Involvement of high mobility group box 1 in the auto-inflammatory process in systemic lupus erythematosus
Schaper, Fleur

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High Mobility Group Box 1 (HMGB1) skews macrophage polarization and negatively influences phagocytosis of apoptotic cells

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SUBMITTED
ABSTRACT

Introduction
Decreased phagocytosis of apoptotic cells plays an important role in the pathogenesis of Systemic Lupus Erythematosus (SLE). This can lead to secondary necrosis and release of nuclear proteins, such as High Mobility Group Box 1 (HMGB1). We hypothesized that increased HMGB1 levels, as present in SLE, skew macrophage differentiation towards M1-like phenotypes and thereby diminish uptake of apoptotic cells. Therefore, the effect of HMGB1 on macrophage polarization and on phagocytic capacity of differentiated macrophages was investigated.

Methods
SLE patients with quiescent disease (SLEDAI ≤4) and healthy controls (HC) were included. Monocytes and differentiated M1 and M2 macrophages were assessed for expression of M1 and M2 markers and for phagocytic capacity. HMGB1 was added during differentiation and during phagocytosis.

Results
Expression of CD86 (M1) was not different, while CD163 (M2) was significantly lower on SLE monocytes. After differentiation no differences regarding surface receptor expression and phagocytic capacity were observed between M1 and M2 macrophages from SLE patients and HC. Addition of HMGB1 during M2 differentiation resulted in high IL-6 and TNF-α mRNA expression and reduced phagocytic capacity of apoptotic cells. Furthermore, adding HMGB1 to apoptotic Jurkat cells diminished phagocytosis of these cells.

Conclusion
Circulating monocytes from SLE patients display a M1-like phenotype compared to HC, however in vitro differentiation abolishes this difference. HMGB1 skews differentiation of M2-like macrophages towards M1-like phenotype and subsequently reduces phagocytosis of apoptotic cells. These data implicate that the phenotype of monocytes or macrophages is determined by their environment, such as presence of cytokines and HMGB1.
INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory disorder. It can affect the skin, joints, kidneys, lungs, nervous system, serous membranes, and other organs of the body. Immunologic abnormalities, especially production of a large number of antinuclear autoantibodies, are a prominent feature of the disease (1). Phagocytosis and clearance of immune complexes, apoptotic cells, and necrotic cell-derived material is an essential function of macrophages and has been shown to be decreased in patients with SLE (2). The decreased phagocytic capacity of macrophages has been implicated in the pathogenesis of SLE, because it can lead to prolonged exposure to auto-antigens and hence promote autoimmunity (2). The underlying mechanisms responsible for the decreased phagocytic capacity of macrophages in SLE patients are not completely known and both intrinsic factors such as polymorphisms in ITGAM gene (3,4) and extrinsic factors may contribute to this defect. For example, lower serum levels of complement can contribute to a decreased phagocytosis (5). Cytokines and other pro-inflammatory molecules might also influence efficiency of phagocytosis. Increased levels of the pro-inflammatory molecule High Mobility Group Box 1 (HMGB1) have been found in serum of SLE patients (6). HMGB1 is a nuclear DNA binding protein that is secreted by activated cells and released into the extracellular milieu from apoptotic and necrotic cells (7). As there is impaired clearance and accumulation of apoptotic debris in SLE this can lead to increased HMGB1 levels locally, for instance in kidney and skin, as well as systemically (8-12).

