EXTENDED REPORT

Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells

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Ann Rheum Dis 2006;**65**:216–221. doi: 10.1136/ard.2005.037143

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Accepted 3 July 2005
Published Online First 13 July 2005

Background: It has been suggested that defective handling of apoptotic cells by macrophages plays a key role in the development of systemic lupus erythematosus (SLE). The relative contribution of intrinsic defects and serum factors remains controversial.
Objective: To compare monocytes from SLE patients, patients with rheumatoid arthritis, and healthy controls for their ability to differentiate in vitro into macrophages and to bind/engulf apoptotic cells.
Methods: Peripheral blood derived monocytes from healthy donors or from patients with SLE or rheumatoid arthritis were allowed to differentiate into macrophages. The in vitro uptake of apoptotic cells by macrophages was evaluated by a flow cytometry assay that allowed discrimination between binding and internalisation.
Results: Monocytes from SLE and rheumatoid patients showed a striking defect in adherence to plastic compared with healthy donors. Absence or heat inactivation of serum resulted in a reduction in the binding and engulfment of apoptotic cells by macrophages. Macrophages from rheumatoid and SLE patients had similar percentages of apoptotic cells bound to their surface compared with normal controls. However, macrophages from SLE patients showed a significant defect in the internalisation of apoptotic cells compared with those from healthy controls, even in the presence of normal human serum.
Conclusions: Monocytes from patients with SLE and rheumatoid arthritis have a similar defect in their capacity to adhere to plastic. However, only macrophages from SLE patients showed an impaired ability to engulf apoptotic cells, which indicates that an intrinsic cellular defect may be responsible for this phenomenon.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a complex pathogenesis that is only partly understood.[1] Several lines of evidence suggest that macrophages play an important role in the disease process, along with other components of the immune system. It has been suggested that the clearance by macrophages of cells undergoing programmed cell death, also known as apoptosis, may be crucial to avoid an immune response to nuclear antigens that become exposed on the cell surface in the process of apoptosis.[2 3] The clearance of apoptotic cells by macrophages may also generate protective anti-inflammatory signals.[4] Furthermore, defective clearance of apoptotic cells by macrophages has been found in both animals[5–8] and humans with SLE.[9–12]

Several phagocyte receptors and bridging molecules, including the complement system, have been implicated in the complex process of recognition and subsequent engulfment of apoptotic cells by macrophages.[1 13 14] In this context there are several reports suggesting that monocytes and macrophages from patients with SLE may have intrinsic defects, as they can display abnormal differentiation/survival, signalling abnormalities, and altered expression of cell surface molecules potentially involved in the clearance of apoptotic cells.[15–19] However, the immunosuppressive treatments most SLE patients receive—corticosteroids in particular—may also account for the altered function of macrophages, including their capacity to phagocytose apoptotic cells in vitro.[20 21] Corticosteroid treatment is also known to alter the expression of several adhesion molecules that have been implicated in the process of apoptotic cell clearance.[19 21–23]

In the present study, we analysed the ability of circulating monocytes from patients with SLE and rheumatoid arthritis, treated with similar doses of corticosteroids, to adhere to plastic and differentiate into macrophages in vitro, compared with monocytes from healthy controls. We also investigated the interaction between apoptotic cells and macrophages using a flow cytometry assay that allowed us to discriminate between binding of apoptotic cells to macrophages and true engulfment. Finally, we tested the effect of serum factors and complement in this phagocytic assay.

METHODS

Patients

Blood from patients with SLE or rheumatoid arthritis was obtained from our outpatient clinic. All cases of SLE and rheumatoid arthritis met the diagnostic criteria of the American College of Rheumatology.[24 25] The study received approval from the Hammersmith Hospital research committee and informed consent was obtained from all donors.

