

Reduced expression of CD44 on monocytes and neutrophils in systemic lupus erythematosus: relations with apoptotic neutrophils and disease activity

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Abstract

Background—Increased numbers of apoptotic neutrophils, and impaired monocyte/macrophage clearance of apoptotic cells, have been demonstrated in systemic lupus erythematosus (SLE). CD44 is implicated in the clearance of apoptotic neutrophils.

Objective—To determine the expression of CD44 on peripheral blood monocytes and neutrophils in SLE, and examine the relations with disease activity and numbers of circulating apoptotic neutrophils.

Methods—Peripheral blood was sampled from 31 patients with SLE, 19 healthy normal subjects, and 19 patients with rheumatoid arthritis (RA). Monocyte and neutrophil density of surface CD44 expression was determined by immunofluorescence labelling and flow cytometry, and results expressed as mean channel fluorescence (MCF) values. Neutrophil apoptosis was measured by morphology in 15 patients with SLE, nine with RA, and six normal subjects.

Results—Monocyte CD44 expression was significantly lower in SLE (median MCF 4.71) than in healthy normal subjects (median MCF 5.61) and controls with RA (median MCF 5.39). Neutrophil CD44 expression was also significantly lower in SLE (median MCF 1.95) than in healthy normal subjects (median MCF 2.37) and controls with RA (median MCF 2.60). Monocyte, but not neutrophil, CD44 expression correlated negatively with the percentage of apoptotic neutrophils. There was no significant correlation of monocyte or neutrophil CD44 expression in SLE with disease activity or damage.

Conclusions—Monocyte and neutrophil CD44 expression is reduced in SLE, and this may contribute to the impaired recognition and clearance of apoptotic neutrophils by monocyte derived macrophages.

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Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease in which tissue damage can best be explained by the production of pathogenic autoantibodies and their participation in immune complex formation. The description of nuclear autoantigen expression (including Ro, La, and dsDNA) on

cytoplasmic blebs of apoptotic cells provides an attractive explanation for the unique clustering of autoantibodies in SLE.¹

Apoptosis is a carefully governed process which brings about cell death in a regulated physiological context without release of inflammatory mediators. During this process enzymatic internucleosomal splicing of nucleic acids leads to the generation of nucleoproteins, which are packaged into membrane bound blebs or apoptotic bodies. Apoptotic cells and bodies are normally rapidly recognised and cleared, especially by local monocyte derived macrophages, thus limiting any possibility of antigenic exposure. Therefore it can be proposed that failed monocyte/macrophage clearance of apoptotic cells will lead to secondary necrosis and release of novel clusters of autoantigens uniquely able to trigger and/or sustain autoimmunity in SLE.

Mechanisms underpinning the specific recognition, engulfment, and clearance of apoptotic cells by macrophages are being defined, and a number of different molecules may have a role, including complement components and receptors,^{2,3} the phosphatidylserine receptor, and the vitronectin receptor.⁴ There is evidence to suggest that decreased phagocytosis of apoptotic cells does occur in SLE.⁵⁻⁸ These studies show deficiency in the phagocytic capacity of mononuclear phagocytes for apoptotic lymphocytes, possibly related to faulty recognition of phosphatidylserine. Our group has shown an increase of circulating apoptotic neutrophils, correlated with disease activity, in SLE. Surface expression of the glycoprotein Fas (APO-1, CD95), the key signalling molecule for apoptosis in neutrophils, was raised above normal in SLE, but also in rheumatic and non-rheumatic inflammatory disease controls. The latter, however, did not show an increase in circulating apoptotic neutrophils; one explanation for these findings is failure to clear apoptotic neutrophils in SLE.⁹

CD44 is a glycoprotein cell surface receptor for the extracellular matrix molecules hyaluronan, fibronectin, collagen, and fibrin. It is widely expressed in a number of tissues, including leucocytes, keratinocytes, chondrocytes, many epithelial cell types, and some endothelial and neural cells.¹⁰ A number of different functions have been ascribed to CD44, including cellular adhesion and migration, lymphocyte activation and proliferation, and tumour cell metastasis.

