Apoptotic cell clearance in Systemic Lupus Erythematosus (SLE)
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Opsonization of late apoptotic cells by Systemic Lupus Erythematosus (SLE) autoantibodies inhibits their uptake via an FcγR-dependent mechanism

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Conditionally accepted by Arthritis Rheum.
CHAPTER 9

ABSTRACT

Objective: Decreased clearance of apoptotic cells is suggested to be a major pathogenic factor in systemic lupus erythematosus (SLE). We investigated whether binding of SLE-autoantibodies to apoptotic cells influences phagocytosis of these cells by macrophages.

Methods: Apoptosis was induced in human T-cell (Jurkat) and keratinocyte (HaCaT) cell lines by UVB exposure. Binding of purified IgG from 26 SLE patients and 15 controls to apoptotic cells was assessed by flowcytometry and westernblotting. Phagocytosis of IgG-opsonized apoptotic cells by monocyte-derived macrophages (MDM) was assessed by light-microscopy. Similar experiments were performed with a monoclonal antibody against SSA/Ro and IgG fractions from patients with Sjögren’s syndrome (n=5) and rheumatoid arthritis patients (n=5).

Results: IgG fractions from all 26 SLE patients bound to late apoptotic (LA) but not to early apoptotic (EA) cells. IgG fractions isolated from SLE patients with different autoantibody profiles showed comparable levels of binding. Control IgG did not bind. Opsonization of apoptotic cells with IgG fractions from SLE patients resulted in a significant inhibition of phagocytosis as compared to control IgG fractions. A monoclonal antibody directed against SSA/Ro and IgG isolated from 5 ANA-positive patients with Sjögren’s syndrome were also able to elicit these effects, whereas IgG from 5 ANA-negative patients with rheumatoid arthritis did not. The inhibitory effect of patient IgG was abolished by blocking either Fcγ-receptors (FcγR) or the constant region of IgG, using a specific Fc-blocking peptide.

Conclusion: Autoantibodies from SLE patients are able to opsonize apoptotic cells and inhibit their uptake by macrophages via an FcγR-dependent mechanism.
INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the presence of autoantibodies directed against nuclear and cytoplasmic antigens, in combination with diverse clinical disease manifestations. Several studies have indicated an important role for apoptotic cells in the induction of autoimmunity in SLE. Injection of large numbers of apoptotic cells in experimental animal models induced autoimmunity, suggesting that self-tolerance can be broken by accumulation of apoptotic cells (1;2). In addition, studies have indicated that phagocytosis of apoptotic cells is impaired in SLE (3;4). During the apoptotic process, intracellular autoantigens may become exposed, in altered or unaltered form, at the outer surface of apoptotic cells (5-8). Decreased clearance of apoptotic cells results in prolonged exposure of these cell surface expressed autoantigens to the immune system in SLE, which might explain why autoantibodies develop against these intracellular antigens. Disturbance in the clearance of apoptotic cells, therefore, is considered one of the pathophysiological mechanisms underlying breakdown of tolerance and, subsequently, induction of SLE (9).

Breakdown of tolerance and production of autoantibodies, generally, precedes the onset of clinical manifestations in SLE (10). Little is known about the role of apoptotic cells during the course of the disease. In particular, its significance for boosting the autoimmune response and inducing lesions has not been studied. Also, the mechanisms involved in the reduced clearance of apoptotic cells have not yet been unraveled. Whether macrophages display intrinsic defects in the clearance of apoptotic cells in SLE is still debated (4;11-13). Alternatively, reduced clearance might result from serum factors. We recently showed that reduced complement levels, as present in patient sera during active disease, correlate with decreased apoptotic cell clearance in vitro (13). Furthermore, the presence of autoantigens on the apoptotic cell surface could, in theory, be a binding site for autoantibodies. Binding of autoantibodies to apoptotic cells will enable interaction with Fcγ-receptors (FcγR) on macrophages and, thereby, alter the route of phagocytosis as compared to non-IgG-opsonized apoptotic cells.

In this study, we investigated whether autoantibodies derived from SLE patients bind to apoptotic cells. In addition, we explored whether binding of autoantibodies to apoptotic cells affects their uptake by monocyte-derived macrophages (MDM).

MATERIALS AND METHODS

Patient selection

Patients eligible for this study fulfilled at least 4 American College of Rheumatology (ACR) criteria for SLE (14). The local ethics committee of the University Medical Center Groningen approved use of patient sera for the study. IgG fractions were isolated from serum of 26 consecutive patients (48.6±12.2 years) who were ANA positive as tested by indirect immunofluorescence and positive for various autoantigenic specificities as shown in Table 1.