The main clinical characteristics of the patients are shown in table 1. Similar proportions of SLE and rheumatoid patients were considered to have active disease on the basis of the SLE disease activity index (SLEDAI)[26] and the 28 joint disease activity score (DAS28),[27] respectively. Every time we

Abbreviations: apJK, apoptotic Jurkat cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; DAS28, 28 joint disease activity score; HIS, heat inactivated human serum; IQR, interquartile range; NHS, normal human serum; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index

Table 1 Characteristics of patients and healthy controls

	SLE	RA	Healthy controls
Number	34	20	15*
Median age (years) (range)	41 (18 to 64)	53.5 (27 to 67)	34 (23 to 61)
Sex ratio (F/M)	33/1	19/1	13/2
Active disease† (%)	11 (32)	7 (35)	
Severe disease flare (%)	3 (9)	2 (10)	
Corticosteroid treatment			
Number of patients (%)	30 (88)	14 (70)	
Median dose (mg/day) (range)	7.5 (2.5 to 40)	7 (3.75 to 20)	
Immunosuppressive drugs			
Number of patients (%)	20 (59)	15 (75)	
Methotrexate	0	13	
Azathioprine	14	1	
Hydroxychloroquine	6	1	
Other drugs	3‡	4§	
Circulating leucocytes, median ($\times10^9$/l)¶ (25% to 75%)	6.15 (4.7 to 7.4)	7.30 (5.3 to 8.6)	
Circulating monocytes, median ($\times10^9$/l)¶ (25% to 75%)	0.34 (0.24 to 0.49)	0.60 (0.49 to 0.79)	
ESR (min/first hour)¶ (25% to 75%)	25 (10 to 34)	27 (10 to 38)	

*Four healthy controls (all women) participated to two (in three cases) or four (in one case) adhesion experiments.
†As defined by a SLEDAI ⩾10 for SLE patients and DAS28 ⩾4.0 for rheumatoid arthritis patients.
‡Pulsed cyclophosphamide intravenously every three months (n = 2), mycofenolate mofetil (n = 1).
§Sulfasalazine (n = 3), leflunomide (n = 1).
¶On day of study.
DAS28, 28 joint disease activity score; ESR, erythrocyte sedimentation rate; F, female; M, male; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

carried out an assay, we included one healthy donor, one to three SLE patients and one or two rheumatoid arthritis patients who were sex and when possible age matched. In addition to the clinical assessment of disease activity, the full blood count and erythrocyte sedimentation rate were recorded.

Isolation of peripheral blood monocyte derived macrophages

Human monocytes were isolated from venous blood. Citrated venous blood was sedimented for 30 minutes in 6% dextran (T500 Amersham Pharmacia Biotech, Amersham, UK) and the leucocyte-rich plasma layered on a discontinuous 70% Percoll gradient (Amersham Pharmacia Biotech). The monocyte enriched fraction (>85% pure) was collected, washed three times in Hank's balanced salt solution (HBSS, Gibco BRL, Life Technologies Laboratories, Paisley, UK), and adhered to 48-well plates (2×10^6 cells per well) in X-VIVO 10 medium (Whitaker, Walkersville, Maryland, USA) containing 10% heat inactivated pooled human serum (HIS). After different incubation times at 37℃, non-adherent cells were removed and fresh medium added. The cells were allowed to mature into macrophages over an eight to nine day period, with the medium refreshed on days 3 and 7.

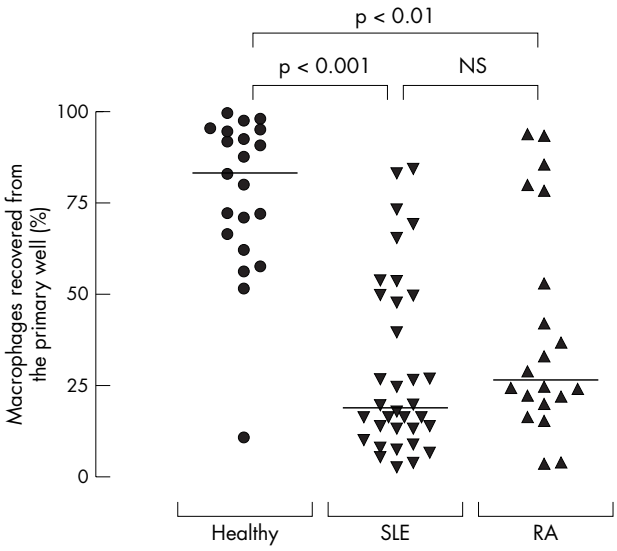

Figure 1 In vitro adhesion defect of monocytes isolated from patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The monocytes were cultured in the presence of pooled heat inactivated human serum and allowed to adhere to plastic (primary well); after 12 hours the non-adherent cells were replated into a new well (secondary well). Macrophages from both wells were collected on days 9–10. The data are expressed as the proportion of macrophages collected from the primary well compared with the total; bars = median.

