the role of complement needs to be further investigated in a more complex setup, to allow firm conclusions about the effect of autoantibodies and complement on the interaction of apoptotic cells and DC *in vivo*.

Apart from autoantibodies and their complexes, high concentrations of certain cytokines may play a role in SLE pathogenesis. Produced in abnormally high amounts by activated plasmacytoid DC, IFN-α causes many of the symptoms associated with recurrent flares of the disease[46]. Likewise, IL-17 and the axis of Th17 differentiation have been shown to be important in SLE. Patients with SLE showed elevated levels of IL-17 in serum and plasma, and an increased number of IL-17-producing cells is present in the peripheral blood of patients with SLE[10,47,48,49]. Enhanced Th17 differentiation in SLE not only leads to the presence of a highly inflammatory T cell subset, but also promotes the survival of B cells and their differentiation into antibody-producing plasma cells[50]. Recently, members of the IL-1 family have also been implicated in SLE pathogenesis[36,37]. We therefore analyzed the influence of IFN-α, IL-17, and Il-1β. Whereas IL-17 and IL-1β had no significant effect on the interaction of DC and apoptotic Jurkat T cells, IFN-α substantially decreased the suppressive effect of apoptotic cells on DC with regard to secretion of TNF and IL-6. The secretion of IL-12 (p40) was not significantly influenced, pointing to a specific interference of both signaling pathways rather than a general downmodulation of DC activation. This effect of IFN-α is consistent with findings by Blanco, *et al*[11], who identified IFN-α as the molecule causing the maturing effect of SLE serum on DC. Our results demonstrate that apart from causing an activation of DC, the presence of IFN-α can interfere with the suppressive effect apoptotic cells have on DC and thus potentially disturb one of the crucial mechanisms for maintaining self-tolerance to autoantigens in the periphery. Physiologically, IFN-α is released during viral infections. In this context, most dying cells that DC encounter will be infected cells, expressing many viral proteins and TLR stimuli. IFN-α in this situation might help to overcome the naturally suppressive effect of apoptotic cells and mount an efficient immune response against the virus. High levels of IFN-α in the absence of viral infection, on the other hand, are likely to interfere with the presentation of self-antigens in a nondanger context, thus potentially allowing the activation of T and B cells specific for antigens derived from apoptotic cells. Our findings further highlight the important role of IFN-α during the early stages of SLE pathogenesis and its potential role as a therapeutic target.

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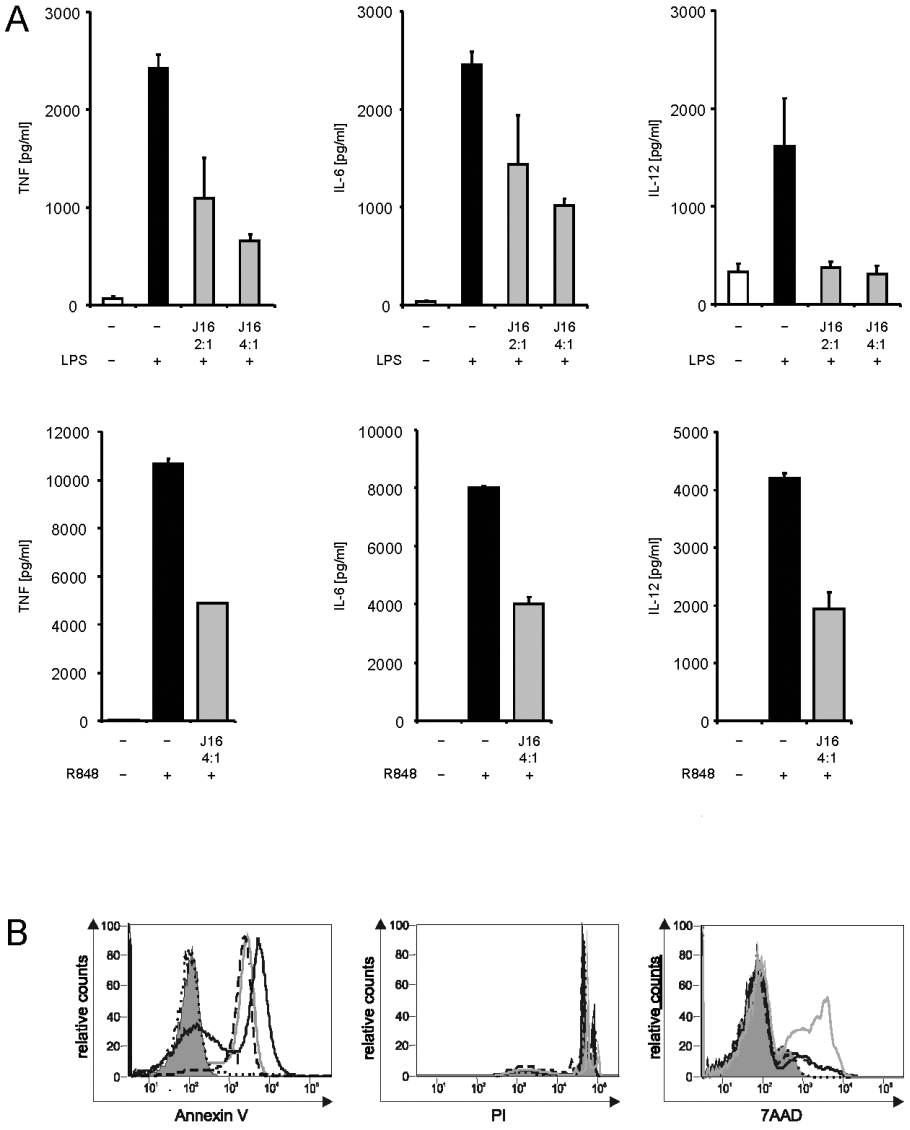

APPENDIX 1.