From this group, 3 patients were selected who had distinct autoantibody profiles. These patients solely tested positive for autoantibodies directed against SSA (patient S), nRNP (patient R) or dsDNA in combination with Sm (patient D),
respectively (Table 1). Autoantibodies to double stranded DNA (dsDNA) were assessed by FARR assay (Diagnostic Products Corporation, Los Angeles, USA), anti-cardiolipin IgG was tested using Varelisa™ Cardiolipin IgG EIA kit (Sweden Diagnostics, Freiburg, Germany) and reactivity to other antigens was tested by the InnoLIA blot system (Innogenetics NV, Zwijndrecht, Belgium).

Furthermore, sera from 5 rheumatoid arthritis (RA) patients (49.2±8.7 years) and 5 patients with primary Sjögren’s syndrome (50.4±18.6 years) were selected and IgG fractions isolated as described above. IgG fractions from the RA patients tested negative for antinuclear antibodies (ANA) and for reactivity against any of the antigens indicated in Table 1. IgG fractions from the patients with Sjögren’s syndrome all tested positive for reactivity against SSA and in 2 out of 5 cases against SSB but did not react with other antigens indicated in Table 1.

**Chemicals and antibodies**

Bovine Serum Albumin (BSA) and Lymphoprep were from Fresenius (Kabi, Norway), Natriumcitrate from Merck (Darmstadt, Germany). Mouse monoclonal antibody directed against β-actin was obtained from ICN (Aurora, OH, USA), polyclonal rabbit anti-hIgG FITC-labeled F(ab)2 fragments and polyclonal rabbit anti-human-Ig-HRP-labeled antibodies were from Dako (Glostrup, Denmark). Polyclonal-goat anti-mIg(H+L)-PE-labeled-IgG was obtained from SouthernBiotech (Birmingham, UK). Antibody directed against nuclear matrix protein p84 was from Novus Biogicals Inc (Littleton, USA). FcγR blocking antibodies, mouse anti-CD16-IgG clone AT10 and anti-CD64-IgG clone 10.1 were from BD Pharmingen (San Diego, USA) and mouse anti-CD32 clone 3G8 was from Serotec (Oxford, UK). IgG blocking peptide, TG19320 (15), was kindly provided by Dr. Fassina, XEPTAGEN (Pozzuoli, Italy).

**Apoptosis induction**

Jurkat cells (a human T cell line) were irradiated with 8.3 mJ/cm² ultraviolet B (UVB) using a TL12 broad band UVB lamp (Philips, Best, The Netherlands) to induce apoptosis. Sub-confluent monolayers of HaCaT cells (human keratinocyte cell line) were irradiated with 19.8 ml/cm² UVB. Apoptosis induction of freshly isolated neutrophils was achieved either by aging for 72-hours or by UV irradiation as described before (16).

To inhibit apoptosis using a general caspase inhibitor we incubated cells 1 hr before induction of apoptosis with two concentrations of ZVAD (Sigma, St Lious MO, USA) (10 and 20 μM, respectively) in culture medium, and subsequently tested cells for apoptosis induction by the methods indicated below.

**Apoptosis detection**

Progression of apoptosis was assessed after incubating cells for 4 up to 48 hours at 37°C in 5% CO₂ followed by annexin V staining and propidium iodide (PI) uptake as described before (17). Furthermore, cell DNA content was determined as described by Nicoletti (18;19). Early apoptotic cells (EA) were defined as cells binding
AnnexinV but not taking up PI. Late apoptotic (LA) cells were positive for both AnnexinV binding and uptake of PI.

**IgG purification and binding assay**

IgG fractions were isolated using a Hitrap protein G column (Amersham, Uppsala, Sweden), and checked for endotoxin contamination. Apoptotic cells were washed with 1% BSA/PBS, resuspended in 1% BSA/PBS (2x10⁶ cells/ml) and incubated with 10 μg/ml purified IgG for 30 min at 37 °C in 5% CO₂. After washing, cells were incubated with 5 μl rabbit anti-hIgG-FITC for 30 min at RT in the dark, and subsequently analyzed by flow cytometry.

In order to test permeability of LA Jurkat cells for IgG molecules, cytoskeleton β-actin was used as an intracellular marker. IgG-permeable cells were defined as cells binding mouse-anti-β-actin-PE. LA Jurkat cells were double stained with anti-β-actin (1:200 diluted in 1% BSA/PBS) combined with IgG isolated from healthy controls or patients S, R or D (10μg/ml). After washing, cells were incubated with a combination of F(ab)₂ rabbit-anti-human IgG-FITC (5 μl undiluted on the pellet) and rabbit-anti-mouse IgG-PE (5 μl of 1:10 dilution on the pellet) for 30 min at RT in the dark and analyzed by flow cytometry.