Plasticity and functional polarization are hallmarks of the mononuclear phagocyte system. Cytokines and microbial products profoundly and differentially affect the function of mononuclear phagocytes. In response to interferons (IFNs) or Toll-like receptor (TLR) ligands macrophages undergo M1 (classical) activation while interleukin-4 (IL-4) or IL-10 signalling leads to M2 (alternative) activation. These two stages represent extremes of a continuum in macrophage activation states (13). M1 macrophages are implicated in initiating and sustaining inflammation and are defined by expression of CD86, iNOS, and production of the inflammatory cytokines IL-6 and tumour necrosis factor (TNF)-α. M2 or M2-like macrophages are associated with resolution or smouldering chronic inflammation. M2 macrophages are characterized by high expression of functional receptors such as the mannose receptor (MR or CD206) and the scavenger receptor CD163, and by production of IL-10 (14). Furthermore, it has been shown that M2-like macrophages have a greater capacity to phagocytose cells in vitro compared to M1 cells (15,16). M2 macrophages preferentially internalize early apoptotic cells compared to late apoptotic cells and necrotic cells (17).
Although distinct M1 or M2 macrophages can be induced in vitro, it is more likely that mixed phenotypes will be present in vivo, depending on the local cytokine milieu (18). In SLE, factors produced by macrophages and factors that are known to influence macrophage function are up- or downregulated due to inflammation. For instance, elevated serum levels of IL-6 and IL-10 are found in SLE patients (19,20) and expression of CD14, mannose receptor and MHCII on macrophages is decreased (21). The cytokine milieu in SLE is also characterized by increased levels of TNF-α, GM-CSF and IFN-γ (21), which represents a pro-inflammatory M1 milieu. However, high serum levels of IL-10 and immune complexes skew macrophages towards a M2 phenotype (18,19). Thus, available data do not conclusively point towards preponderance of one specific subtype of macrophages. Although abnormalities in monocyte function and phenotype have been described (21-24) little is known about M1/M2 macrophages in SLE.

HMGB1 can induce the secretion of pro-inflammatory cytokines IL-6 and TNF-α in monocytes (25) which is associated with a M1 phenotype. Therefore we hypothesised that increased levels of HMGB1 might lead to skewing of monocyte differentiation into M1 macrophages. This might explain the decreased phagocytic capacity of macrophages in SLE because M1 macrophages are less efficient phagocytes. To investigate whether the M1 phenotype is already present in monocytes of SLE patients, we determined expression of M1 and M2 markers on peripheral blood monocytes of SLE patients. Moreover, differentiated macrophages of SLE patients were investigated for M1 and M2 markers and for their ability to phagocytose apoptotic cells. Finally, we tested whether HMGB1 is a potential factor in skewing differentiation of macrophages towards the M1 phenotype resulting in diminished phagocytic capacity.

METHODS

Patients and controls

Consecutive SLE patients (n=48) visiting the outpatient clinic and age- and sex matched healthy controls (n=43) were included in the study. SLE patients fulfilled the criteria of the American College of Rheumatology for SLE (26) and disease activity at time of blood sampling was assessed by SLEDAI-2K (SLE Disease Activity Index). Patients with quiescent disease (SLEDAI 0-4) were included. Clinical data and medication use was retrieved from medical records. Levels of anti-dsDNA were measured by EliA assay (ThermoFisher Scientific, Nieuwegein, the Netherlands). Monocyte number and complement factors (C3, C4) were determined by routine techniques. Serum HMGB1 levels were measured by Western Blotting as described before (6). Serum soluble CD163 levels were determined by ELISA (IQ Products,
The Netherlands), according to the manufacturer’s instructions. The study was approved by the Institutional Review Board (M07.052492) of the UMCG and informed consent was obtained from all patients and HC. Characteristics of all patients and controls are summarized in table 1.