Toll-like receptor (TLR)-induced activation of dendritic cells (DC) is suppressed by preincubation with apoptotic cells. A. Primary human DC were incubated with apoptotic Jurkat T cells at the indicated ratios of apoptotic cells: DC for 4 h prior to stimulation with the TLR 4 ligand lipopolysaccharide (LPS; 1 ng/ml) or the TLR 7/8 ligand R848 (1 μg/ml). Secretion of tumor necrosis factor (TNF), interleukin 6 (IL-6), and IL-12 (p40) were measured by ELISA 18 h after stimulation. Error bars represent SE of the mean of triplicate wells. Data are representative of more than 3 experiments. B. Apoptosis kinetics of Jurkat T cells after UV-C irradiation (filled gray space: background; dotted line: 0 h; black line: 2 h; dashed line: 4 h; gray: 6 h). Staining from left to right: Annexin V-FITC, Nicoletti (PI), 7-AAD.

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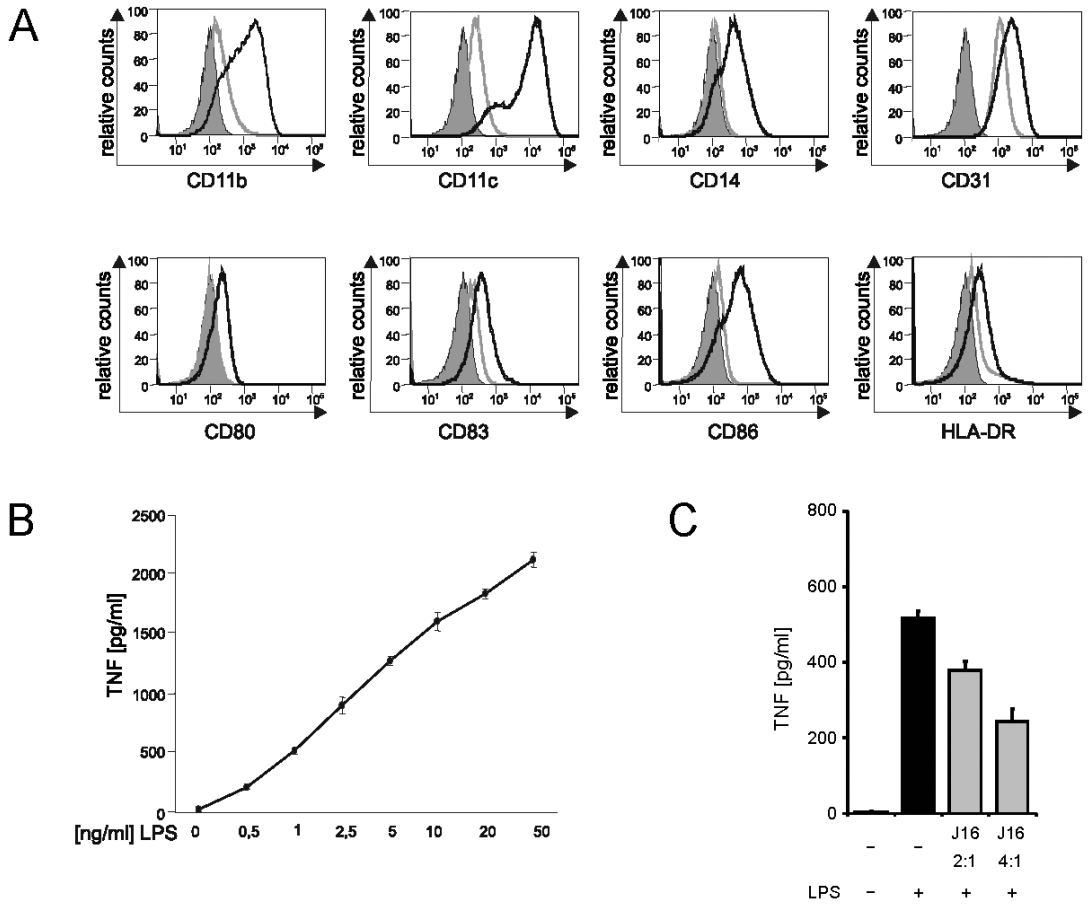

APPENDIX 2.

U937 cells resemble immature dendritic cells in their expression of surface markers, ability to produce tumor necrosis factor (TNF) after lipopolysaccharide (LPS) stimulation and their responsiveness to the suppressive effect of apoptotic cells. A. U937 cells were stained for expression of surface markers reflecting lineage expression before and after phorbol myristate acetate (PMA)–induced differentiation and analyzed by flow cytometry. Filled gray space: isotype; gray line: d3 undifferentiated; black line: d3 differentiated with 10 ng/ml PMA. B. PMA-differentiated U937 cells were stimulated with LPS. Secretion of TNF was measured by ELISA 18 h after stimulation. C. PMA-differentiated U937 were incubated with apoptotic Jurkat T cells at the indicated ratios of apoptotic cells: U937 cells 4 h prior to activation with LPS (5 ng/ml). Error bars represent the mean ± mean deviation of duplicate wells. Data are representative of more than 3 experiments. TNF secretion in (B) and (C) was measured by ELISA 18 h after stimulation.

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