**Plasma membrane extraction and western blotting**

Non-irradiated, EA, and LA Jurkat cells were resuspended (30x10⁶ cells /ml) in buffer containing 10 mM Tris/HCl (pH 7.3), 1.5 mM MgCl₂, 10 mM NaCl, and protease inhibitors (Complete™, Roche, Indianapolis, USA), and incubated for 1 hr on ice. Cell membranes were disrupted by douncing, and subsequently, intact cells and nuclei were pelleted by centrifugation (1200 rpm, 5 min). Supernatant was centrifuged in a high speed centrifuge (12000 rpm, 30min) and pelleted plasma membranes were resuspended in 10 mM Tris/HCl pH 7.3.

Plasma membrane fractions (1.8 μg protein/lane) were analyzed using standard western blotting technique. In short, after transfer to a nitrocellulose membrane, membrane was blocked using 5% milk suspension, washed and incubated with 15 μg/ml IgG derived from controls or SLE patients for 1 hour. After washing, the membrane was incubated (30min, RT) with a secondary antibody (rabbit anti-human IgG-HRP labeled antibody (diluted 1:2500 in block buffer)), and analyzed using LumilightPLUS (Roche, Indianapolis, USA).

Nuclear fractions were isolated using the NE-PER Nuclear extraction kit (Pierce, Rockford IL, USA) according to the manufacturers description.

**Monocyte-derived macrophage (MDM) culture**

Peripheral blood mononuclear cells were isolated from healthy controls as described before (13).

**Phagocytosis assay**

Phagocytosis assay was performed as described before (17). In short, UVB irradiated apoptotic cells were incubated with 10 μg/ml IgG fractions (see above), and cells were washed twice to remove non binding IgG. Subsequently, cells (2x10⁶
cells/well for Jurkat cells, 1x10^6 cells/well for HaCaT cells) were incubated with MDM for 30 min at 37°C in 5% CO₂, in the presence of 30% normal human serum (NHS). Subsequently, staining procedures were performed and preparations scored for phagocytosis of apoptotic cells as described previously (17). Only apoptotic cells engulfed for at least three quarters were considered internalized. All experiments were performed in duplicate and scoring was blinded. Phagocytosis was expressed as phagocytosis index (PhI), which is the number of apoptotic cells internalized by 100 MDM, or as percentage inhibition compared to phagocytosis of non-opsonized control apoptotic cells.

Monoclonal antibody directed against SSA clone 2G10 (kindly provided by Prof. L.A. Aarden, Sanquin Research, Amsterdam, The Netherlands) was used for opsonization at a concentration of 10 μg/ml as described for IgG fractions. In all experiments comparing effects of patient IgG with control IgG, MDM from the same culture and serum from the same donor were used in order to prevent differences resulting from culture or serum conditions.

**Blocking FcγR-mediated uptake of opsonized apoptotic cells**

Apoptotic cells were opsonized with SLE- and control IgG as described above. Subsequently, cells were washed and incubated for 30 min at RT with varying concentrations of TG19320 (0-100μg/ml) in 1% BSA/PBS. This allowed binding of TG19320 to the Fc part of IgG, supposed to block subsequent binding to FcγR. After washing, cells were used in the phagocytosis assay as described above.

In addition, blocking antibodies were used to block the various subclasses of FcγR on MDM, that is CD16, CD32, and CD64. MDM were preincubated with 5 μg/ml blocking antibody diluted in 1%BSA/PBS for 30 min at 4 °C. For double blocking of both CD32 and CD64, 5ug/ml of each antibody was used. Unbound antibody was removed by washing, and MDM were subsequently allowed to take up opsonized apoptotic cells as described above.

**Statistics**

Results are indicated as mean±SEM. Levels of statistical significance were calculated using GraphPad Prism™ (version 4.0; GraphPad software). Comparison between groups was done using Mann-Whitney U test. Correlations were calculated using Spearman rank correlation test. A p-value <0.05 was considered significant.

**RESULTS**

**Apoptosis induction by UVB irradiation in Jurkat and HaCaT cells**

Apoptosis was induced in a human T cell (Jurkat) line using UVB irradiation. In addition, as photosensitivity is one of the manifestations of SLE, we studied UVB-induced apoptosis in a keratinocyte cell line (HaCaT). After UVB irradiation and 4 hours culture, the majority (54.0±5.1% (mean ± SD, n=3)) of Jurkat cells were early apoptotic (EA) (Annexin V^-/Propidium Iodide (PI)-) while low levels (8.5 ± 2.5%) of late apoptotic (LA) cells (Annexin V+/PI+) were present (Fig 1A). At that time point, DNA content was decreased in 35.5 ± 3.1% of Jurkat cells. Twenty-four hours after UVB irradiation, 47.3±2.7% of Jurkat cells were LA, with 60.6±6.3% of cells
**Inhibition of apoptotic cell uptake by autoantibodies**