Table 1: Characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Patients</th>
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<tbody>
<tr>
<td>Number</td>
<td>43</td>
<td>48</td>
</tr>
<tr>
<td>Age</td>
<td>40 (21-63)</td>
<td>48 (24-78)</td>
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<tr>
<td>Female (%)</td>
<td>36 (84%)</td>
<td>45 (93%)</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>2 (0-4)</td>
<td></td>
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<tr>
<td>Anti-dsDNA, IU/ml</td>
<td>4 (0-318)</td>
<td></td>
</tr>
<tr>
<td>C3, g/l</td>
<td>1.00 (0.5-2.16)</td>
<td></td>
</tr>
<tr>
<td>C4, g/l</td>
<td>0.17 (0.05 – 0.49)</td>
<td></td>
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<tr>
<td>Monocytes, (10^9/l)</td>
<td>0.34 (0.06 -0.97)</td>
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<tr>
<td>HMG-B1 (intensity)</td>
<td>1.7 (1.1-3.5)</td>
<td>3.4 (1.1 - 43.5)</td>
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<tr>
<td>ACR criteria, n (% of total number of pts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malar rash</td>
<td>11 (23%)</td>
<td></td>
</tr>
<tr>
<td>Discoid rash</td>
<td>21 (44%)</td>
<td></td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>16 (32%)</td>
<td></td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>7 (15%)</td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
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<td>Serositis</td>
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<tr>
<td>Renal disorder</td>
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<td>Neurologic disorder</td>
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<td>Hematologic disorder</td>
<td>36 (75%)</td>
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<td>Immunologic disorder</td>
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<tr>
<td>Antinuclear antibody</td>
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<td>Prednisolone use, n (%)</td>
<td>18 (37%)</td>
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</tr>
<tr>
<td>Daily median dose, mg</td>
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<td>Hydroxychloroquine use, n (%)</td>
<td>30 (61%)</td>
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<tr>
<td>Daily median dose, mg</td>
<td>400 (200-600)</td>
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<tr>
<td>Azathioprine use, n (%)</td>
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<tr>
<td>Daily median dose, mg</td>
<td>125 (50-150)</td>
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</tbody>
</table>

Unless otherwise indicated, data are expressed as median (range).
**In vitro differentiation and polarization of M1-like and M2-like macrophages**

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, monocytes were allowed to adhere to culture plates for a minimum of 2 hours, after which the non-adherent cells were removed. Adherent cells were maintained for 5 days in RPMI 1640 medium (Lonza, Walkersville, MD, USA) with 10% filtered fetal calf serum (FCS) and 10 mg/ml gentamicin (Invitrogen, USA), supplemented with 50 ng/ml macrophage colony stimulating factor (M-CSF, R&D systems) for differentiation into macrophages. Medium was replaced every two to three days. Subsequently, macrophages were polarized towards M1-like and M2-like phenotypes using 100 U/ml IFN-\(\gamma\) (PeproTech, USA) and 1 ng/ml lipopolysaccharide (LPS) (Sigma, Germany) or 20 ng/ml IL-4 and 10 ng/ml IL-10 (PeproTech) or 50 ng/ml M-CSF for an additional 48 hours as described before (27).

**Cells surface staining of monocytes and polarized macrophages**

200 µl EDTA-whole blood cells were stained with anti-CD14 Pe-Cy7, anti-CD163 PE (Biolegend), anti-CD86 FITC (IQ-Products), or isotype matched control antibodies for 30 minutes. Next, red blood cells were lysed using FACS lysing solution (BD, Belgium) diluted 1:10 with demineralized water. Cells were washed with PBS supplemented with 1% BSA (Sigma Aldrich, Netherlands). Subsequently four-color flow cytometry was performed on a FACS Calibur, and monocytes were gated by size based on the forward-sideward scatter pattern.

Cultured macrophages were washed with warm PBS, and harvested using 0.01% Trypsin (ICN Biomedicals, Netherlands). Afterwards cells were incubated with anti-CD86 FITC or anti-CD163 PE or isotype matched controls for 30 minutes and washed again with PBS supplemented with 1% BSA. Four-color flow cytometry was performed on a FACS Calibur, and mean fluorescence intensity (MFI) expression levels were determined using Cell Quest software (BD, USA) and WinList 6.0 software. Delta MFI was calculated as measured MFI, corrected for isotype control.