Apoptotic cells

The human Jurkat T cell line was cultured in RPMI 1640 (Gibco BRL) containing 10% heat inactivated fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin and L-glutamine (Sigma-Aldrich, Poole, UK). Before induction of apoptosis, Jurkat T cells were resuspended in RPMI 1640 (Gibco BRL) containing 0.4% BSA at 1×10^7 cells/ml and incubated for five minutes at room temperature with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's protocol (5 µM per 1×10^7 cells). Cells were subsequently washed with 10% FCS RPMI. Apoptosis was induced by ultraviolet irradiation (254 nm; 800 mJ/cm^2) followed by incubation at 37℃ for 2.5 h in RPMI 1640/0.4% BSA. This resulted in a population that was >35% apoptotic as assessed by Diff-Quick (Dade Behring, Duedingen, Switzerland) on cytospin and less than 5% necrotic by Trypan blue (Sigma-Aldrich) staining. Similar data were obtained by Annexin V/propidium iodide staining (BD Biosciences Pharmingen, San Diego, California, USA).

Flow cytometry based phagocytosis assay

CFSE labelled apoptotic Jurkat T cells (apJK) (1.25×10^6 cells/well) were fed to the monocyte derived macrophages in the absence or presence of 15% HIS or 15% normal (non-heat-inactivated) human sera (NHS) in X-VIVO 10 medium. The plate was spun at $50\times g$ for three minutes. After incubation

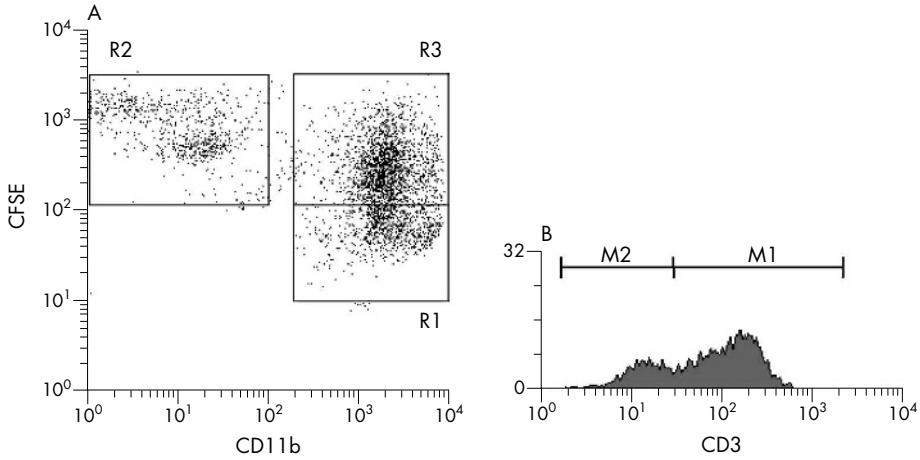

Figure 2 Flow cytometry analysis of macrophages ($CD11b^+$) from a healthy donor incubated with CFSE labelled apoptotic Jurkat cells (apJK) ($CFSE^+CD3^+$) in the presence of normal human serum. Cells were labelled with anti-CD11b-PE and anti-CD3-TC. (A) Macrophages ($CD11b^+$ cells) that had bound and/or engulfed apJK are $CFSE^+$ (R3), while free macrophages were $CFSE^-$ (R1). $CD11b^-CFSE^+$ cells are apJK cells that had not been ingested. (B) CD3 staining of cells gated in R3. Macrophages that had completely phagocytosed apJK were $CD3^-$ (M2), while macrophages that had apJK bound on their surface were $CD3^+$ (M1). CFSE, carboxyfluorescein diacetate succinimidyl ester.

for one hour at 37°C, most of the non-ingested apJK were removed by repeated washing with cold PBS. The remaining firmly adherent cells were trypsinised (5× Trypsin, GIBCO BRL), harvested in PBS/1% BSA and stained with mouse anti-human CD11b mAb (ICRF44-phycoerythrin (PE), BD Biosciences Pharmingen) and mouse anti-human CD3 mAb (S4.1-Tri-Color™ (TC); Caltag, Burlingame, California, USA). Flow cytometry was carried out on a FACScalibur™ instrument operating with CELLQuest™ software (Becton Dickinson, Mountain View, California, USA). Data were analysed using WinMDi software (version 2.8, The Scripps Research Institute, La Jolla, California, USA).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism (version 2.0; GraphPad, San Diego, California, USA). Non-parametric tests were applied throughout, with differences considered significant for p values <0.05. Dunn's post test was used for multiple comparisons. Two way analysis of variance was used in appropriate cases. Data are expressed as median (25–75% interquartile range (IQR)) or as mean (SEM).