**Figure 1:** Apoptosis induction by UVB in Jurkat and HaCaT cell lines. A) Annexin V/Propidium Iodine (PI) staining (upper panel) and DNA content (lower panel) in Jurkat cells before (viable) and at 4 and 24 hrs after UVB irradiation. B) Annexin V/Propidium Iodine (PI) staining (upper panel) and DNA content (lower panel) in HaCaT cells before (viable) and at 8 and 24 hrs after UVB irradiation. Percentages of apoptotic cells in the different compartments are indicated. Upper panel: x-axis AnnexinV and y-axis Propidium Iodide staining(PI), lower panel: x-axis log of PI value. C) Inhibition of apoptosis of Jurkat and HaCat cells by a caspase inhibitor. X-axis indicates concentration of caspase inhibitor (ZVAD) used and y-axis indicates the percentage of cells which are Annexin V+. Open symbols indicate cells not exposed to UVB, closed symbols cells exposed to UVB.

Having a subdiploid DNA content. HaCaT cells were more resistant to UVB, and needed twice the UVB dose used for Jurkat cells to induce apoptosis. After 8 hours, only 11.2±2.3% and 20.4 ± 4.1% of HaCaT cells were EA and LA, respectively. DNA content was decreased in 16.5±2.1% of HaCaT cells (Fig 1B). After 24 hrs 58.4±4.5% of HaCaT cells were LA while less than 10% of cells were EA. According to DNA content, 55.1% of HaCaT cells were apoptotic after 24 hrs (Fig 1B), which further increased to 76.3% at 48 hrs (data not shown). Additionally, a caspase inhibitor blocked the induction of apoptotic cells to a background level of approximately 10% dead cells also seen in non-irradiated cultures (Fig 1C).
Binding of SLE antibodies to apoptotic cells

IgG was isolated from sera of 26 SLE-patients with various autoantibody profiles (Table 1) and 15 healthy controls. Binding of IgG to Jurkat cells irradiated and incubated for 4 (EA)- and 24 hours (LA), respectively, was tested by flowcytometry. As only a minority of HaCaT cells were apoptotic after 8 hours, we only tested IgG binding to HaCaT cells that had been incubated for 24 hrs after UVB irradiation. As only a part of the cells were apoptotic, percentages of cells binding IgG were multiplied by the MFI of the positive peak resulting in the more representative binding index (BI). No significant binding of patient or control IgG could be detected to viable or EA Jurkat cells (data not shown). Patient IgG of all patients showed increased binding to both LA Jurkat and LA HaCaT cells as compared to control IgG, p<0.001 (Fig2A). Both for patient and control IgG binding to LA Jurkat cells correlated highly with

Table 1: Autoantibody specificities to various antigens present in sera of 26 SLE patients used for this study.

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Autoantibody reactivity as found in 26 SLE patients. Patient S, R and D have been selected for the presence of autoantibodies to SSA (52 and 60 kDa), nRNP and dsDNA/Sm, respectively, without the concomitant presence of other autoantibodies. “+” indicates presence and “-” indicates absence of a particular autoantibody specificity. Numbers in dsDNA column indicate the levels of anti-dsDNA expressed in WHO-units (28) as detected by FARR assay (>10 was regarded as ds-DNA positive). aC1 antibodies are expressed in GPL units.
Figure 2: Binding of SLE autoantibodies to apoptotic Jurkat and HaCaT cells. A) Binding of IgG fractions from 26 SLE patients with autoantibodies to multiple intracellular antigens and 15 healthy controls to LA Jurkat cells (left panel) and LA HaCaT cells (right panel). Left axis: binding is expressed as binding index (BI): % positive cells x MFI of the positive peak. Right axis: binding is expressed as the total MFI. Box and whiskers plot showing median with minimal and maximal values. B) Binding of IgG fractions from patients S (anti-SSA), R (anti-nRNP) and D (anti-dsDNA and anti-Sm) and control IgG to LA Jurkat cells (left panel) and LA HaCaT cells (right panel). Binding is expressed as BI. Results are the mean of 4 independent experiments. *, p<0.05; ***, p<0.0001 C) Double staining of LA Jurkat cells with a monoclonal antibody to β-actin as an intracellular marker for IgG-permeability (y-axis) and healthy control or SLE IgG (x-axis). D) Western blot analysis detecting binding of a control IgG and IgG from patient S, R and D, respectively, to membrane fractions derived from viable (V), EA and LA Jurkat cells (1.8μg/lane). Molecular sizes are indicated to the left.
(r=0.89, p<0.0001) with that to LA HaCaT cells. To study possible differences in binding capacity between patients with distinct autoantibody profiles, 3 patient IgG fractions were selected. These patients were known to have autoantibodies directed against the following SLE-antigens; SSA (patient S), nRNP (patient R) or dsDNA in combination with Sm (patient D), without reactivity against other nuclear and/or cytoplasmic antigens tested (Table 1). Autoantibodies from patients S, R and D showed increased binding to LA Jurkat and LA HaCaT cells compared to control IgG (Fig 2B). Significantly more IgG from patient S (BI: 7.2 ± 3.1 (x1000), p=0.032), R (BI: 11.7±4.1 (x1000), p=0.029) and D (BI: 11.3±3.7 (x1000), p=0.029) bound to LA cells compared to control IgG (BI: 2.1±3.1 (x1000)). Percentages of LA cells binding IgG from these patients varied between 21% - 23% for Jurkat cells and 40-60% for HaCaT cells (data not shown).