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Hart *et al* have shown that phagocytosis of apoptotic neutrophils, but not lymphocytes, by human monocyte derived macrophages was augmented rapidly after ligation of CD44 by bivalent antibodies *in vitro*.¹¹ This suggests that CD44 has a role in the clearance of apoptotic neutrophils by monocyte derived macrophages.

We proposed the hypothesis that deficient monocyte or neutrophil surface expression of CD44 in SLE could provide an explanation for the impaired recognition and clearance of apoptotic neutrophils, resulting in the increased circulating numbers of apoptotic neutrophils seen.

Patients and methods

PATIENTS AND CONTROLS

All patients with SLE fulfilled American College of Rheumatology (ACR) criteria for diagnosis,¹² and were recruited from outpatient clinics and rheumatology inpatient wards. Disease controls with rheumatoid arthritis (RA) were also recruited. Patients receiving parenteral corticosteroids, cyclophosphamide, or tumour necrosis factor blocking agents were excluded from the study to avoid the unpredictable effect of large boluses of these drugs before blood sampling. Ethical approval was granted and written informed consent obtained from all patients. Normal healthy volunteers were recruited from hospital staff.

Thirty one Caucasian patients with SLE (28 female, 3 male, median age 50), 19 Caucasian patients with RA (17 female, 2 male, median age 51), and 19 Caucasian normal healthy volunteers (17 female, 2 male, median age 48) were studied.

CLINICAL INFORMATION

Clinical details were recorded after full history and examination and review of hospital charts. Drug treatment, including prednisolone dosage, was recorded for all patients. For patients with SLE the history or absence of each ACR diagnostic criterion was recorded. Disease activity was assessed by the SLAM (Systemic Lupus Activity Measure) and the BILAG (British Isles Lupus Activity Guide) scores.¹³ Organ damage was assessed by the American College of Rheumatology/Systemic Lupus International Collaborating Clinics (ACR/SLICC) score.¹⁴ RA disease activity was

assessed by the Modified Disease Activity Score (DAS28).¹⁵ Full blood picture, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), and complement C3 and C4 fractions were measured in all patients. Serum antibodies to dsDNA were measured by the Farr technique in the patients with SLE.

IMMUNOFLUORESCENCE LABELLING

Blood samples were collected in EDTA and immunolabelled within two hours. Aliquots of 100 μ l whole blood were incubated for 15 minutes at 4°C with 10 μ l of FITC labelled monoclonal antihuman pan-reactive CD44 (Clone DF1485, Dako). FITC labelled IgG (Dako) was incubated with a control sample to allow subtraction of non-specific staining. The cells were washed once with phosphate buffered saline (PBS), then red cells were lysed with Immunolyse (Beckman Coulter) according to the manufacturer's instructions. The cells were washed three times with PBS and then suspended in 300 μ l of 1% paraformaldehyde in PBS before flow cytometric analysis.

FLOW CYTOMETRY

Flow cytometric analyses were performed on a Coulter EPICS ELITE instrument, standardised for inter- and intra-run variability by calibration with Immunocheck fluorospheres (Coulter). Monocytes and neutrophils were identified by forward and side light scatter properties. Five thousand cells were analysed for each sample, and the percentage of cells expressing CD44, and density of surface expression (expressed as mean channel fluorescence (MCF) values) determined after subtraction of non-specific staining as identified by the isotype control histogram (fig 1).

NEUTROPHIL ISOLATION AND MORPHOLOGY

Peripheral blood neutrophils were isolated as previously described.¹⁶ Briefly, heparinised whole blood samples were centrifuged at 400 *g* for 20 minutes, and the cell layer obtained mixed with warm dextran and saline and allowed to stand for red cell sedimentation to occur. The leucocyte fraction obtained was washed once in PBS, then layered onto gradients of 55%, 70%, and 81% Percoll solution (Pharmacia) and centrifuged at 750 *g* for 20 minutes at room temperature. The neutrophil fraction was carefully aspirated from the