**Cell sorting, RNA isolation, cDNA synthesis and qRT-PCR**

PBMC isolation was done as described above in a representative subpopulation of 19 patients (median age: 46 years, median SLEDAI: 2) and 16 age and sex matched HC. Monocytes were isolated by negative sorting (characterized as CD3- CD56- CD19-) by FACS cell sorting on a MoFlo XDP cell sorter. Purity was > 95%. After sorting, monocytes were lysed and RNA was isolated using RNeasy Plus Micro Kit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. cDNA was synthesized from total RNA using M-MLV Reverse Transcriptase and oligo(dT) 24 (Life Technologies, USA). One µl cDNA sample was
analysed in duplicate for amplification by the Taqman real time PCR system (ABI Prısm 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). Taqman primer/probe sets for glyceraldehyde 3-phosphatedehydrogenase (GAPDH), IL-6, TNF-α, IL-10, TLR2 and -4, CD86, CD163, and mannose receptor (CD206) were used (Applied Biosystems). Ct (threshold cycle) values were determined using the software program SDS 2.4 (Applied Biosystems). The amount of target was normalized to an endogenous reference (GAPDH) and expressed as relative expression ($2^{-\Delta Ct}$).

**Phagocytosis assay on coverslips**

Jurkat cells were irradiated with ultraviolet B (UVB; 20W, 170 mJ/cm2) using a TL12 lamp (Philips, Best, The Netherlands) for 35 minutes to induce apoptosis, and were subsequently incubated for 4 hours at 37°C and 5% CO2 to obtain early apoptotic cells. The phagocytosis assay was performed with polarized macrophages on coverslips as described earlier (28). Preparations were scored blindly by two independent observers at 400x magnification using light microscopy. Phagocytosis was expressed as phagocytosis index (PI) indicating the number of Jurkat cells internalized by 100 macrophages. All experiments were performed in triplicate.

**Phagocytosis assay measured by flow cytometry**

The phagocytic capacity of macrophages was also analysed by flow cytometry. In this assay, apoptotic Jurkat cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, USA), and added to polarized macrophages for 45 minutes at 37°C and 5% CO2. As a control, the assay was also performed at 4°C. Afterwards, the medium was aspirated, adhered cells were washed and trypsinised as described above. Cells were stained with anti-CD11b- PE (BD Biosciences) as a macrophage marker. Macrophages that had phagocyted apoptotic Jurkat cells were defined as cells that were positive for both CFSE and CD11b. Two populations could be distinguished by CFSE fluorescence dependent on phagocytic capacity of macrophages, indicating good and moderate phagocytosis. In the results section, MFI of CSFE fluorescence and percentage of strong double positive cells is reported (R3, gating strategy shown in supplementary figure 1).

**Effect of HMGB1 on differentiation of macrophages and subsequent phagocytosis**

To study the effect of HMGB1 (Sigma, St. Louis, MO, USA) on differentiation, macrophages were cultured with M-CSF for 6 days. Next, medium was replaced by Opti-MEM medium and HMGB1 (0.1, 0.5 and 1 µg/ml) was added to macrophages
for 4 hours for mRNA measurement or for 24 hours for phagocytosis assay. For mRNA measurement, cells were lysed with TRIzol reagent according to the manufacturer’s instructions and RNA was isolated. DNAse treatment (Ambion, Huntingdon, Cambridgeshire, UK) was performed. cDNA synthesis and subsequent RT-qPCR were performed as described above for sorted monocytes. Phagocytic capacity of macrophages after incubation with HMGB1 was tested by flow cytometry as described above.

**Effect of preincubation of apoptotic Jurkat cells with HMGB1 on subsequent phagocytosis**

To study the effect of HMGB1 on phagocytosis, apoptotic Jurkat cells were incubated with 1 µg/ml HMGB1 for 30 minutes at 37°C and 5% CO2 prior to phagocytosis by M-CSF differentiated macrophages. Phagocytosis assays using coverslips and flow cytometry were performed as described above.

**Statistical analysis**

Data are presented as median (range) unless stated otherwise. Statistical calculations were performed used Graph Pad Prism version 5.00 (Graph Pad software Inc, USA). Differences between patients and controls were calculated using a Mann-Whitney test. Paired samples were compared using Wilcoxon Signed Rank Test. P values <0.05 were considered significant.

