Aberrant Phenotype and Function of Myeloid Dendritic Cells in Systemic Lupus Erythematosus

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Aberrant Phenotype and Function of Myeloid Dendritic Cells in Systemic Lupus Erythematosus

Dacheng Ding, Hemal Mehta, W. Joseph McCune, and Mariana J. Kaplan

Systemic lupus erythematosus (SLE) is characterized by a systemic autoimmune response with profound and diverse T cell changes. Dendritic cells (DCs) are important orchestrators of immune responses and have an important role in the regulation of T cell function. The objective of this study was to determine whether myeloid DCs from individuals with SLE display abnormalities in phenotype and promote abnormal T cell function. Monocyte-derived DCs and freshly isolated peripheral blood myeloid DCs from lupus patients displayed an abnormal phenotype characterized by accelerated differentiation, maturation, and secretion of proinflammatory cytokines. These abnormalities were characterized by higher expression of the DC differentiation marker CD1a, the maturation markers CD86, CD80, and HLA-DR, and the proinflammatory cytokine IL-8. In addition, SLE patients displayed selective down-regulation of the maturation marker CD83 and had abnormal responses to maturation stimuli. These abnormalities have functional relevance, as SLE DCs were able to significantly increase proliferation and activation of allogeneic T cells when compared with control DCs. We conclude that myeloid DCs from SLE patients display significant changes in phenotype which promote aberrant T cell function and could contribute to the pathogenesis of SLE and organ damage. The Journal of Immunology, 2006, 177: 5878–5889.

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; DC, dendritic cell; RA, rheumatoid arthritis; SLEDAI, SLE disease activity index; 6-MP, 6-mercaptopurine; MMF, mycophenolate-mofetil.

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The Journal of Immunology

Materials and Methods

Patient selection

Patients with SLE and rheumatoid arthritis (RA) fulfilled the American College of Rheumatology criteria for these diseases (37–39) and were recruited from the outpatient rheumatology clinic and inpatient services at the University of Michigan, and from the Michigan Lupus Cohort (Ann Arbor, MI). Healthy controls were obtained by advertisement. SLE activity
was assessed by the SLE disease activity index (SLEDAI) (40). Patient and control cells were paired and studied in parallel. Overall, 146 SLE patients, 76 healthy controls and 35 RA patients were studied. Information regarding the demographics, disease activity, and use of medications is provided in Table I.

Reagents
Human rIL-4, TNF-α, and IL-2 were purchased from PeproTech. Human GM-CSF was a gift from Berlex. rIFN-α and IL-2 were obtained from Schering. X-vivo 10 serum-free medium was from BioWhittaker. LPS and PHA were purchased from Sigma-Aldrich. The following anti-human mAbs conjugated to FITC, PE, CyChrome, and allophycocyanin were used: anti-CD1a, CD3, CD4, CD8, CD11c, CD14, CD25, CD40L, CD69, CD80, CD83, CD86, HLA-DR, and isotype controls (all obtained from BD Biosciences). The pan T cell isolation kit was obtained from Miltenyi Biotec. CFSE was obtained from Molecular Probes.

Generation of monocyte-derived DCs
Myeloid DCs were generated from human peripheral monocytes as previously described (41). In brief, human PBMC were separated by standard density gradient centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) and resuspended at 6 x 10^7 cells/ml in RPMI 1640 with l-glutamine and 10% FBS. The cells were transferred to tissue culture plates and allowed to adhere to the plastic surface for 1 h at 37°C. Nonadherent cells were then removed by washing with PBS. Monocyte recovery rate after adherence was >89% in both healthy controls and SLE patients. The adhered monocytes were further cultured for 5–7 days in DC induction medium (serum-free X-vivo-10 containing 20 ng/ml GM-CSF and 5–10 ng/ml IL-4). In some experiments, cells were further purified using metrizamide gradient (Sigma-Aldrich). The purity of myeloid DCs obtained under our experimental conditions was >90%, as confirmed by flow cytometric analysis (data not shown). At days 5–7, cells were harvested for analysis or stimulated with DC maturation stimuli (41) (0.5–2 μg/ml LPS and/or 10–100 ng/ml TNF-α) for 48 h. In additional experiments, freshly isolated monocytes were cultured for 7 days with GM-CSF, with or without IL-4, in the presence or absence of 100 or 1000 U of rIFN-α.