RESULTS

Adhesion defect of monocytes from patients

In the initial experiments, monocyte enriched cell fractions were allowed to adhere for one hour before washing, as described previously.[28] However, it was noticed that after one hour the patient monocytes had adhered poorly, leading to loss of most of them on washing. Thus the experimental protocol was modified as described below. Freshly isolated monocyte enriched cells were allowed to adhere overnight instead of for one hour. The non-adherent cells were collected and replated into a new well to allow an even longer period of adhesion (two days) before washing. These wells were defined as secondary wells. The original well was washed and fresh medium was added; this was later referred to as the primary well. All the wells had their media changed on day 3. On days 9–10 the number of fully differentiated macrophages in each well (primary and secondary) was counted and by pooling the two wells more than 1500 macrophages were usually recovered. Slightly more macrophages were recovered on average from healthy donors (median = 7400 (25–75% IQR, 4710 to 10 500)) than from patients with SLE (3500 (1500 to 12 850)) or rheumatoid arthritis (5000 (2850 to

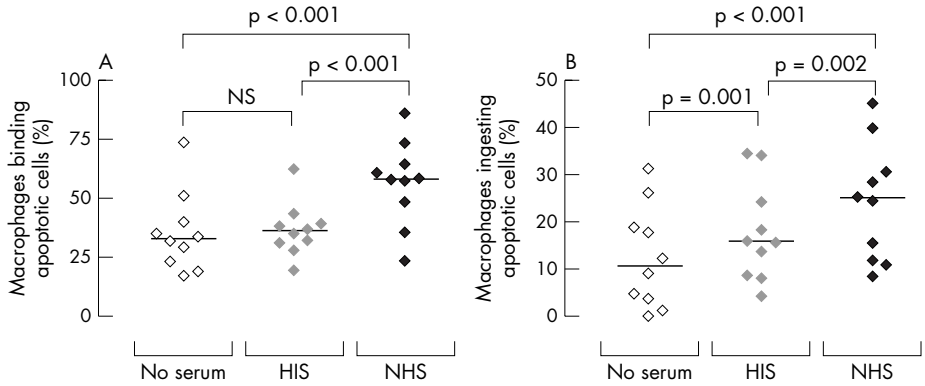

Figure 3 Serum dependent uptake of apoptotic cells by macrophages. Macrophages from healthy controls were incubated with apoptotic Jurkat cells (apJK) in the presence of normal human serum (NHS), heat inactivated human serum (HIS), or no serum. (A) The percentage of macrophages binding apJK was significantly greater when apJK had been incubated with NHS. There was no significant difference between HIS and no serum. (B) Percentage of macrophages phagocytosing apJK: the percentage of uptake was markedly increased when apJK had been incubated with NHS. Heat inactivation of serum did not reduce the engulfment of apJK to the same extent as the lack of serum. Bars = median.

8500)), most probably reflecting the smaller number of circulating monocytes found in these patients. The most striking difference between these three groups was the well from which the majority of macrophages were recovered. The percentage of macrophages collected from the primary wells was significantly lower in the SLE and rheumatoid patients than in the healthy controls (fig 1). There was no significant difference in the proportion of macrophages recovered from the primary wells between SLE and rheumatoid patients. There was no correlation with disease activity and no differences between patients treated with a ''low'' (up to 10 mg/kg) versus a ''high''(>10 mg/kg) daily dose of prednisolone.

The macrophages obtained from the different wells (primary and secondary) were analysed separately and were found to show similar phenotypic characteristics with respect to morphology on cytospin, surface marker expression, and phagocytic properties. On the basis of these observations, for each individual only the results obtained from the well containing the largest number of macrophages are presented.

Effect of serum and heat labile serum components on control macrophages

Figure 2 shows a representative example of the flow assay used to assess binding or ingestion of apoptotic cells. Free macrophages and non-ingested apJK were gated as $CD11b^+CFSE^-CD3^-$ (R1) and $CD11b^-CFSE^+CD3^+$ (R2), respectively; while macrophages with bound or ingested apJK were $CD11b^+CFSE^+$ (R3). To discriminate between binding and ingestion of apJK, CD3 staining of $CD11b^+CFSE^+$ macrophages (R3) was analysed (fig 2B). Macrophages with apJK bound to their surface were $CD3^+$ (M1), while macrophages that had phagocytosed apJK were $CD3^-$ (M2). The staining with anti-CD3 allowed us to quantify the percentage of binding and ingestion of apJK by macrophages.