**RESULTS**

**Membrane and mRNA expression of markers in peripheral monocytes**

Absolute numbers of peripheral blood monocytes of SLE patients were determined by routine measurements. The median number of monocytes was 0.34 (10⁹/l) with a range of 0.06 – 0.97 (10⁹/l), which is in the normal range of 0.3-0.9 (10⁹/l), indicating there was no monocytopenia (table 1).

There was no difference in expression of CD86 (M1 marker) on peripheral blood monocytes of patients compared to HC (figure 1A). However, a lower expression of CD163 (M2 marker), expressed as delta MFI, on monocytes of SLE patients was observed (p<0.05) (figure 1B). Accordingly, sCD163 was increased in serum of SLE patients (figure 1C, p<0.001). To further characterize the monocytes, cells were isolated by negative sorting (characterized as CD3-, CD56-, CD19-) and analysed by RT-qPCR to determine mRNA levels of CD86, TLR2, TLR4, TNF-α, IL-6 (M1) and CD163, CD206, and IL-10 (M2). As shown in figure 1D, relative expression of CD206 was very low in monocytes of both patients and controls. In contrast to the observed difference in the expression at protein level, there was no difference in CD163
mRNA expression between patients and controls (figure 1D). Furthermore, for the other receptors no differences between monocyte mRNA levels of patients and HC were found (figure 1D). However, both IL-6 and IL-10 mRNA levels were significantly upregulated in monocytes of SLE patients (p<0.001), whereas TNF-α mRNA levels were similar to levels detected in monocytes of HC. To investigate possible effects of medication patients were stratified into two groups: immunosuppressive therapy

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**Figure 1:** Expression of M1 and M2 makers on peripheral monocytes from healthy controls (HC, open circles) and Systemic Lupus Erythematosus (SLE, black circles) patients.  
A: Expression of CD86 (M1 marker) measured by flow cytometry in HC (n= 27) and SLE patients (n= 27) expressed as ΔMFI (mean fluorescence intensity), which is MFI corrected for the isotype control  
B: Expression of CD163 (M2 marker) measured by flow cytometry in HC (n= 27) and SLE patients (n= 27) expressed as ΔMFI (mean fluorescence intensity), which is MFI corrected for the isotype control  
C: Soluble CD163 (ng/ml) levels measured by ELISA in serum of HC (n=19) and SLE patients (n=21)  
D: mRNA levels of TNF-α, Interleukin-6 (IL-6), IL-10, CD86, CD163, Toll like receptor 2 (TLR2), TRL4 and CD206 analysed by RT-qPCR, from monocytes isolated by negative sorting (characterized as CD3- CD56- CD19-) in HC (n= 16) and SLE patients (n= 19)  
* P <0.05 and *** P <0.001
HMGB1 affects macrophage polarization and inhibits phagocytosis

As phagocytic capacity and expression of surface markers on in vitro differentiated M1 and M2 macrophages was similar for patients and HC the effect of HMGB1 was studied for potential influence on macrophage polarization and phagocytosis. Confirming earlier studies, increased serum levels of HMGB1 were observed in SLE patients (table 1, p<0.01). HMGB1 was added to M-CSF differentiated macrophages (including prednisolon, azathioprine or combinations) or hydroxychloroquine only. There were no differences between patient groups for M1 and M2 markers investigated. Moreover, there were no differences for M1 and M2 markers between patient groups which were negative for anti-dsDNA or positive.

**Membrane marker expression on in vitro differentiated macrophages**

Monocytes from both SLE patients and HC were polarized in vitro towards M1 or M2 phenotypes, and membrane expression of CD86 (M1 marker) or CD163 (M2 marker) was analysed. There was no difference in expression of CD86 or CD163 on macrophages from patients and HC (figure 2). As expected, M2-like macrophages, polarized either with IL-4 and IL-10 or M-CSF, showed higher expression of CD163 compared to M1 polarized macrophages (p<0.05), both in patients and HC. CD86 was higher expressed on M1 macrophages (p<0.001) compared to M2-like polarized macrophages (figure 2). Double staining of CD86 and CD163 (using quadrant dotplots) confirmed that there was no difference in expression between macrophages from patients and controls (data not shown) and that differentiation was successful.