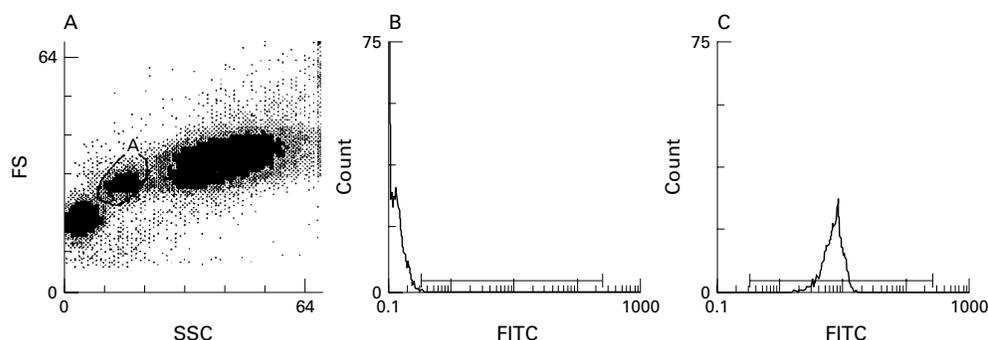


Figure 1 (A) Flow cytometry scatter plot demonstrating the three leucocyte populations—from left to right: lymphocytes, monocytes (gated), and neutrophils; (B) gated monocyte IgG isotype control histogram; (C) gated monocyte CD44 staining histogram—in this sample 99.3% of gated cells express CD44 with a mean channel fluorescence of 6.74.

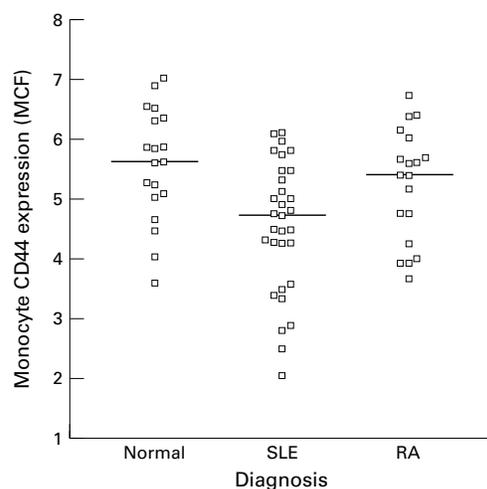


Figure 2 Monocyte CD44 expression in normal subjects, patients with systemic lupus erythematosus (SLE), and patients with rheumatoid arthritis (RA). Each point represents one patient, and the median value for each group is shown.

70%/81% interface, and washed in PBS before preparation of cytocentrifuge slides. Slides were stained with modified Wright's stain, and neutrophils with morphological features of apoptosis identified and counted under high power view in 200 cells. Samples of isolated neutrophils were available from 15 patients with SLE, nine with RA, and six normal subjects.

STATISTICAL ANALYSIS

Mann-Whitney U tests were performed to analyse the significance of differences between the groups. Spearman's correlation coefficients were calculated to determine the relations of monocyte and neutrophil density of surface CD44 expression with percentage apoptotic neutrophil count, and measures of disease activity, damage, and treatment. *p* Values <0.05 were deemed significant. These calculations were performed with the SPSS for windows v10 statistical package.

Results

MONOCYTE CD44 EXPRESSION

CD44 was expressed by over 99% of monocytes in patients with SLE, patients with RA, and normal subjects. Monocyte density of surface CD44 expression (fig 2) was significantly reduced in SLE (median MCF 4.71, interquartile range (IQR) 3.56–5.46) compared with normal subjects (median MCF 5.61, IQR 5.02–6.35, *p*=0.002), and in patients with SLE compared with patients with RA (median MCF 5.39, IQR 4.23–6.01, *p*=0.046). There was no significant difference of monocyte density of surface CD44 expression when normal subjects were compared with the patients with RA.