Immunofluorescence staining, FACS, and fluorescent microscopy analysis
DCs were washed with PBS/0.2% BSA and FcRs were blocked by incubating cells for 20 min in PBS with 40% control human sera or with anti-FcγR (Miltenyi Biotec). Cells were then incubated for 30 min at 4°C with different mixtures of Abs: 1) CD11c-FITC, CD1a-allophycocyanin, CD83-PE, and CD86-PE/Cy5, and 2) CD11c-FITC, CD14-allophycocyanin/Cy5, CD86-PE/Cy5 and HLA-DR-allophycocyanin. After incubation for 30 min with these Abs at 4°C, cells were washed, fixed in PBS/1% paraformaldehyde, and analyzed by FACS by gating the CD11c+ population and excluding CD14+ cells.

Drug treatment
Monocytes were cultured as stated above to induce DC differentiation, in the presence or absence of graded concentrations of indomethacin (0.01–1 μg/ml), hydroxychloroquine (0.02–2 μg/ml), hydrocortisone (0.01–1 μM), 6-mercaptopyrione (6-MP) (0.01–1 μM) and mycophenolate-mofetil (MMF) (0.04–4 μg/ml; all obtained from Sigma-Aldrich) or vehicle (42–46). A stock solution of 6-MP was prepared in dimethylformamide at a concentration of 10 mg/ml. The stock solution was diluted in assay diluent (80% culture medium/20% ethanol) to yield a 6-MP working solution of 80 μg/ml or less as indicated. The working solution of 6-MP as well as the other materials prepared in assay diluent were then further diluted 1/40 into the cell cultures for the tests. The final concentrations of ethanol (0.1%) or dimethylformamide (<0.02%) do not yield significant effects on the cell cultures (11, 47). Indomethacin was prepared in a concentration of 500 mM in absolute ethanol, then diluted to final concentrations in the cell culture medium (11). Control cells were treated with an equal volume of the solvent. MMF was prepared as previously described (43). At day 7, DCs were washed and analyzed by flow cytometry for expression of differentiation (CD1a) and maturation (CD83, CD86, HLA-DR) markers.

RNA extraction and quantitative real-time RT-PCR
Total RNA was isolated from myeloid DCs using the RNeasy kit with DNase I digestion (Qiagen) to remove possible genomic DNA contamination, and reverse transcribed to cDNA using the SuperScript III first-strand synthesis system (Invitrogen Life Technologies) with Oligo(dT)30 primer. For real-time detection of target and reference gene expression, eight pair primers and probes were designed as follows: 83 forward (F): 5'-ACCTCGTGAGGTTCCCTACACGGT-3', CD83 probe: 5'-GCCTGCAGGTTCCCTACACGGT-3'; CD83 reverse (R): 5'-TCCTTGAGGTTCCCTACACGGT-3', CD86 forward (F): 5'-CTTCTGAGGTTCCCTACACGGT-3', CD86 probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD86 reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD80 forward (F): 5'-ATCCGGAGGTTCCCTACACGGT-3', CD80 probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD80 reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD20 forward (F): 5'-ATCCGGAGGTTCCCTACACGGT-3', CD20 probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD20 reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD1a forward (F): 5'-ATCCGGAGGTTCCCTACACGGT-3', CD1a probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD1a reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD11c forward (F): 5'-ATCCGGAGGTTCCCTACACGGT-3', CD11c probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD11c reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD14 forward (F): 5'-ATCCGGAGGTTCCCTACACGGT-3', CD14 probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; CD14 reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'; 18S forward (F): 5'-GTCCTGAGGTTCCCTACACGGT-3', 18S probe: 5'-GTCCTGAGGTTCCCTACACGGT-3'; 18S reverse (R): 5'-GTCCTGAGGTTCCCTACACGGT-3'.

