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SLE serum deposits C4d on red blood cells, decreases red blood cell membrane deformability, and promotes nitric oxide production

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Abstract

Objective—Systemic lupus erythematosus (SLE) is characterized by intravascular activation of the complement system and deposition of complement fragments (C3 and C4) on plasma membranes of circulating cells, including red blood cells (RBC). The aim of this study was to address whether this process affects the biophysical properties of RBC.

Methods—Serum and red blood cells were isolated from patients with SLE, and healthy controls. RBC from healthy O Rh negative individuals were incubated with SLE or control serum. We used flow cytometry to assess complement fragment deposition on RBC. RBC membrane deformability was measured using 2D microchannel arrays. Protein phosphorylation levels were quantified by western blot.

Results—Incubation of healthy donor RBC with sera from patients with SLE but not control sera led to deposition of C4 fragments on the RBC. Complement decorated RBC exhibited significant decrease in both membrane deformability and flickering. Sera from SLE patients triggered a transitory Ca^{++} influx in RBC that was associated with decreased phosphorylation of β-spectrin, and increased phosphorylation of band 3, two key proteins of RBC cytoskeleton. Finally, SLE but not control sera led to the production of nitric oxide (NO) by RBC.

Conclusion—Our data suggest that complement activation in patients with SLE leads to calcium dependent cytosketeletal changes in RBC that render them less deformable, likely impairing their flow through capillaries. This phenomenon may negatively impact the delivery of oxygen to the tissues.

Introduction

During systemic lupus erythematosus (SLE) flares, circulating immune complexes lead to intravascular complement consumption and deposition of nascent C3 and C4 fragments on red blood cell (RBC) membranes (1–3). Although this phenomenon is well established, very

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little is known about the effect of complement fragment deposition on RBC physiology in SLE.

In a recent communication, we showed that deposition of C3 and C4 complement fragments on RBC *in vitro*, promoted a significant decrease in RBC membrane deformability (4), which influences the ability of RBC to change shape in response to external forces (5,6). When RBC enter the microcirculation where the gas exchange takes place, the cell diameter changes rapidly from 7.5–8 μm to 4.5–5 μm. The dynamic, energy-dependent link between cell membrane and skeleton is essential for the ability of RBC to pass rapidly through capillaries without cell fragmentation and critical for maintenance of adequate tissue perfusion (7,8).

Based on our previous *in vitro* findings, we hypothesized that deposition of complement fragments on circulating RBC in SLE leads to impaired RBC deformability. Herein, we present evidence for the first time that incubation of RBC from healthy donors with sera from SLE patients resulted in complement fragment deposition, significant changes in the phosphorylation status of skeletal proteins, and decrease in RBC membrane deformability. Moreover, RBC with membrane bound complement produce increased amounts of nitric oxide (NO), an important mediator of pathophysiologic processes characterizing the immune system deregulation in SLE.

Patients and Methods

Subjects

Thirty-nine patients who fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (9) and twelve healthy donors donated 20 mL of blood for our studies (53 unique blood collections from SLE patients and 17 from controls). The serum was collected in serum-separator tubes and RBC in heparin-lithium tubes. The patients were 95% female with a mean age of 37.5 (23–59) years old. The controls were all female and had a mean age of 35 (20–52) years old. 56% of SLE patients and 58% of control were white; 23% of SLE patients and 25% of control were African American; and 20% of SLE patients and 17% of controls belonged to other races.

The disease activity was calculated using the SLE disease activity index (SLEDAI) (10). 68% of the patients were taking oral prednisone at a mean dose of 23 (2–60) mg per day. One patient was on intravenous high dose of methyl prednisone (1000 mg). Other immunosuppressive medications the patients were on at the time of the study: hydroxychloroquine (66%); azathioprine (19%); mycophenolate mofetil (30%). One patient was on intravenous monthly cyclophosphamide, one patient was taking cyclosporine and three patients were taking weekly methotrexate at the time of the study. Prednisone was held for at least 12 hours prior to the blood draw. The institutional review board of Beth Israel Deaconess Medical Center approved the study protocol and informed consents were obtained from all the study subjects.

Reagents

Antibodies (Abs) were obtained as follows: nonimmune IgG1, (BD Biosciences, San Jose, CA); anti-C4c mAb (A211, Quidel, San Diego, CA), anti-CR1 mAb 1F11 (a gift of Henry Marsh, PhD, Celldex Immunotherapeutics, Needham, MA), anti-actin, anti-phospho-serine mAb (Abcam, Cambridge, MA) secondary antibodies: AlexaFluor-488 goat anti–mouse IgG, AlexaFluor-594 goat anti–rabbit IgG, and AlexaFluor-594 goat anti–mouse IgG (Invitrogen, Carlsbad, CA); horseradishperoxidase (HRP)-goat anti–mouse IgG, and HRP-donkey anti–rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Reagents were obtained as follows: Hank's Buffer Saline Solution (HBSS) (Invitrogen), protease inhibitor

cocktail (Roche, Nutley, NJ); IgG-free BSA (Jackson ImmunoResearch). DAF-FM diacetate, Fluo-4 AM, and eosin 5 maleimide were purchased from Invitrogen.