NEUTROPHIL CD44 EXPRESSION

CD44 was expressed by over 99% of neutrophils in patients with SLE, patients with RA, and normal subjects. Neutrophil density of surface CD44 expression (fig 3) was significantly reduced in SLE (median MCF 1.95,

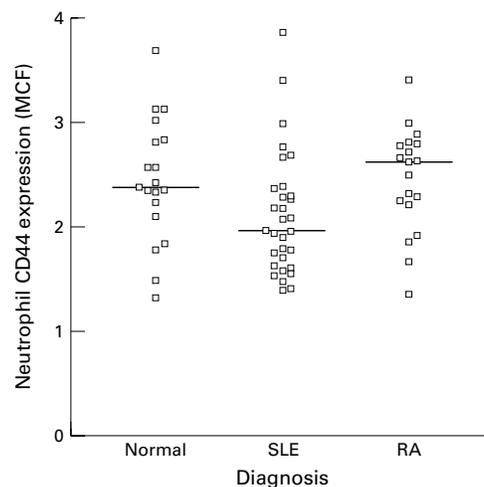


Figure 3 Neutrophil CD44 expression in normal subjects, patients with systemic lupus erythematosus (SLE), and patients with rheumatoid arthritis (RA). Each point represents one patient, and the median value for each group is shown.

IQR 1.61–2.35) compared with normal subjects (median MCF 2.37, IQR 2.09–2.83, *p*=0.025), and in patients with SLE compared with patients with RA (median MCF 2.60, IQR 2.19–2.78, *p*=0.016). There was no significant difference of neutrophil density of surface CD44 expression when normal subjects were compared with the patients with RA.

NEUTROPHIL MORPHOLOGY

There were significantly increased apoptotic neutrophils, as assessed by morphology, in the SLE group (median 4.00%, IQR 2.00–5.00%) compared with normal subjects (median 1.00%, IQR 0.75–2.00%, *p*=0.002) and patients with RA (median 1.00%, IQR 1.00–2.75%, *p*=0.007). There was no significant difference in the percentage of apoptotic neutrophils between the normal and RA groups. A significant negative correlation of monocyte density of surface CD44 expression with percentage apoptotic neutrophil count (Spearman's correlation, $r_s = -0.41$, *p*=0.026) was seen (fig 4). Neutrophil density of surface

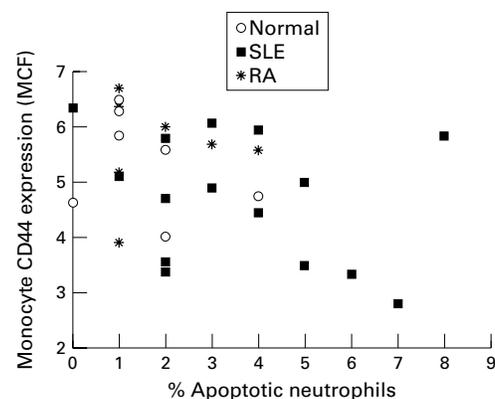


Figure 4 Scatter plot showing the negative correlation of monocyte CD44 expression with percentage apoptotic neutrophils ($r_s = -0.41$, *p*=0.026). Data from patients with systemic lupus erythematosus (SLE), patients with rheumatoid arthritis (RA), and normal subjects are included, and each point represents one patient. MCF = mean channel fluorescence.

CD44 expression did not correlate with percentage apoptotic neutrophil count. There was no significant difference in monocyte or neutrophil density of surface CD44 expression between those patients who had apoptotic neutrophil counts determined, and those who did not.

SLE DISEASE ACTIVITY AND DAMAGE

The median number of ACR criteria (for diagnosing SLE) fulfilled was 6 (IQR 6–8). The total number of patients with SLE (n=31) fulfilling each ACR criterion was as follows: malar rash 18, discoid rash 6, photosensitivity 25, oral ulcers 19, arthritis 13, serositis 6, renal disorder 7, neurological disorder 5, haematological disorder 27, immunological disorder 24, anti-nuclear antibody 29. The median SLAM score was 8 (IQR 6–11). The total BILAG score was calculated by attributing scores of 4, 3, 2, and 1 to letters a, b, c, and d, respectively for each group of disease manifestations. Median total BILAG score was 11 (IQR 9–15). There were no significant correlations between monocyte or neutrophil density of surface CD44 expression and SLAM or BILAG scores.

There was no correlation between ESR, CRP, dsDNA, C3 or C4 levels and monocyte or neutrophil density of surface CD44 expression. The median ACR/SLICC damage score was 1 (IQR 0–1). There was no correlation of damage score with monocyte or neutrophil density of surface CD44 expression.