### Table I. Demographic and clinical characteristics of human subjects studied

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<th>Variable</th>
<th>Lupus</th>
<th>Control</th>
<th>RA</th>
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<tbody>
<tr>
<td>Number studied</td>
<td>146</td>
<td>76</td>
<td>35</td>
</tr>
<tr>
<td>Age (mean ± range)</td>
<td>44.5 ± 11.5</td>
<td>37 ± 13.5</td>
<td>51 ± 14.4</td>
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<tr>
<td>Females (n (%))</td>
<td>130 (89)</td>
<td>55 (72.3)</td>
<td>18 (51.5)</td>
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<tr>
<td>Males (n)</td>
<td>16 (11)</td>
<td>21 (27.6)</td>
<td>17 (48.5)</td>
</tr>
<tr>
<td>Lupus disease activity (mean ± SEM)</td>
<td>4.5 ± 3</td>
<td>77</td>
<td>69</td>
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<tr>
<td>SLEDAI ≤ 2 (%)</td>
<td>110</td>
<td>55</td>
<td>18 (51.5)</td>
</tr>
<tr>
<td>SLEDAI &gt; 2 (%)</td>
<td>16 (11)</td>
<td>21 (27.6)</td>
<td>17 (48.5)</td>
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<tr>
<td>Medications</td>
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</tr>
<tr>
<td>Antimalarials (%)</td>
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<tr>
<td>Azathioprine (%)</td>
<td>5.47</td>
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<tr>
<td>Cyclophosphamide (%)</td>
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<td></td>
</tr>
<tr>
<td>Methotrexate (%)</td>
<td>1.36</td>
<td>51.4</td>
<td></td>
</tr>
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<td>Mycophenolate mofetil (%)</td>
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<tr>
<td>Prednisone (&lt;0.5 mg/kg/day) (%)</td>
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<td>Prednisone (0.5–1 mg/kg/day) (%)</td>
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<tr>
<td>Prednisone (&gt;1 mg/kg/day) (%)</td>
<td>4.1</td>
<td>8.5</td>
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</table>
ABNORMAL PHENOTYPE AND FUNCTION OF LUPUS DCs

5′-CTTGCCGGTGATGTCCTCTCTTC-3′; CD86 F: 5′-GACAG GCATTGGTCAAGCCTACGT-3′; CD86 R: 5′-TCTCAAGTTGGCGCCACGCT-3′; PBGD probe 5′-GGGTACCCACGCGAATCAC-3′; PBGD-F 5′-GGCAATGCGGCTGCAA-3′; PBGD-R 5′-GGGTACCCACGCGAATCACAG; Drα GAGCGCGCGT; Drα F: 5′-TCTGGCGTGGTCGCAGAGAC; CD14 probe: 5′-ATATCGACCATG

Probes were labeled with 5′-6-FAM/3′-TAMRA (Integrated DNA Technologies). HotStar Taq polymerase (Qiagen) was used. PCR was performed using a conventional MyCycler Thermal Cycler (Bio-Rad) in a total reaction mixture of 20 μl containing 50 ng of cDNA, 1× HotStar TaqPCR buffer, and 400 nM probe/primer mixtures. After denaturation at 95°C for 15 min, 55 cycles were performed at 95°C for 15 s, followed by 60°C for 1 min. Comparative cycle threshold method with PCR efficiency correction, also known as Pfaffl’s method, was used for quantification, as previously described (48). The expression levels of the target genes were adjusted to the expression levels of the housekeeping genes PBGD and β-actin.