Binding of apJK to the macrophages occurred in the absence of serum, and was not different with heat inactivated serum (fig 3A). However, heat labile factors markedly increased the binding. Consistent with this observation, the uptake of apoptotic cells was serum dependent (fig 3B). In the absence of serum the engulfment was minimal and increased markedly when a pool of serum from healthy donors was added. Heat inactivation of the serum markedly reduced the proportion of macrophages ingesting apoptotic cells, indicating that complement may be involved in this process. Interestingly, other heat resistant serum factors must also contribute to this, as phagocytosis in the presence of HIS was higher than without serum. Adding HIS or NHS from healthy donors to the macrophages from rheumatoid and SLE patients influenced the binding and internalisation of the apoptotic debris in the same way (data not shown).

Impaired engulfment of apoptotic cells by macrophages from SLE and rheumatoid patients

The flow cytometry method we used allowed discrimination between apJK bound to and ingested by macrophages. No differences were observed in the binding of apJK to macrophages from SLE and rheumatoid patients compared with the healthy controls (fig 4A). However, there was a small but significant difference in the percentage of macrophages that had engulfed apJK between SLE patients and controls (fig 4B). Macrophages from rheumatoid patients also showed slightly reduced ingestion compared with the controls and a higher rate of ingestion compared with SLE patients; however, these differences did not reach statistical significance. Among SLE patients no significant correlation between the impaired phagocytosis and disease activity or prednisolone treatment was found, suggesting an intrinsic defect in the macrophages in this disease.

DISCUSSION

In the present report we showed a marked in vitro adhesion defect of peripheral blood derived monocytes from patients with SLE and rheumatoid arthritis. This defect could be partly rectified by allowing the cells to adhere to the plastic for longer. In addition, using a novel flow cytometry assay that allowed us to measure the ability of monocyte derived macrophages to either bind or internalise apoptotic cells, we found that monocyte derived macrophages from SLE and rheumatoid patients had a similar binding ability for apoptotic cells as macrophages from healthy controls. Nevertheless macrophages from SLE patients showed impaired engulfment of apoptotic cells.

The delayed adhesion to plastic that we observed in monocytes from SLE or rheumatoid patients is in agreement with previous reports showing defective in vitro maturation and survival of monocytes and macrophages from patients with SLE and rheumatoid arthritis and from animal models of autoimmunity.[15–17] Abnormal patterns of adhesion molecule expression on these cells, or defective cytokine expression following their adhesion to plastic, have also been reported.[9 12 15–17 19 23] In addition, soluble factors present in

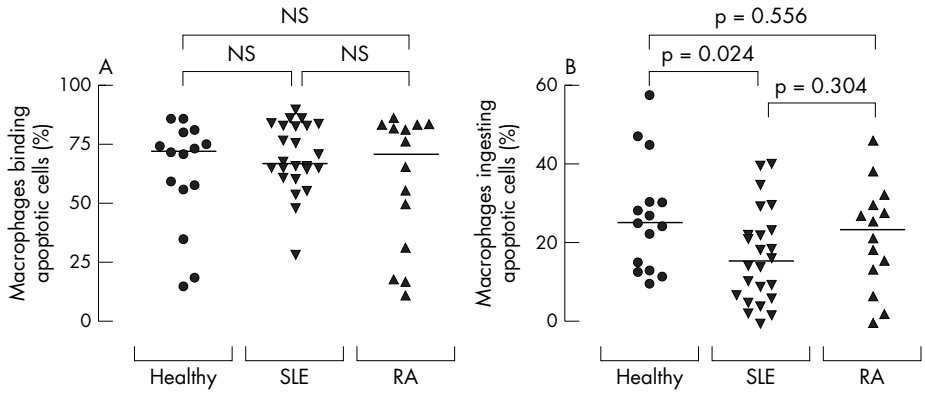

Figure 4 Impaired ingestion of apoptotic cells by macrophages from patients. Macrophages from healthy controls (n = 15), patients with systemic lupus erythematosus (SLE) (n = 24), and patients with rheumatoid arthritis (RA) (n = 14) were incubated with apoptotic Jurkat cells (apJK) in the presence of normal human serum. (A) Binding of apoptotic cells by macrophages from SLE and RA patients was similar and did not differ significantly from that of healthy controls. (B) Ingestion of apoptotic cells by macrophages was significantly reduced in SLE patients compared with healthy controls. Bars = median.