RBC preparation and deposition of complement fragments

Blood was drawn from healthy adult volunteers in accordance with the guidelines of the Institutional Review Committee of Beth Israel Deaconess Medical Center and after informed consent was obtained in accordance with the Declaration of Helsinki. RBC from healthy universal donors (O Rh negative) were obtained by finger prick (~50μl), resuspended in 1mL of HBSS with Ca^{++} and Mg^{++} ($HBSS^{++}$) (Invitrogen), and washed twice before use. RBC were then resuspended in $HBSS^{++}$ with 0.5% IgG-free bovine serum albumin (BSA) (Jackson ImmunoResearch, West Grove, PA) at a hematocrit of 20%. RBC were incubated with 20% serum from either SLE patients or normal donors (matched for sex, age and race to the SLE patient) for 15 minutes at 37°C, and then washed twice and resuspended in $HBSS^{++}$.

Serum depletion of C4

One mL of control or SLE sera were incubated with 100 μl of protein A-Sepharose beads (Pierce, Rockford IL) coupled to either IgG control or anti-C4 mAb for 30 minutes at 4°C. To prevent complement activation due to immobilized IgG, EDTA was added to the sera to a final concentration of 5 mM for 10 minutes prior to immune depletion. At the completion of the reaction, Sepharose beads were removed by centrifugation and initial sera concentrations of Ca^{++} (1.5 mM) and Mg^{++} (1 mM) were restored.

Immunofluorescence microscopy

RBC isolated from healthy universal donors were incubated for 30 minutes at 37°C with 20% sera from healthy control or SLE patients diluted in HBSS with Ca^{++} and Mg^{++} ($HBSS^{++}$). Cells were then washed twice and incubated with 10 μg/mL anti-C4c mAb for 10 minutes in $HBSS^{++}$ with 0.5% BSA (staining buffer), washed, and stained with AlexaFluor-594 goat anti–mouse Ab for an additional 15 minutes. After staining, cells were washed twice, mounted in fluorescence mounting media (DakoCytomation, Carpinteria, CA), and imaged using an Olympus B×62 fitted with a cooled Hamamatsu Orca AG camera. The microscope, filters, and camera were controlled by iVision v. 4.0.9 (Biovision Technologies, Exton, PA).

Measurements of RBC membrane flickering

Phase contrast, time-lapse images of RBC seeded on microscope slides were recorded for 10 seconds representing 250 frames using a 100× UPlanApo phase contrast objective on an Olympus B×62 microscope (Olympus, Center Valley, PA). Changes in intensity due to oscillation of RBC membranes (flickering) were measured using iVision v. 4.0.9. At the end of each recording, an intensity projection step of the image stack was included to identify and exclude RBC that drifted during recording.

Flow cytometry

Fresh or serum-exposed RBC were incubated for 15 minutes withAb (as noted in the figure legends) in staining buffer at 4°C, followed by two washes and incubation for 15 minutes with AlexaFluor–488 labeled secondary Ab specific for each primary Ab at a dilution recommended by the manufacturer. After incubation with secondary Ab, RBC were washed once and analyzed using a LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). In all experiments, at least 10,000 events were recorded and analyzed (FlowJo, v. 9.0.1, TreeStar, Ashland, OR).

Eosin-5-maleimide (EMA) labeling: RBC were incubated with 25% control or SLE sera for 30 minutes at 37°C. RBC were washed and incubated in the presence of $HBSS^{++}$ with EMA at a final concentration of 0.1 mg/ml for 20 minutes in the dark at room temperature. RBC were then washed four times with 1000 μl of HBSS with Ca^{++} and Mg^{++} and analyzed by flow cytometry as described above.

Analysis of RBC calcium influx

RBC (~10^8) were preloaded with Fluo-4 AM for 15 minutes at room temperature, washed, and resuspended in HBSS with Ca^{++} and Mg^{++}. RBC were allow to sit at room temperature for an additional 10 minutes and washed again to remove any uncleaved Fluo-4 AM. Due to the ATP-depleting effect of the acetoxymethyl group, all experiments were performed within one hour from Fluo-4 AM loading of RBC. Fluorescence levels of RBC were acquired for 15 seconds using a LSRII flow cytometer (BD Biosciences) to establish a baseline for intra-RBC Ca^{++} concentration. Fifty μl of control or SLE sera (12.5% of the final volume) were then added to the RBC, and the fluorescence intensity associated with intra-RBC Ca^{++} concentration was recorded for an additional 3 to 5 minutes. Data were exported as FSC 3.0 files without the time dimension and analyzed using the kinetic module of FlowJo 9.0.1 (Treestar, Inc.).