No correlations were seen between apoptotic neutrophil count and BILAG, SLAM, SLICC, ESR, or complement components. The correlation between dsDNA titre and apoptotic neutrophil count in SLE was of borderline significance ($r_s=0.513$, $p = 0.051$).

RHEUMATOID ARTHRITIS DISEASE ACTIVITY

The median DAS28 score was 4.73 (IQR 4.24–6.14). There was no correlation between the DAS28 score, CRP, or ESR levels and monocyte or neutrophil density of surface CD44 expression.

TREATMENT

Nineteen patients with SLE (61%) were receiving oral corticosteroids; the median dose was 10 mg (IQR 7.5–15.0). The prednisolone dose did not correlate with monocyte or neutrophil density of surface CD44 expression in SLE. There was no significant difference in monocyte or neutrophil density of surface CD44 expression between patients with SLE receiving corticosteroids, and those not. Other drugs recorded were hydroxychloroquine (n=18), non-steroidal anti-inflammatory drugs (NSAIDs) (n=10), azathioprine (n=6), and methotrexate (n=2). There was no difference in monocyte density of surface CD44 expression between patients with SLE taking any of these drugs. Neutrophil density of surface CD44 expression was higher in patients with SLE taking NSAIDs (median MCF 2.36, IQR 2.09–2.81) than in those not taking them (median MCF 1.78, IQR 1.56–2.12,

$p=0.004$). There was no difference in neutrophil density of surface CD44 expression between patients with SLE taking any other drugs.

Seven patients with RA (37%) were receiving oral corticosteroids; the median dose was 10 mg (IQR 5.0–12.0). The prednisolone dose did not correlate with monocyte or neutrophil density of surface CD44 expression in RA. There was no significant difference in monocyte or neutrophil density of surface CD44 expression between patients with RA receiving corticosteroids, and those not. Other drugs recorded were NSAIDs (n=11), methotrexate (n=3), sulfasalazine (n=1), hydroxychloroquine (n=1), leflunomide (n=1), D-penicillamine (n=4). There was no significant difference in monocyte or neutrophil density of surface CD44 expression between patients with RA taking any of these drugs.

Discussion

As far as we know this is the first reported study to demonstrate reduced density of surface expression of CD44 on circulating neutrophils and monocytes in SLE. The experimental design using fresh whole blood for immunofluorescence labelling was chosen to avoid the possible artefacts associated with cell separation and culture techniques.¹⁷ As CD44 may have a role in regulating the phagocytosis of apoptotic neutrophils by monocyte/macrophages, this study provides a possible explanation for the impaired recognition and clearance of apoptotic neutrophils by monocyte/macrophages in SLE.

The increased apoptotic neutrophils, as assessed by morphology, seen in this study are in keeping with previous studies showing increased apoptotic leucocytes in SLE,^{8, 18, 19} including a study by our group demonstrating increased apoptotic neutrophils, as assessed by annexin V immunofluorescence, in 50 patients with SLE.⁹ This is concordant with the hypothesis that impaired clearance of apoptotic neutrophils contributes to the pathogenesis of SLE.

A number of different molecules mediating recognition and clearance by monocyte/macrophages have been described and it seems likely that clearance may be mediated using a combination of these molecules. These include engagement of lectins, CD51/61 ($\alpha v\beta 3$ vitronectin receptor),^{20, 21} CD36 (thrombospondin receptor),^{22, 23} and a phosphatidylserine receptor.⁴ Complement components and receptors (C1q, CR3, CR4) have also been proposed as important contributors to apoptotic cell clearance.^{2, 3} C1q deficiency is strongly associated with the development of SLE,²⁴ and the binding of C1q to phagocytic cell surfaces has been shown to enhance phagocytosis.²⁵ These studies suggest that C1q deficiency contributes to autoimmunity by impairment of the clearance of apoptotic cells. Serum factors other than complement components have been implicated in the clearance of apoptotic cells. CRP has been shown to bind to nuclear components such as snRNPs and chromatin,^{26, 27} and when complexed to activate complement by the classical pathway.²⁸ This

binding may facilitate the clearance of apoptotic cells. Patients with SLE with active disease have inappropriately low levels of CRP,²⁹ and this may contribute to the impaired clearance of apoptotic cells. Reduced monocyte surface expression of CD14 has been described in SLE, and this may also contribute to the deficient clearance of apoptotic cells.^{30–31} Different populations of macrophages use different recognition receptors,⁴ and separate populations of white cells may use specific clearance pathways.