**Phagocytosis of apoptotic Jurkat cells by polarized macrophages**

Aberrant clearing of apoptotic material by macrophages plays an important role in the pathogenesis of SLE. Therefore, phagocytic capacity of polarized macrophages was assessed by two different methods. M2-like macrophages, polarized either with IL-4 and IL-10 or M-CSF, showed a higher phagocytic capacity compared to M1 macrophages as shown on representative pictures of coverslips (figure 3A). Comparing phagocytosis index (PI) of macrophages obtained from controls and patients respectively showed no differences in M1 macrophages (p=0.4), and M2-like macrophages (M-CSF, p=0.7, figure 3B). M2-like macrophages from SLE patients displayed a higher PI (IL-4 and IL-10, p=0.004, figure 3B). These data were confirmed by flow cytometry demonstrating no differences in the percentages of CD11b and CFSE positive cells (figure 3C) between HC and SLE patients in both M1 and M2-like macrophages. Moreover, the MFI of CFSE, representing the phagocytosis of CFSE-labelled apoptotic Jurkat cells, was similar (figure 3D).
during the last 4 or 24 hours. mRNA analysis demonstrated that 4 hours after HMGB1 addition both IL-6 and TNF-α mRNA levels were significantly upregulated in a dose dependent manner (figure 4A and B). Addition of HMGB1 during the last 24 hours resulted in a dose-dependent decrease of phagocytosis of apoptotic cells.

Figure 2: Expression of CD86 and CD163 on polarized macrophages from healthy controls (HC, open circles) and Systemic Lupus Erythematosus (SLE, black circles) patients.

A: Expression of CD86 (M1 marker) measured by flow cytometry in HC (n= 16-19) and SLE patients (n= 10-21) on M1 and M2-like polarized macrophages. Data are expressed as ΔMFI (mean fluorescence intensity), which is MFI corrected for the isotype control

B: CD163 (M2 marker) measured by flow cytometry in HC (n= 15-19) and SLE patients (n= 10-21) on M1 and M2-like polarized macrophages. Data are expressed as ΔMFI (mean fluorescence intensity), which is MFI corrected for the isotype control
HMGB1 affects Macrophage polarization and phagocytic activity.

A: Images showing different states of macrophage polarization.

B: Graph showing phagocytosis index (PI) with different conditions.

C: Graph showing percentage of positive cells with different conditions.

D: Graph showing CFSE (MFI) with different conditions.
**Figure 3:** Phagocytosis measured on coverslips and by flow cytometry by macrophages from (HC, open circles) and Systemic Lupus Erythematosus (SLE, black circles) patients. 

A: Representative examples of phagocytosis of apoptotic Jurkat cells on coverslips by M1 and M2-like polarized macrophages from HC. 

B: Phagocytosis index (PI) of M1 and M2-like polarized macrophages in HC (n=11-12) and SLE patients (n=13-19). 

C: Phagocytosis of apoptotic Jurkat cells labelled with CFSE as measured by flow cytometry and expressed as percentage of CD11b and CFSE double positive cells in M1 and M2-like polarized macrophages of HC (n=15) and SLE patients (n=16). 

**Figure 4:** HMGB1 (High Mobility Group Box 1) affects macrophage polarization and subsequent phagocytosis.

A-B: mRNA levels of Interleukin-6 (IL-6) (A) and TNF-α (B) of M2-like macrophages (n=9) treated with HMGB1 (0.1, 0.5 and 1 µg/ml) during the last 4 hours of differentiation. Data are presented as fold increase compared to untreated sample. Statistics were performed on relative expression levels. 