Analysis of RBC NO production

RBC were preloaded with DAF-FM diacetate for 30 minutes at room temperature, washed, and resuspended in HBSS with Ca^{++} and Mg^{++}. RBC were then analyzed following the same protocol as described above for Ca^{++} influx measurements. Certain NO detection experiments were run for 40 minutes post serum challenge.

Western blot

RBC (3 μL) incubated with normal or SLE serum were lysed in 100 μL of 1× reducing-loading buffer (Invitrogen) and boiled for 4 minutes. Samples were run on 10% Bis-Tris gels (Invitrogen), transferred to nitrocellulose paper (Pierce), and blocked with 6% nonfat dry milk (Biorad, Hercules, CA) in Tris buffer with Tween 0.1% for 1 hour at room temperature. Membranes were then incubated with Ab as noted in figure legends for 30 minutes at room temperature, washed, and incubated with HRP-conjugated appropriate secondary Ab for an additional 30 minutes. Nitrocellulose membranes were washed extensively in Tris buffer with Tween 0.1% and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and developed using LAS 4000 imaging system (FujiFilm, Edison, NJ). The intensity profiles of the protein bands were analyzed using Quantity One v. 4.5.2 (BioRad) and plotted using GraphPad v. 4.0 (GraphPad Software, Inc. La Jolla, CA)

Fabrication of the 2D filters

To measure the ability of RBC to undergo capillary-like deformations, we fabricated a pseudo-2D analog of the 5 μm Nuclepore filter comprising an array of posts with 5 μm openings (channels) between them. The design and fabrication of this microchannel and configuration of the experimental setup have been described previously in detail (11). Briefly, we patterned a silicon wafer with an image of the 2D filter in sunken-relief using a direct laser writer (Heidelberg DWL 66, Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, Germany) and reactive ion etching (Bosch process, Unaxis SLR 770 ICP Deep Silicon Etcher, Unaxis USA Inc, St. Petersburg, FL) at the Cornell NanoScale Science & Technology Facility (Cornell University, Ithaca, NY. Immediately following assembly, the devices were filled with 1% solution of IgG-free BSA (Jackson ImmunoResearch Laboratories) in PBS buffer containing 0.01% sodium azide. We found that devices

fabricated this way can be stored at 4°C and at 100% humidity for at least 6 weeks without any measurable loss of functionality.

Measuring RBC deformability by 2D filters

To establish the flow of RBC through the 2D filter device the outlet of the 2D filter device was connected to a waste reservoir (60 mL syringe) (BD Biosciences) with a 60 cm long piece of PE-60 tubing (Instech Laboratories Inc, Plymouth Meeting, PA) filled with $HBSS^{++}$. The difference in level of liquid in the inlet of the 2D filter device and the level of liquid in the waste reservoir provided the driving pressure difference. The zero pressure difference corresponded to the absence of movement of RBC within the device. RBC (8 μl, 20% hematocrit) were loaded into the inlet reservoir and allow to enter the network (~ 1 minute) by lowering the waste reservoir tubing. Once RBC entered the capillary area, the waste reservoir tubing was reconnected to the waste reservoir at a height that allowed RBC to pass through the 25 μm length of the capillary in about 3 seconds. The passage of RBC through the 2D network was recorded using a 40×0.75 Ph2 Plan Fluorite objective on a TE300 Nikon inverted microscope, using a Retiga Exi (QImaging, Canada) CCD camera controlled by iVision (Biovision) at a rate of 10 frames/second. The time-stacks files were analyzed frame by frame and the results expressed as seconds necessary for a RBC to pass through a microchannel.

Results

Sera from SLE patients deposit C4d complement fragments on normal RBC

In order to evaluate the effect of complement deposition on RBC functions, we incubated normal RBC from universal donors (O Rh negative) with sera from 4 healthy controls or 10 SLE patients. In Figure 1A, we show that incubation of RBC with SLE sera promotes significant ($p=0.016$) C4d deposition on RBC membranes in contrast to sera from matched healthy controls. We then asked whether the deposition of complement fragments on RBC membranes is due to activation of the classical complement pathway by the immune complexes present in sera from SLE patients. To answer this question, we repeated the incubation of RBC from healthy universal donors with sera from control or SLE patients in the presence or absence of the Ca^{++} chelator, EGTA. As shown in Figure 1B, lower panel, removal of Ca^{++} by 10 mM EGTA inhibited significantly the deposition of C4d on RBC membranes promoted by SLE serum. Thus, our results suggest that activation of the classical complement pathway by either immune complexes present in SLE sera or anti-RBC Abs is responsible for the deposition of C4d on RBC membranes. To discriminate between these two mechanisms, we incubated RBC with sera from SLE patients and measured the levels of IgG and IgM on their membranes. Our results show that SLE sera did not promote significant IgG deposition on RBC membranes (Figure 1A supplemental), whereas two out of the five SLE sera tested led to IgM deposition on RBC membranes (Figure 1B supplemental). We concluded, therefore, that the observed deposition of C4d on RBC membranes is likely due to activation in solution of complement by circulating immune complexes (12, 13), followed by passive deposition of C4d on by-standing RBC (14). In certain cases, it is possible that non-hemolytic, complement fixing, anti-RBC IgM antibodies in SLE patients (15) deposit complement fragments directly on the surface of RBC. Finally, although in SLE complement is primarily activated through the classical pathway, the lectin pathway may also contribute to the generation of C4 fragments.