As LA cells are permeable to PI, IgG of SLE patients could possibly bind to autoantigens localized intracellularly in LA cells. To evaluate whether IgG bound intracellularly or also extracellularly, we used an antibody directed against a strongly expressed cytoskeleton protein, β-actin. LA Jurkat cells were double-stained with control IgG or IgG from patient S, R and D in combination with the anti-β-actin antibody. As expected, viable cells only stained positive for β-actin after cell membrane permeabilization making this a suitable antibody to study IgG-permeability of LA cells. Double staining of LA Jurkat cells revealed that approximately 40% of LA Jurkat cells were permeable for IgG, defined as anti-β-actin positive (Fig 2C). IgG derived from healthy controls did not stain LA cells as described before. Using patient IgG from patient S, R and D, approximately 40% of cells stained double positive. Nevertheless, around 15% percent of LA cells were only positive for the patient IgGs, indicating that IgG binding to these cells solely bound to membrane-expressed antigens. Mean fluorescence intensity of the double positive and single positive populations of Jurkat cells were comparable.

Additionally, we studied the ability of IgG fractions derived from patient S, R, and D to react with plasma membrane fractions isolated from viable, EA, and LA Jurkat cells. No contamination of nuclei in the plasma membrane fractions was detected by westernblot, using a nuclear matrix protein (p84) as a nuclear marker (data not shown). Control IgG was only reactive to a protein of approximately 60kDa in the viable plasma membrane fraction. This reaction was not specific as it was also detectable using the secondary antibody only. Compared to control IgG, patient IgG fractions recognized several proteins in plasma membrane fractions of LA cells, incidentally one protein in EA Jurkat cells (patient S), and none in viable cells (Fig 2D). Proteins of similar size could also be detected in nuclear extracts when run side by side (data not shown). For patient S, the expected 52 and 60 kDa SSA bands were detected, although many more proteins were recognized. Patient R mainly recognized a protein of approximately 70 kDa, expected to be nRNP, although other proteins were also weakly detected. Proteins of 52, 60 and 70 kDa proteins were not recognized by patient D, in accordance with diagnostic testing, for autoantibodies. However, patient D also recognized multiple other proteins in membranes of apoptotic cells.
Inhibition of apoptotic cell uptake by autoantibodies

Monocyte-derived macrophages (MDM) were used to assess the uptake of LA Jurkat and HaCaT cells opsonized with IgG fractions from SLE patients, in the presence of normal human serum. Preincubation of LA HaCaT cells with control IgG fractions resulted in a phagocytosis index (PHI) comparable to control PBS incubated cells (median±SEM: 69.0 ± 1.9 for control IgG vs 78.0±0.0 for PBS). Opsonization of LA HaCaT cells with IgG fractions from SLE patients decreased the PHI to 38.0±3.4 (Fig 3A). Phagocytosis was inhibited by 51.3±4.3 % after pre-incubation with IgG fractions from SLE patients compared to 11.9±2.7 % when IgG fractions of healthy controls were used for pre-incubation (Fig 3B). Similar levels of inhibition were obtained when late apoptotic neutrophils, either aged or UVB irradiated, were opsonized with IgG isolated from SLE patients (data not shown). Level of inhibition of phagocytosis of opsonized LA cells correlated between Jurkat and HaCaT cells (r=0.56, p=0.0009). No correlation was found between the presence and levels of anti-cardiolipin antibodies, one of the known phospholipid autoantibodies, and levels of phagocytosis (data not shown). However, levels of inhibition of phagocytosis correlated with the binding indices (BI) of the IgG fractions both for LA Jurkat cells (r=0.42, p=0.02) and LA HaCaT cells (r= 0.52, p=0.0007).