C: Phagocytosis of apoptotic Jurkat cells by M2-like macrophages (n=9) treated with HMGB1 (0.1, 0.5 and 1 µg/ml) during the last 24 hours of differentiation measured by flow cytometry and expressed as mean fluorescence intensity (MFI) of CFSE. 

D: Phagocytosis of apoptotic Jurkat cells by M2-like macrophages (n=9) treated with HMGB1 (0.1, 0.5 and 1 µg/ml) during the last 24 hours of differentiation measured by flow cytometry and expressed as percentage of CD11b and CFSE double positive cells. 

* P <0.05, ** P <0.01 and *** P <0.001
HMGB1 affects macrophage polarization and phagocytic activity

Figure 5: HMGB1 (High Mobility Group Box 1) inhibits phagocytosis of apoptotic Jurkat cells. A: Phagocytosis of apoptotic Jurkat cells with and without pre-incubation of HMGB1 (1 µg/ml) by M2-like macrophages. Representative example of coverslips with and without pre-incubation of HMGB1 with apoptotic Jurkat cells is shown. B: Phagocytosis of apoptotic Jurkat cells with and without pre-incubation of HMGB1 (1 µg/ml) by M2-like macrophages. Phagocytosis Index (PI) of apoptotic cells by M2-like polarized macrophages (n=8) is depicted. C: Phagocytosis of apoptotic Jurkat cells with and without pre-incubation of HMGB1 (1 µg/ml) by M2-like macrophages as measured by flow cytometry. MFI of CFSE of M2-like polarized macrophages (n=8) is depicted. * P <0.05 and ** P<0.01

(figure 4C and D) as measured by flow cytometry. Incubation with HMGB1 showed a dose-dependent decrease on both MFI of CFSE and percentage of CD11b- and CFSE positive cells (figure 4C and D).

The capacity of HMGB1 to directly influence phagocytosis of apoptotic cells by M-CSF differentiated macrophages was studied by pre-incubating apoptotic Jurkat cells with HMGB1. Representative pictures of coverslips show decreased uptake of apoptotic cells (figure 5A), quantified by a decrease in PI (figure 5B, p<0.05). Results were confirmed by flow cytometry (figure 5C), where pre-incubating Jurkat cells with HMGB1 led to a decrease in the uptake of apoptotic cells represented by a decrease in the MFI of the CFSE signal (p<0.001).
DISCUSSION

In the present study we show that peripheral blood monocytes from SLE patients displayed a more M1-like phenotype compared to HC. CD163 membrane expression on monocytes was lower and mRNA expression levels of IL-6 and IL-10 were increased in monocytes from SLE patients compared to HC. These differences disappear after in vitro differentiation towards a M1-like or M2-like macrophage phenotype. However, adding HMGB1 during differentiation of M-CSF differentiated macrophages resulted in upregulation of IL-6 and TNF-α mRNA levels and reduction in phagocytic capacity. Finally, pre-incubation of apoptotic cells with HMGB1 also resulted in reduced phagocytosis.

Expression of CD163 was significantly lower on circulating monocytes from SLE patients, while there was no difference in CD86 expression. CD163 is a scavenger receptor and can be shed from the cell surface into its soluble form (29). Increased levels of sCD163 in serum of SLE patients have been described before (30,31) which we confirmed in our patient cohort. Thus, lower levels of membrane CD163 on monocytes in SLE patients could be explained by CD163 shedding leading to increased sCD163 levels. Moreover, increased serum levels of sMer and sAxl, both also associated with phagocytosis, were also increased in serum of SLE patients (30). It has been demonstrated that immune complex stimulation of Fc-receptors can lead to activation of metalloproteinases resulting in shedding of CD163 (32). This could explain why lower membrane expression of the CD163 protein was observed, without differences at the mRNA level. Taken together, these results demonstrate that circulating monocytes from SLE patients display a pro-inflammatory M1-like phenotype.

Monocytes of both patients and controls showed the same capacity to differentiate into either pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. This could indicate that the cells themselves are not intrinsically different but that differences are induced by the environment. This supports the hypothesis that environmental mediators play an important role in polarization and macrophage function, as proposed previously by others (13,18).