Cytokine determination

Cytokines were measured using two different methods. The human cytokines IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IFN-γ, GM-CSF, and TNF-α were simultaneously quantified in duplicate using the human cytokine multiplex kit (Linco Research). Supernatants obtained from day 7 (unstimulated DCs) or day 9 (DCs treated with maturation stimuli for 48 h) cultures were aliquoted and stored at −80°C until used. Sera from the same patients were also stored. Cytokine concentrations were determined following manufacturer’s instructions. In brief, standards and samples were added into the appropriate wells. Mixed beads were added to each well and the plate was incubated with agitation for 1 h at room temperature. Plate was washed and a detection Ab mixture was added to each well and incubated for 30 min at room temperature. Streptavidin-PE was added to each well containing the detection Ab mixture. The plate was then incubated with agitation on a shaker for 30 min at room temperature, washed, and sheath fluid was added for 5 min. Plate was read on Luminex 100 (Luminex) and the concentration reported as picograms per milliliter.

In addition, the BD CBA Human Inflammation kit (BD Biosciences) was used to quantitatively measure IL-8, IL-1, IL-6, IL-10, TNF-α, and IL-12p70 protein levels in sera and supernatant samples, following manufacturer’s instructions. In brief, mixed capture beads were added to assay tubes. Human inflammation standard dilutions and test samples were added to the assay tubes. Samples were incubated for 1.5 h at room temperature and protected from direct exposure to light. Wash buffer was added and tubes were centrifuged at 200 × g for 5 min. Supernatants were discarded leaving 100 μl of liquid in each assay tube. Human inflammation PE detection reagent was added to the tubes and samples were incubated for 1.5 h at room temperature, washed, and analyzed by BD Cytometric Bead Array Software.

T cell proliferation and determination of T cell activation

T cells were isolated by negative selection using magnetic beads and instructions provided by the manufacturer (pan T cell isolation kit; Miltenyi Biotec). Purity was >95%, as assessed by CD3 expression. T cell proliferation was analyzed using the intracellular fluorescent dye CFSE. With each cell division, the CFSE fluorescence intensity of the cells is reduced by half (49-51). T cells from healthy controls were labeled with 2 μM CFSE in RPMI 1640/10% FBS for 2 min at room temperature. After washing to remove unbound fluorescent dye, 4 × 10^5 T cells were cocultured with allogeneic DCs from SLE or healthy controls. Conditions included unstimulated DCs or DCs stimulated with LPS and TNF-α for 48 h, as described above. Cells were cocultured at a T cell/DC ratio of 5:1 to 10:1. After 1 and 5 days, cells were harvested and stained with mouse anti-human CD3-PE, CD8-APC, and CD45-PerCP. The percentage of T cells that had undergone specific numbers of cell divisions (G1-G5) or no cell divisions (G0) was calculated (52, 53). Controls included PHA-stimulated T cells and unstimulated T cells.

Results

Myeloid lupus DCs display abnormal levels of differentiation and maturation markers and have abnormal responses to maturation stimuli

Confirming a previous study (54), no morphologic differences in the capacity of monocytes to differentiate into DCs were found between SLE and controls, using light and fluorescent microscopy (Fig. 1). After 5–7 days of culture, DCs from SLE patients and healthy controls were found to be increased in size and developed typical dendrites. To assess whether DCs from SLE individuals and controls differ in their differentiation and maturation potentials, monocyte-derived DCs from 31 individuals with SLE, 8 patients with RA, and 20 healthy controls obtained at day 7 were stained with anti-human Abs to CD1a to (evaluate DC differentiation) (31) and CD83 (a marker of DC maturation) (55), and expression of these markers was measured by FACS. As shown in Fig. 2, A–C, SLE patients, have increased numbers of cells expressing the differentiation marker CD1a, and decreased numbers of cells expressing the maturation marker CD83 (p < 0.05). No

Statistical analysis

The difference between means was analyzed using paired t test or one-way ANOVA with post hoc analysis and Bonferroni correction. Spearman and Pearson’s correlation were used to assess correlation between different variables. Analyses were performed with SPSS version 11.5. A value of p < 0.05 was considered to be statistically significant.