SLE serum decreases RBC membrane deformability

Since deposition of C3 and C4 fragments on RBC membranes leads to significant decrease in membrane deformability (4), we asked whether SLE serum-dependent deposition of C4d on normal RBC membranes would induce a similar decrease in membrane deformability.

RBC from universal donors were exposed to control or SLE sera from patients with mild to moderate disease activity. We then evaluated RBC membrane deformability by using a microfluidic device (11,16) (Figure 2A, top panel) and measuring the time necessary for RBC to pass through a microchannel. As a positive control for complement-mediated decreased RBC membrane deformability, we deposited complement fragments on RBC membranes by activating the alternative complement pathway using cobra venom factor and C7-deficient serum, as previously described (Figure 2B) (4). Our results (Figure 2C and summary graph 2D) show that incubation with SLE sera significantly delayed the passage time of RBC through the microchannels when compared to incubation with control sera (t_{sle}= 26.56 +/−20.83 s, vs. t_c=12.65 +/−4.35 s $p<0.001$). It has to be noted that there was significant heterogeneity in the effect of SLE serum on individual RBC. In addition, there was no clear correlation between patient's disease activity score or specific disease manifestations and the extent of loss of RBC membrane deformability induced by SLE serum.

Next, we asked whether circulating RBC from SLE patients also displayed the same phenotype. We analyzed 3 patients with active SLE (as defined by SLEDAI>4), 2 patients with inactive disease (SLEDAI<4) and 9 healthy controls. All patients with active disease showed significant decrease in membrane deformability expressed as increased passage time (17.86s +/− 3.01) when compared to normal control (10.11s +/− 1.59) or patients with inactive disease (11.325s +/− 0.46, p=0.001).

Complement deposition inhibits RBC membrane flickering

Besides membrane deformability, the ability of RBC to pass through capillaries is also impacted by RBC membrane flickering (17), a continuous oscillation of the RBC membranes at amplitudes varying between 20–400 nm and at frequencies between 0.2–20 Hz (18). We analyzed RBC membrane flickering by measuring changes in light scattering at the surface of RBC using time-lapse phase contrast microscopy, at a frame rate of 20 frames/second, as previously described (19). RBC from universal donors were incubated with sera from control (n=2) or SLE patients (n=3) and the extent of C4d deposition on RBC membrane was quantified before time-lapse recording of membrane flickering by fluorescence microscopy. As shown in Figure 3, we found an inverse correlation between the amount of C4d deposition on RBC membranes and the amplitude of membrane flickering. Changes in flickering amplitude were expressed as standard deviation (SD) of flickering values. The decrease in flickering amplitude of SLE sera-treated RBC (n=40 analyzed RBC) varied between 10–80% compared to the amplitude of normal serum treated RBC (n=35 analyzed RBC). Cells that showed an almost complete inhibition of membrane flickering (Figure 3C) represented about 5% of the total RBC population (Fig. 3D, summary graph). Most of the RBC displayed intermediate C4d staining (Figure 3B) that resulted in inhibition of flickering amplitude between 10–45%. Our results suggest that besides affecting the deformability of RBC membranes, SLE serum-induced complement deposition is associated with significant decrease in RBC membrane flickering.

SLE serum promotes RBC Ca^{++} influx and changes in phosphorylation levels of β-spectrin and band 3

RBC membrane deformability depends on the Ca^{++}-dependent phosphorylation status of cytoskeletal proteins such as β-spectrin and band 3 (20–22). We therefore asked whether serum from SLE patients induces a Ca^{++} influx in RBC that would initiate the events responsible for the observed decrease in RBC membrane deformability. RBC from healthy universal donors were preloaded with Fluo-4 AM for 15 minutes, washed, and incubated with sera from either healthy control or SLE patients, and changes in the intra-RBC Ca^{++} levels were recorded by time-lapse flow cytometry. In Figure 4A and 4B, we show that sera

from SLE patients, not from healthy controls, triggered a sustained (over 30 minutes) RBC Ca^{++} influx.

RBC cytoskeleton is comprised of elongated α- and β-spectrin tetramers associated with short actin filaments and accessory proteins that form a dense elastic network on the cytoplasmic side of plasma membrane (reviewed in (23)). Decrease in serine phosphorylation levels of β-spectrin was shown to correlate positively with reduced RBC membrane deformability (20). Therefore, based on our finding regarding the effect of SLE serum on RBC membrane deformability, we hypothesized that SLE serum promotes de-phosphorylation of β-spectrin. RBC from healthy universal donors were incubated with control or SLE sera, lysed and phosphorylation levels of β-spectrin were measured by western blot using anti-phospho-serine mAb. Our results (Figure 4C and D) show that compared to sera from healthy donors, sera from SLE patients decreased serine phosphorylation levels of β-spectrin, thus providing a mechanistic explanation for the decreased membrane deformability induced by SLE sera and measured by 2D microchannel array (Figure 2).