sera from SLE patients have been shown to affect the ability of monocytes from these patients—and even from healthy controls—to differentiate into macrophages.[12 29] However, in the present study monocytes were cultured and tested in the presence of pooled normal serum and thus soluble factors could not fully account for the differences between patients and healthy controls. Besides the differences in the adhesion properties, fewer macrophages were recovered from SLE patients than from healthy donors, which may reflect a higher rate of apoptosis in lupus phagocytes even in the presence of normal serum.[15]

Treatment in general and corticosteroids in particular may well be responsible, at least in part, for the adhesion defect. Indeed, an adhesion defect was not only found in SLE patients but also, and to the same extent, in rheumatoid patients who were receiving similar doses of corticosteroids. This finding is consistent with previous reports showing that corticosteroids can influence the level of adhesion molecule expression on monocytes and macrophages [19 22 23] and the ability of these cells to adhere to epithelia in vivo.[30] On the other hand, given the small number of patients with no treatment (one patient with rheumatoid arthritis and three with SLE), we cannot exclude the possibility that monocytes from SLE and rheumatoid patients share common functional abnormalities independently of the treatments. A potential implication of this adhesion defect would be that monocytes from patients with rheumatoid arthritis and SLE are less capable of migrating into tissues when needed, resulting in a delayed resolution of the inflammatory lesions and possible exacerbation of the disease.

An intrinsic defect in the ability of monocyte derived macrophages from SLE patients to engulf apoptotic cells was suggested by the impaired internalisation observed in the presence of normal human serum. Interestingly, previous reports showed that the ability of normal human macrophages to interact with apoptotic cells was defective in the presence of SLE serum compared with normal serum.[12] Here, we found that serum factors, including serum heat labile factors such as complement components, indeed play an important role in the interactions of apoptotic cells with macrophages. However, the defective internalisation of apoptotic cells observed with macrophages from SLE patients in the presence of normal serum is in agreement with previous reports showing that SLE macrophages have an impaired capacity to interact with apoptotic cells.[9 10 12 15] It has been suggested that the defective interaction (binding or internalisation or both) between SLE macrophages and apoptotic cells could reflect, at least in part, a reduced density of differentiated macrophages in SLE patients.[15] In our study, by allowing the circulating monocytes to adhere in vitro for a longer period (up to two days) we were able to obtain comparable numbers of differentiated macrophages from SLE patients, rheumatoid patients, and healthy controls. In these experimental conditions we found, in agreement with previous observations,[15] that the percentage of macrophages binding apoptotic cells was similar between SLE patients, rheumatoid patients, and controls. Nevertheless, macrophages from SLE patients showed a significantly reduced internalisation of the bound apoptotic debris compared with macrophages from the controls. The ability of macrophages from rheumatoid patients to engulf apoptotic cells was greater, although not significantly so, than that of macrophages from SLE patients, and both groups were on similar doses of corticosteroids. However, there were clear differences in the use of other immunosuppressive drugs between the rheumatoid arthritis and the SLE groups. There is evidence from in vitro studies that these drugs may have an effect on the macrophage function and thus they could contribute to the observed macrophage abnormalities.[31 32] The uptake by macrophages from rheumatoid patients was not different from that of healthy controls. Thus our results suggest that the phagocytic defect of macrophages from SLE patients is likely to be related to the disease itself and not to treatment. Overall our data, in accordance with previous findings,[9] suggest that abnormalities of the monocyte-macrophage lineage are likely to play an important role in the pathogenesis of SLE.

ACKNOWLEDGEMENTS

We would like to thank the patients and healthy donors for their participation in this study. All the clinicians in the Rheumatology Unit are acknowledged for the recruitment of patients. This work was supported by the Wellcome Trust (071467). SWT was supported by grants from the Dutch Kidney Foundation, the Prins Bernhard Cultuurfonds, and a fellowship from the Leiden University Medical Centre. PQ was supported by grants from the Institut Français pour la Recherche Scientifique et Médicale (INSERM) and the French Académie Nationale de Médecine. LFJ is an Arthritis Research Campaign funded research fellow.

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