This study provides evidence of decreased surface expression of CD44 on lupus monocytes and neutrophils. This reduced CD44 expression may contribute to impaired clearance by interfering with binding and/or phagocytosis at both the neutrophil and monocyte/macrophage surface. CD44-induced clearance may be mediated by the triggering of intracellular pathways, leading to increased phagocytosis. CD44 ligation in lymphocytes has been shown to induce tyrosine phosphorylation of p56lck and other intracellular proteins,³² and to raise cAMP concentrations.³³ Additionally, CD44 may form associations with cytoskeletal molecules to facilitate clearance.³⁴ However, the exact mechanism by which CD44 regulates clearance remains to be elucidated.

No correlations of CD44 expression with disease activity or damage scores were seen, suggesting that the reduced CD44 expression is not solely related to disease activity or damage. The association of higher neutrophil CD44 expression in patients with SLE taking NSAIDs is interesting, though it is difficult to speculate on the significance or mechanism of this. It is hard to distinguish disease activity from treatment effects in a disease such as lupus, though SLAM and BILAG scores were not significantly different between patients with SLE taking NSAIDs and those not. The lack of correlation with prednisolone treatment or dosage is in keeping with one study which showed no effect of pulse methylprednisolone on CD44 expression on synovial or peripheral blood neutrophils in RA.³⁵

The negative correlation of monocyte density of surface CD44 expression with apoptotic neutrophil count is in keeping with the hypothesis that reduced CD44 expression on lupus monocyte/macrophages may impair their ability to recognise and/or phagocytose apoptotic neutrophils. This correlation includes data from all three studied groups, suggesting that, as expected, the role of CD44 in the clearance of apoptotic neutrophils is not confined to patients with SLE. The lack of correlation between neutrophil density of surface CD44 expression and apoptotic neutrophil count suggests that reduced CD44 expression on neutrophils is not simply caused by membrane changes during apoptosis.

Estess *et al* have reported increased CD44 mediated, T lymphocyte adhesion (or “rolling”) to a model of vascular endothelium in a population of paediatric patients with active SLE.³⁶ We also determined the density of surface CD44 expression on lymphocytes and found this to be significantly lower in SLE than

in normal controls, though not significantly different from RA controls (data not shown). These two studies are not, however, directly comparable, as Estess *et al* used a functional assay of T lymphocyte adhesion in a flow chamber (another of the many functions attributed to CD44), whereas we measured the density of cell surface CD44 expression, in a very different (adult) SLE population.

Increased expression of CD44 has been described on synovial lymphocytes,^{37–38} and synovial monocyte/macrophages in RA,^{39–40} and CD44 blocking or depleting treatment has been suggested as a possible treatment for RA.^{41–44} CD44 expression is also up regulated on a number of tumour cell types, and anti-CD44 treatment has been studied in the treatment of malignant disease.⁴⁵

Why should CD44 expression be reduced on SLE leucocytes? A possible explanation is the effect of the surrounding cytokine environment. SLE is a disease associated with raised circulating levels of interleukin 10 (IL10).⁴⁶ Osada *et al* have shown that IL10 down regulated the expression of CD44 on epidermal Langerhans cells in a concentration dependent manner.⁴⁷ In contrast, tumour necrosis factor α (TNF α) up regulated the CD44 expression. Conceivably, these cytokines would have similar effects on CD44 expression on other cell types, though no data on leucocytes are presently available. This might also explain the increased CD44 expression on synovial cells in RA, an environment characterised by raised TNF α levels.

Possible new treatments of SLE may involve augmenting the clearance of apoptotic cells, possibly by devising means to up regulate the surface expression of deficient, clearance related molecules. It might be speculated that because of the association of raised CD44 in rheumatoid synovium and some tumours, increasing CD44 expression, generally, could theoretically increase the risk of developing rheumatoid-like disease or even malignancy. Equally, any anti-CD44 treatment proposed for RA or malignancy has the theoretical risk of inducing lupus-like side effects, and should be carefully monitored for such.

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