Conversely, decreased RBC membrane deformability was recently shown to be associated with increased levels of band 3 tyrosine phosphorylation (24). Therefore, we tested the effect of SLE sera on the tyrosine phosphorylation levels of RBC band 3 by flow cytometry using eosin 5 maleimide (EMA). The binding of EMA to lysine 430 on the extracellular loop of band 3 parallels tyrosine phosphorylation levels of band 3 through a mechanism that is currently poorly understood (24). In accordance with deformability data and β-spectrin phosphorylation results, incubation of RBC from normal universal donors with SLE sera (n=23) significantly increased (p=0.035) the tyrosine phosphorylation of RBC band 3 compared to healthy control serum (n=7) (Figure 4E and cumulative 4F).

To further prove that the effect of SLE serum on RBC membranes is due to complement deposition, we depleted the serum of an SLE patient of C4 by immuno-depletion and measured its biological efficacy in inducing band 3 phosphorylation. Our results show that depletion of SLE sera of C4 (Figure 5A) resulted in a diminished deposition of C4 fragments on RBC membrane compared to the original SLE sera and modest phosphorylation of band 3 (Figure 5B).

Overall, these results suggest that SLE serum induced decrease in RBC deformability is mediated by altered Ca^{++} dependent phosphorylation of cytoskeletal proteins.

SLE serum induces the production of nitric oxide by RBC

RBC express functional endothelial nitric oxide synthase (eNOS, NOS3), which allows them to generate biologically active nitric oxide (NO) (25). RBC eNOS is activated by shear stress-generated Ca^{++} influx, L-arginine, and phosphorylation *via* PI3K (26,27). We therefore hypothesized that Ca^{++} influx triggered by sera from SLE patients would increase RBC production of NO. RBC from normal universal donors were preloaded with DAF-FM diacetate and incubated with sera from healthy control or SLE patients. Our results (Figure 6A and 6B) show that sera from SLE patients promoted a significant increase (p= 0.028) in intra-RBC production (MFI= 151.4+/−47.53) of NO when compared to sera from healthy individuals (MFI=104.8 +/−4.42), suggesting that in SLE, complement-decorated RBC could represent a significant source of intravascular NO.

In conclusion, we present evidence for the first time that in SLE, complement fragment deposition on RBC membrane alters the phosphorylation pattern of spectrin and band 3, decreases membrane deformability and flickering, and promotes RBC production of NO.

Discussion

SLE is characterized by ongoing activation of complement followed by deposition of complement fragments on circulating RBC (28). Here we present evidence that *ex vivo* deposition of C4d on RBC significantly decreases RBC membrane deformability and flickering, thus negatively impacting the ability of RBC to flow through capillary-like microchannels. The underlying mechanism for this phenomenon is SLE serum induced Ca^{++} influx that changes the phosphorylation status of the key cytoskeletal proteins β-spectrin and band 3. In addition, SLE serum incubated RBC generate NO in significant amounts.

The complete range of functional repercussions of the observed alterations in RBC deformability by SLE serum remains to be determined. Diminished RBC membrane deformability may result in decreased ability of RBC to deliver O_2 in certain tissues with narrow capillaries (4–6μm in diameter) such as the brain and muscle (29,30). Our observation that SLE RBC have decreased membrane deformability may explain, together with chronic anemia, some of the constitutional symptoms of patients with SLE such as chronic fatigue (31) and cognitive dysfunction (32,33).

Another important observation in this study is that complement decorated RBC produce significant amounts of NO. RBC-generated NO has been recently recognized as a key signaling molecule, directly affecting vascular endothelial cells and smooth muscle cells during physiological conditions. (34). Moreover, NO was also identified as a significant factor in promoting signaling abnormalities in SLE T cells, and inducing mitochondrial proliferation and hyperpolarization (35,36). Mechanistically, extracellular NO and its derivatives, such as peroxynitrate, have been directly implicated in the pathogenesis of SLE by modifying the functions of key enzymes and altering the immunogenicity of self-antigens (reviewed in (37)). Thus, our findings regarding complement-induced RBC NO production strongly suggest that RBC may play a significant role in SLE pathophysiology.

In conclusion, we describe herein the effect of SLE serum on RBC membrane deformability and NO production. The altered RBC functional properties that we described could explain several of the systemic and local manifestations of SLE. Therefore, RBC may not only prove to be a good SLE biomarker but also an important therapeutic target for intercepting abnormal signaling events that characterize SLE immunopathology.