Both types of M2-like macrophages showed a greater phagocytic capacity when compared to M1-like phenotypes, confirming previous observations (15,16). Several different types of M2 macrophages have been described, depending on the polarizing stimulus. The most efficient clearance of apoptotic cells occurs by cells differentiated with IL-10 and M-CSF, which have high expression of CD206 and CD163 (33). There is no general consensus regarding surface markers on M2 macrophages but both CD163 and CD206 are generally considered to be M2 markers (13). Both types of M2 macrophages in our study expressed high levels of CD163 and CD206 (data not shown) suggesting efficient induction of phagocytes as described by others (33).
While previous studies have reported defects in phagocytic capacity of monocyte derived macrophages from SLE patients, we could not confirm this (34-36). The main difference between previous studies and ours might be that in our phagocytosis assays serum from a single healthy donor was used, instead of autologous serum or pooled serum from either patients or controls. Furthermore, apoptotic cells used in our phagocytosis tests were Jurkat cells, while other groups have also used autologous cells for the assay. Finally, other studies have used monocytes from both active and inactive patients, while this study only used monocytes from inactive patients. Standard conditions were chosen to explore whether decreased phagocytic capacity observed in SLE patients is caused by an intrinsic defect in monocytes and macrophages from these patients or whether this is due to factors in the local milieu. Previously, it has been shown that decreased complement levels (5) or the presence of auto-antibodies (37) in serum of patients can contribute to a decrease in phagocytic capacity. The possible importance of environmental factors is supported by our results demonstrating that HMGB1 affects M2 polarization by inducing the expression of pro-inflammatory cytokines generally associated with the M1 phenotype. Earlier studies have shown that HMGB1 can activate and induce production of IL-6 and TNF-α in monocytes (7,25) and in macrophages (38-40). While our results support these findings, it also reveals a different aspect of HMGB1 as it shows that HMGB1 can influence the function of macrophages, reflected by a decreased phagocytic capacity. Furthermore, pre-incubation of early apoptotic Jurkat cells with HMGB1 led to a decrease in phagocytosis of these cells. This is in line with the study by Liu et al (41), which showed an inhibitory effect of HMGB1 on phagocytosis of apoptotic neutrophils by peritoneal mouse macrophages and demonstrated that HMGB1 could bind to phosphatidylserine, a strong “eat-me” signal, thereby blocking efficient phagocytosis. As HMGB1 is increased both locally (8-10,12) and systemically in SLE patients (6,42,43), our data indicate that HMGB1 can contribute to the impaired phagocytosis as seen in SLE patients by interfering with recognition of apoptotic cells and by influencing the macrophage itself.

In summary, this study shows that while monocytes from SLE patients differ from HC in vivo, in vitro differentiation into macrophages abolishes these differences. This indicates that there is no intrinsic defect in monocytes and macrophages from these patients. However, local factors, such as cytokines and HMGB1 can skew macrophage polarization towards a pro-inflammatory phenotype and decrease phagocytosis of apoptotic cells and as such might contribute to the pathogenesis of SLE.
Key messages

• Monocytes from SLE patients have lower expression of CD163 and higher mRNA levels of IL-6 and IL-10
• In vitro differentiation into macrophages abolishes differences between SLE patients and HC
• HMGB1 skews differentiation of M2-like macrophages towards M1-like phenotype and subsequently reduces phagocytosis of apoptotic cells

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REFERENCES

HMGB1 affects macrophage polarization and phagocytic activity


SUPPLEMENTARY MATERIAL

Supplementary figure 1: Gating strategy of phagocytosis measured by flow cytometry.
Experiment carried out at 4°C, M1 and M2 differentiated macrophages showed two populations, and cells were divided into two populations, a low and high CFSE signal. MFI of CSFE and percentage of strong positive cells from (R3) were determined. Representative dotplots from a patient is shown.