FIGURE 1. Monocyte-derived DCs from SLE patients display normal morphology. A, Images represent different magnifications of cells from one individual with SLE, after culture in X-vivo medium with IL-4 and GM-CSF for 7 days. Cells display the characteristic membrane and nuclear features of DCs. Arrows point at the cells that are displayed at higher magnification on the left panels. B, Fluorescent microscopy image of one cell from a patient with SLE that displays the classic morphology DCs, at day 7 in culture. The cell expresses both CD1a and CD83.
significant differences were observed in mean fluorescence intensity (data not shown). No significant differences were detected between RA patients and controls in CD1a expression (Fig. 2A) or CD83 expression (Fig. 2B). The differences between SLE and controls were confirmed at the mRNA level by real-time RT-PCR (Fig. 2D). Indeed, lupus patients (but not RA patients) displayed significantly decreased mRNA levels of the monocyte marker CD14 and higher levels of CD1a mRNA at day 7, suggesting an acceleration of the differentiation from the monocyte stage to the myeloid DC stage (Fig. 2D). Baseline CD14 levels in monocytes did no differ between controls and lupus patients (data not shown), suggesting that the differences seen on day 7 DCs were the consequence of accelerated differentiation from the monocyte to the DC stage and not due to baseline down-regulation of CD14 on lupus monocytes. Interestingly, while CD83 protein and mRNA levels were significantly lower in lupus DCs at day 7 (Fig. 2, B–D),
mRNA levels of other specific maturation and differentiation markers (CD86, CD80, and HLA-DR) were significantly higher in SLE than in healthy controls in the absence of exogenous maturation stimuli (Fig. 3A). These findings were confirmed at the protein level (Fig. 3, B and C), both as numbers of cells expressing these markers (data not shown) and as mean fluorescent intensity in each cell. When compared with healthy controls, there were no differences in the expression of maturation markers in RA patients (Fig. 3). These results indicate that differentiation and maturation are enhanced in monocyte-derived DCs from SLE patients, but that the maturation marker CD83 is selectively down-regulated in lupus DCs.

We considered the possibility that an in vivo serum effect could induce early responses in monocytes that would lead/promote the observed differences in maturation. However, when we treated lupus and healthy control monocytes with autologous sera, no differences were found between control and SLE sera on the induction of expression of specific maturation markers (CD80: 11.5 ± 4.6% for untreated control DCs; 10.5 ± 4.1% for control DCs treated with autologous sera; 32.6 ± 11.2% for untreated lupus DCs and 34.2 ± 12% for lupus DCs treated with autologous sera; CD83: 28.2 ± 11.1% for untreated control DCs; 5.35 ± 0.8% for control DCs treated with sera; 14.5 ± 2% for untreated lupus DCs and 2 ± 0.1 for lupus DCs treated with autologous serum, p = NS when comparing lupus vs control sera; results represent mean ± SEM of three SLE patients and four healthy controls). These results suggest that the differences found on maturation markers were intrinsic to lupus monocyte-derived DCs, rather than an exogenous effect secondary to a serum factor or to serum withdrawal.

Furthermore, when we cultured lupus monocytes in GM-CSF and IL-4 in the presence or absence of IFN-α, there were no significant differences in the expression of CD83 between IFN-α-treated or untreated cells, suggesting that adding this cytokine did not alter the phenotypic abnormalities seen in SLE DCs (CD83: 39 ± 10% in IL-4 + GM-CSF; 30 ± 5% in IL-4 + GM-CSF + 100 U of IFN-α; 30 ± 4.4% in IL-4 + GM-CSF + 1000 U of IFN-α; results represent mean ± SEM of eight independent experiments, p > 0.05). Similarly, when lupus monocytes were cultured in GM-CSF without IL-4 but in the presence of IFN-α, this combination of cytokines did not result in changes in CD83 expression (10 ± 4% in GM-CSF alone; 15 ± 5% in GM-CSF + 100 U of IFN-α; 19 ± 5% in GM-CSF + 1000 U of IFN-α; results represent mean ± SEM of eight independent experiments; p > 0.05 between all conditions).