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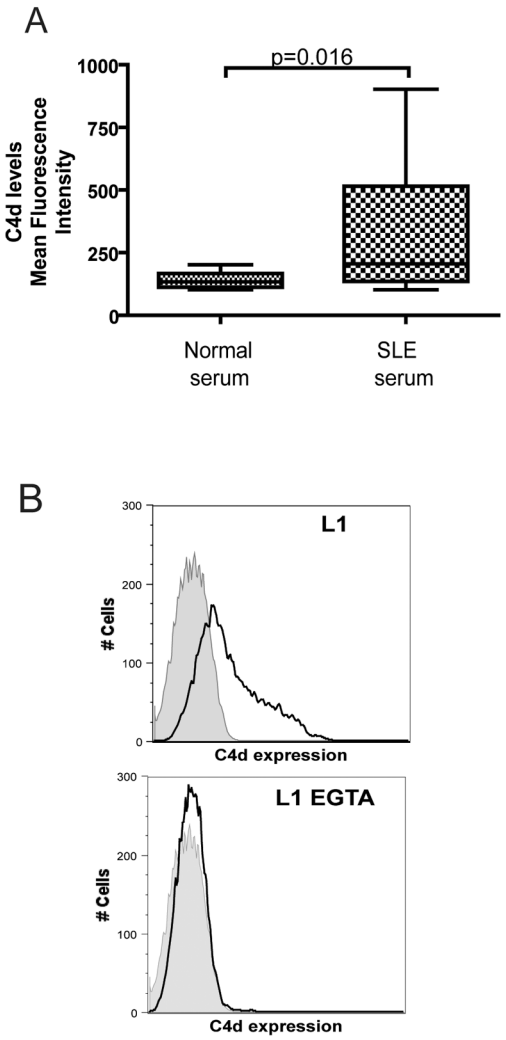

Figure 1.

SLE sera promote C4d deposition on RBC membranes. RBC from healthy individual blood group O Rh negative were incubated with sera from healthy volunteers or SLE patients. The expression of C4d on the surface of RBC was measured with flow cytometry and expressed as mean fluorescent intensity (MFI). A) Cumulative results of C4d deposition on RBC incubated with sera from healthy individuals (n=7) or SLE patients (n= 27). B) Healthy control RBC were incubated with SLE serum in the presence (top) or absence (bottom) of EGTA. A representative (out of three) experiment is shown here.

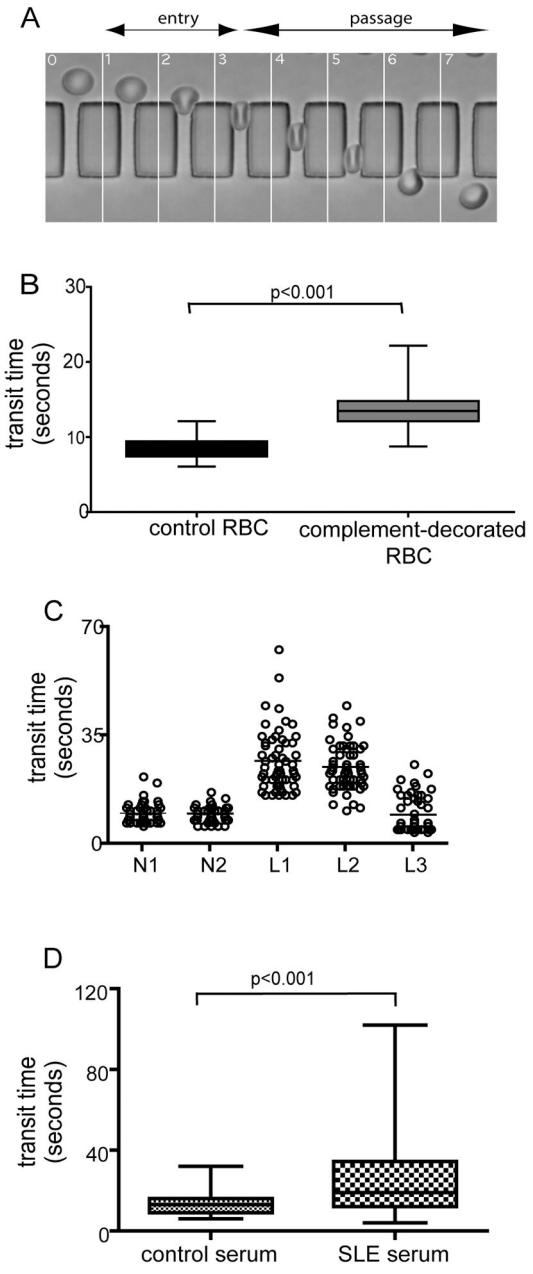

Figure 2.

SLE sera decrease RBC membrane deformability. A) Serial snapshots of an RBC passing through a 2D filter. B) RBC were incubated with C7-depleted serum and cobra venom factor in the presence (control RBC) or absence (complement-decorated RBC) of EGTA. The RBC deformability was then analyzed using 2D filters (see Methods). C) RBC from a healthy universal donor were incubated with sera from healthy individuals (N1, 2), or SLE patients (L1, 2, 3). RBC deformability was measured using 2D filters. Each dot represents one RBC. D) Cumulative results from 4 control and 10 SLE sera.