A single MDM could internalize up to three apoptotic cells (Fig 3C). Even the relatively large LA HaCaT cells were taken up. Opsonization with IgG fractions from SLE patients decreased both the numbers of MDM that had internalized apoptotic cells as the number of apoptotic cells taken up per MDM (data not shown).

To test whether the inhibitory effect was specific for IgG isolated from SLE patients we also isolated IgG from 5 ANA negative RA patients and 5 anti-SSA positive patients with primary Sjögren’s syndrome and performed the phagocytosis assays as described before. IgG isolated from patients with Sjögren’s syndrome was able to inhibit uptake of apoptotic cells to the same extent as IgG isolated from SLE patients (median±SEM, 39.7±3.9 vs. 39.8±3.8%, respectively) (Fig 3D). IgG obtained from RA patients did not bind nor inhibit uptake of late apoptotic cells.

Next, we studied the uptake of LA Jurkat cells and HaCaT cells opsonized with IgG fractions from patients S (SSA), R (nRNP) and D (dsDNA/Sm), respectively. Compared to LA Jurkat cells pre-incubated with PBS (54.8 ± 3.2, n=4) or control IgG(50.5 ± 8.5, n=4), PHI was decreased when LA cells had been pre-incubated with IgG fractions from patients S (30 ± 8.1, n=4), R (26.8 ± 7.6, n=4) or D (34.3 ± 10.2, n=4) (Fig 4A). Compared to control IgG, phagocytosis was inhibited by 40-50 % depending on the patient IgG and nature of the apoptotic cells (Fig 4B).

Additionally, a monoclonal antibody directed against SSA/Ro was used for opsonization of both apoptotic Jurkat and HaCat cells. In phagocytosis assays this monoclonal also inhibited uptake of LA cells by macrophages at a similar level (mean±SD, 39.2±17.8% vs 8.6±9.9% of controls) as patient IgGs (Fig 4C).
Figure 3: Phagocytosis of apoptotic cells opsonized with IgG from SLE patients compared to healthy control IgG. 

**A** Phagocytosis index (PhI) of monocyte-derived macrophages (MDM) incubated with LA Jurkat cells (left panel) and LA HaCat cells (right panel) opsonized with patient IgG (n=26) or healthy control IgG (n=15) as compared to PBS treated cells (n=4).

**B** Percentage inhibition of phagocytosis of LA cells after opsonization with control IgG and patient IgG as compared to PBS treated cells.

**C** Photographs showing phagocytosis of LA Jurkat cells (left panel) and LA HaCat cells (right panel) pre-incubated with either a representative control IgG or patient IgG. * indicate nuclei of MDM (magnification 200x).

**D** Percentage inhibition of phagocytosis of LA Jurkat cells after opsonization with control IgG (n=4) and either SLE (n=4), RA (n=5) or Sjögren’s syndrome (n=5) patient IgGs as compared to PBS treated cells. **, p<0.01; ***, p<0.0001.
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**Figure 4:** Phagocytosis of LA cells opsonized with patient IgG fractions primarily reactive against SSA/Ro(S), nRNP(R) and dsDNA(D), respectively. A) Phagocytosis index (PhI) of LA Jurkat cells (left panel) and LA HaCaT cells (right panel) opsonized with patient IgG primarily reactive to SSA (S), nRNP (R) and dsDNA/Sm (D) as compared to control IgG and PBS pre-treated cells. Results are the mean of 4 independent experiments. B) Percentage of inhibition of phagocytosis of LA Jurkat cells (left panel) and LA HaCaT cells (right panel) opsonized with control IgG or with IgG fractions from patients S, R and D primarily reactive to SSA/Ro, nRNP and dsDNA, respectively, as compared to phagocytosis of PBS treated cells. C) Percentage inhibition of phagocytosis of LA Jurkat cells opsonized with control IgG or with a monoclonal antibody directed against SS/Ro, as compared to phagocytosis of PBS treated cells. *, p<0.05, **, p<0.01.