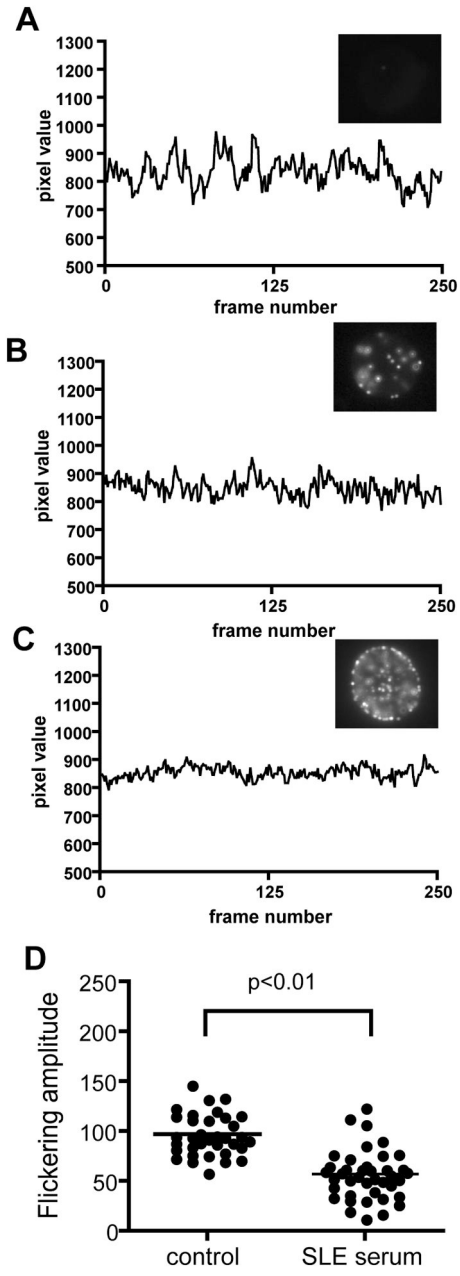

Figure 3.

Sera from SLE patients inhibit RBC membrane flickering. RBC from a healthy universal donor were incubated with serum from SLE patients and stained for C4d deposition as described in Methods. The membrane flickering of RBC displaying no C4d deposition (A, inset), moderate C4d deposition (B, inset) and high C4d deposition (C, inset) is shown here. These are representative results of three independent experiments. D) Cumulative results from 35 RBC incubated with sera from 2 controls and 40 RBC incubated with sera from 3 SLE patients.

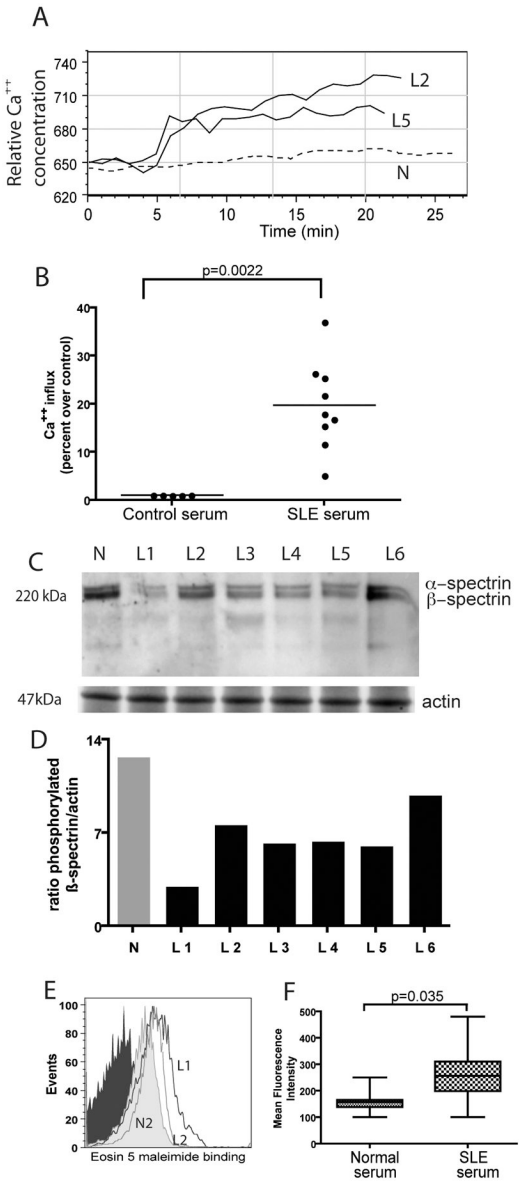

Figure 4.

Sera from SLE patients trigger Ca^{++} influx and change the phosphorylation profile of β-spectrin and band 3 in RBC from healthy donors. A) RBC from a healthy universal donor were preloaded with Fluo-4 AM and incubated with serum from control (N), or SLE patients (L 2,5) and RBC Ca^{++} influx measured by flow cytometry. B) Summary graph showing the Ca^{++} influx triggered by SLE serum displayed as percent over control. The mean value of the fluorescence intensity associated with Ca^{++} concentration over the entire recording was used as representative fluorescence for each sample. C) SLE serum induces de-phosphorylation of β-spectrin. RBC from a healthy universal donor were incubated with serum from a healthy control (N), or SLE patients (L 1–6) for 15 minutes at 37°C, lysed and the phosphorylation levels of β-spectrin detected using anti-phospho serin/threonin mAb. Lower panel represents actin loading control of RBC samples. D) Ratio between phosphorylated β-spectrin and actin levels. E) SLE serum induces phosphorylation of band 3. Band 3 phosphorylation in RBC was measured after staining RBC with EMA as described

in Methods. RBC incubated with normal serum (N2, filled light grey histogram), or SLE sera (L1, L2, open histograms) were stained with EMA and analyzed by flow cytometry. Autofluorescence of unlabeled RBC is shown as filled dark grey histogram. F) Cumulative results of band 3 phosphorylation in RBC incubated with control (n=7) or SLE (n=23) sera.

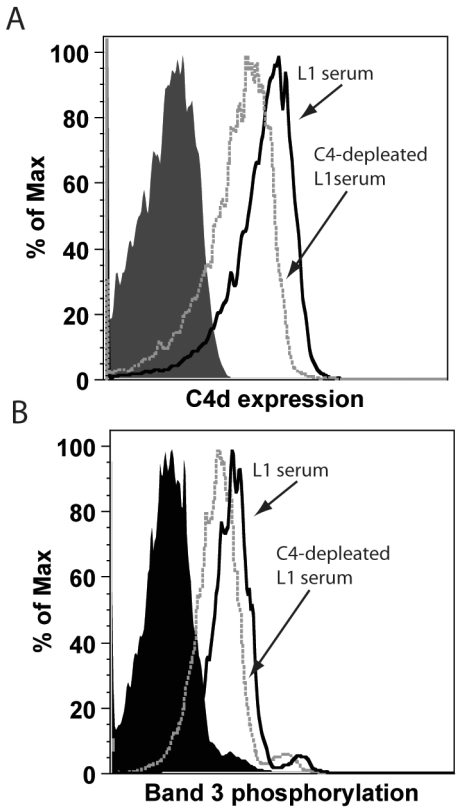

Figure 5.

Depletion of C4 from SLE serum prevents increase in RBC band 3 phosphorylation. SLE serum was incubated with either IgG control (control serum) or anti-C4c mAb coupled to Sepharose beads (C4-depleted serum) in the absence of Ca^{++} and Mg^{++} for 30 minutes. RBC were then incubated with either the control (continuous histogram) or the C4-depleted serum (dotted histogram) and the RBC expression levels of C4d (A) and band 3 phosphorylation (B) were measured by flow cytometry as described above.

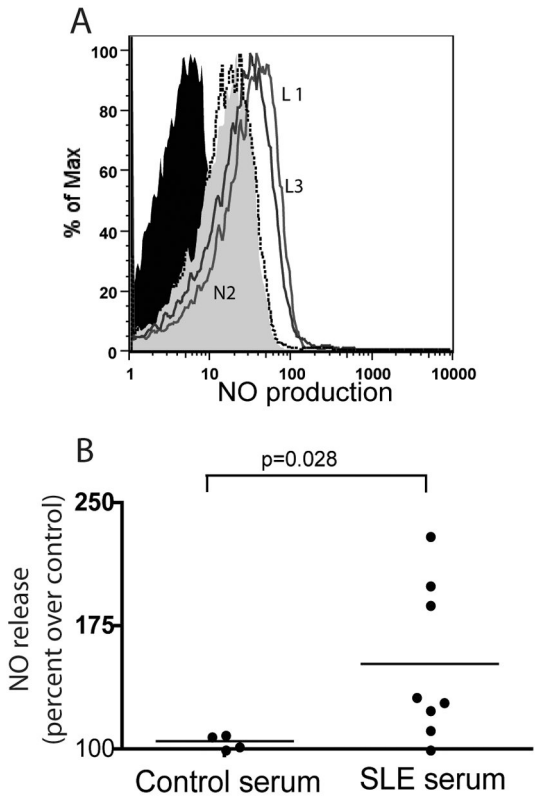

Figure 6.

SLE serum induces the production of nitric oxide (NO) by RBC. A) RBC preloaded with DAF-FM were incubated with serum from normal individuals (N2) or SLE patients (L1, 3) for 15 minutes at 37°C. The fluorescence due to normal serum is shown as light gray histogram. RBC autofluorescence is shown as dark grey histogram. N stands for normal control and L for SLE patient serum. B) Cumulative data of RBC NO production generated with sera from 4 controls and 8 SLE patients displayed as percent over control.