**Phagocytosis of apoptotic cells is dependent on serum concentration, IgG concentration and IgG-Fc-FcγR interaction**

We investigated the effect of normal human serum (NHS) on phagocytosis of apoptotic cells by varying serum concentrations from 50% down to 10%. PhI of LA Jurkat cells incubated with IgG fractions of controls or SLE patients did not change when the serum concentration was reduced from 50% down to 30%. (data not shown). Lower serum concentrations reduced PhI for both LA cells opsonized with IgG fractions of controls and SLE patients. However, the inhibitory effect of opsonizing
Figure 5: Phagocytosis of IgG-opsonized LA cells is dependent on patient IgG concentration and Fc-FcγR interaction. A) Effect of decreasing IgG concentrations, used for opsonizing LA Jurkat cells, on PhI. B) Effect of blocking Fc-FcγR interaction on SLE-IgG mediated inhibition of phagocytosis. Opsonized LA Jurkat cells were pre-incubated with different concentrations of the blocking peptide TG19320 and incubated with MDM. C) Effect of blocking CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI) on phagocytosis of LA Jurkat cells opsonized with patient IgG. Graph indicates percentage inhibition of phagocytosis by MDM of apoptotic cells pre-incubated with either PBS or control IgG (C4,C5) or 5 different patient IgGs, without blocking (PBS, white bars), after blocking the different FcγR (dark grey bars) or after blocking IgG-Fc, using TG19320 peptide (light grey bars). D) Effect of blocking both CD32 and CD64 on phagocytosis of LA Jurkat cells opsonized with two patient IgGs. Apoptotic cells were opsonized with both IgG from patient D (CD32 mediated) and patient 5 (CD64 mediated); without FcγR blockade (white bar), with α-CD32 and α-CD64 alone (dark grey bars), after blocking with both α-CD32 and α-CD64 (light grey bar), and after blockade using IgG Fc-blocking peptide, TG19320. Error bars represent SEM of two independent experiments.
IgG fractions from SLE patients was still detectable at a serum concentration of 20%. At 10% serum concentration, LA cells opsonized with either control IgG or patient IgG were taken up at a comparable but low rate (data not shown).

Subsequently, we assessed the influence of the IgG concentration, as used for opsonizing LA Jurkat cells, on the level of phagocytosis. As expected, concentration of IgG from controls did not influence PhI (Fig 5A). However, diluting IgG fractions from SLE patients increased the PhI to levels comparable to that of control IgG.

To evaluate whether the inhibition of phagocytosis by IgG from SLE patients was dependent on Fc-Fcγ-receptor (FcγR) interaction, we blocked the Fc-portion of IgG using a blocking peptide (TG19320). Pre-incubation of SLE-IgG-opsonized LA Jurkat cells with increasing concentrations of the Fc-blocking peptide increased phagocytosis of these cells to levels similar to control IgG-opsonized LA cells (Fig 5B). In addition, using FcγR blocking antibodies, we blocked the various FcγR on MDM, after which the phagocytosis assay was performed. Phagocytosis of LA Jurkat cells pretreated with PBS or IgG fractions from controls was not influenced by FcγR blockade (data not shown). The inhibitory effect of opsonization with IgG fractions from SLE patients was strongly decreased by FcγR blockade. PhI was comparable to the PhI of LA cells blocked with the IgG-Fc blocking peptide (Fig 5C). Blocking of CD32 resulted in a complete resolution of the inhibitory effect in 2 out of 5 patient IgG fractions tested. In two other patients, this occurred after blockade of CD64. The remaining patient IgG tested required both CD32- and CD64 blockade to abolish its inhibitory effect. Thus, IgG fractions interacted differentially with FcγR. Co-blockade of CD32 and CD64 was performed to test the independent contribution of these receptors to the inhibitory effect. LA Jurkat cells were opsonized with a mixture of IgG from patient D (CD32 mediated) and patient 5 (CD64 mediated). Inhibition of phagocytosis mediated by these two SLE IgGs could only be completely abolished by blocking both CD32 and CD64 (Figure 5D). This confirms the independent involvement of both CD32 and CD64 in inhibiting phagocytosis of SLE IgG opsonized LA cells.

**DISCUSSION**

Although SLE autoantigens are known to be exposed on the surface of apoptotic cells, little is known about binding of IgG from SLE patients to these surface exposed autoantigens, and its subsequent effect on apoptotic cell clearance. In this study, we show that IgG derived from SLE patients not only opsonize late apoptotic cells but also inhibit their uptake by monocyte derived macrophages (MDM).

Thus far, binding of human autoantibodies to nuclear and/or cytoplasmic antigens expressed on apoptotic cells has only been investigated using a limited number of sera (8). Here, we could show, by flowcytometry, that IgG isolated from SLE patients bind to late apoptotic (LA) cells. Because flowcytometry was used to analyze binding, it could be argued that cell permeability, as demonstrated by propidium iodide (PI) staining, of LA cells potentially could allow binding of IgG molecules to autoantigens localized intracellularly. Using an antibody to the cytoskeleton protein β-actin, we could show that approximately 15 percent of LA cells were negative for β-actin staining, and, thus, not permeable for IgG. This strongly
suggests that patient IgG binds to the outer leaflet of the apoptotic cell membrane. Furthermore, IgG fractions of patients with 3 distinct autoantibody profiles all recognized proteins in plasma membrane fractions derived from LA Jurkat cells and hardly any proteins in membrane fractions from EA Jurkat cells, consistent with the flowcytometry experiments. The comparable inhibitory behavior of the IgG fractions isolated from patients with these distinct autoantibody profiles indicates that opsonization of LA cells and its subsequent effect on phagocytosis is not restricted to one particular autoantibody profile.

One could argue that use of transformed cell lines as apoptotic targets may not reflect the physiological situation. Therefore, we also performed these experiments using LA neutrophils. Phagocytosis was similarly inhibited by SLE IgG using these primary cells, indicating that these effects can be extrapolated to the apoptotic non-transformed cells present in human subjects.

Antibodies directed against nuclear antigens are not restricted to SLE patients. In patients with primary Sjögren’s syndrome antibodies directed against SSA/Ro are often found. To establish whether inhibition of apoptotic cell clearance by opsonizing autoantibodies is SLE specific, we also tested IgG fractions isolated from patients with Sjögren’s syndrome positive for anti-SSA/Ro and sera from ANA-negative rheumatoid arthritis (RA) patients for inhibitory effects on phagocytosis. Phagocytosis of LA cells was inhibited to a similar extent by IgG fractions isolated from patients with Sjögren’s syndrome as by IgG fractions from SLE patients but not by IgG from RA patients lacking autoantibodies directed against nuclear or cytoplasmic antigens. Therefore, inhibition of phagocytosis seems to be dependent on the presence of antibodies recognizing surface molecules expressed on apoptotic cells, and is not specific for SLE autoantibodies.

Facilitation of apoptotic cell uptake by anti-phospholipid antibodies has been suggested by Manfredi et al (20). In our study, three of the 26 patient IgG fractions lacked inhibitory capacity and even, to some extent, facilitated uptake of LA Jurkat cells. However, no correlation was found between the presence and levels of IgG anti-cardiolipin antibodies, one of the known anti-phospholipid autoantibodies, and levels of phagocytosis (data not shown). The phagocytosis assay as applied by Manfredi et al used radioactively labeled apoptotic cells and detected levels of the radioactive label in MDM after a one hour contact between the cells. Despite washing MDM monolayers, apoptotic cells binding to MDM but not phagocytosed will not be removed, and were detected as well in their assay. In the current study, we were able to discriminate, by microscopy, uptake of apoptotic cells from binding which might explain differences in results. Recently, Clancy et al also reported an inhibitory effect of anti-SSA and anti-SSB monoclonal antibodies on uptake of apoptotic myocardiocytes (21). That study as well as our study, strongly indicate that the binding of antibodies to apoptotic cells can have a negative effect on their uptake by macrophages.

Some speculation about the mechanisms underlying the reduced uptake of apoptotic cells opsonized by SLE IgG is relevant. As suggested by Clancy et al (21) the antigens on apoptotic cells bound by autoantibodies may themselves be involved in the normal engulfment of apoptotic cells. SLE IgGs might block molecules on the apoptotic cells necessary for recognition and facilitation of their uptake by
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macrophages, like phosphatidylserine (PS) or C1q (22-25). Otherwise, we showed in our study that pre-incubation with a peptide blocking the IgG Fc-region or blockade of FcγRs on the macrophage abrogated the inhibitory effect of the SLE autoantibodies. This finding clearly indicates that FcγR engagement by the antibodies is necessary for the inhibitory effect. Additionally, the involvement of both CD64 and CD32 rules out the possibility that solely engagement of the inhibitory FcγRIIb was responsible for the inhibitory effect seen.

Additionally, signaling via the FcγR may influence the capacity of MDM to internalize additional apoptotic cells (26). Triggering of FcγR receptors influences the signals provided by other receptors involved in uptake. This may lead to altered kinetics of uptake compared to uptake of non-IgG opsonized apoptotic cells. Unfortunately, the intracellular signaling pathways of the most important uptake receptor of apoptotic cells, the PS receptor, has not been unraveled as it seems to lack a distinct intracellular signaling domain. Nevertheless both FcγRs and the PS-receptor (PSR) are thought to use Rho and Rac to elicit actin polymerization (27). The simultaneous signaling of both receptors may result in a disorganized actin reorganization, resulting in decreased uptake. On the other hand, PSR signaling also results in the release of anti-inflammatory mediators, while FcγR engagement is known to trigger pro-inflammatory mediators. How these two pathways are involved in the actual phagocytic process is not known, but they may counteract each other and hamper the uptake of apoptotic cells. Further elucidation of the underlying mechanism should focus on the study of changes in signaling in macrophages interacting with IgG opsonized apoptotic cells. This subject is currently being pursued in our laboratory.

The role of autoantibodies in the clearance of apoptotic cells has, until now, not been explored. Here, we show that autoantibodies inhibit apoptotic cell clearance which might further increase the apoptotic cell load in SLE patients.
Reference List


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