To exclude the possibility that medications could account for the differences in expression of differentiation and maturation markers between controls and SLE patients, monocytes were treated with graded concentrations of drugs commonly used in the management of SLE, including hydrocortisone (for steroids), indomethacin (for non-steroidal anti-inflammatory drugs), chloroquine (for antimalariais), 6-MP (for azathioprine), and MMF; while these cells were being differentiated into DCs in vitro. At doses equivalent to the ones used to treat SLE patients, we did not find any significant changes in the expression of differentiation and maturation markers induced by these drugs (Table II). Further, there were no significant correlations between the use of specific immunosuppressive drugs by SLE patients and the phenotypic differences found in SLE DCs (Table III).

Abnormal differentiation and maturation correlated with specific lupus clinical and serological manifestations. Indeed, decreased CD83 expression in DCs correlated significantly with current or previous evidence of lupus nephritis, and with levels of anti-dsDNA Abs. Increased CD1a” expression in lupus DCs correlated with decreased levels of complement (defined as C3 levels <83 mg/dl and/or C4 levels <13 mg/dl) (Fig. 4B). These correlations were seen both at the protein (Fig. 4) and mRNA level (p < 0.05 for same variables). Increased levels of CD80 at the protein level correlated with hemologic manifestations of SLE (leucopenia and immune-mediated thrombocytopenia) (r = 0.49, p = 0.02) and levels of anti-dsDNA Abs (r = 0.46, p = 0.03), and negatively correlated with skin manifestations of SLE (r = −0.59, p = 0.004). Increased levels of CD86 at the protein level strongly correlated with disease activity (SLEDAI) (r = 0.74, p = 0.001) and with previous or current lupus nephritis (r = 0.45, p = 0.04). The correlations between CD80 and CD86 with these specific clinical variables were also confirmed at the mRNA level (data not shown).

To establish whether DCs from individuals with SLE normally up-regulate maturation markers in response to maturation stimuli, we proceeded to treat day 7 monocyte-derived DCs with LPS and/or TNF-α for 48 h. As shown in Fig. 5C, lupus DCs significantly up-regulate mRNA of the maturation markers CD86, CD80, and CD83. However, when compared with healthy controls, the degree of up-regulation for CD80 and CD86 was blunted in the lupus group both at the protein and mRNA level (p < 0.05) (Fig. 5), using either LPS and/or TNF-α as maturation stimuli. These experiments suggest that lupus DCs have the capacity to respond
to maturation stimuli, as shown by up-regulation of maturation markers; however, the level of up-regulation of these markers is decreased when compared with healthy controls. When compared with healthy controls, there were no statistical differences in the capacity of RA DCs to up-regulate maturation markers after exposure to exogenous maturation stimuli (Fig. 5, A and B).

To exclude the possibility that abnormal differentiation and maturation observed in SLE DCs were consequences of the in vitro conditions used, the expression of maturation and differentiation markers in myeloid DCs directly obtained from peripheral blood was examined. As shown in Fig. 6A, the myeloid DC population obtained directly from lupus blood is also characterized by higher numbers of CD1a+ cells, lower numbers of CD83+ cells, and lower expression of CD86+ cells when compared with healthy controls, further confirming our in vitro findings. In addition, high levels of CD1a+ cells significantly correlated with levels of anti-dsDNA Abs (Fig. 6B) and with hypocomplementemia (p = 0.004). Furthermore, similar to what we found in DCs generated in vitro, there were no significant correlations between the use of specific immunosuppressive drugs and phenotypic abnormalities seen in lupus DCs isolated from peripheral blood (data not shown).

**Lupus DCs secrete higher levels of IL-8**

To evaluate whether SLE DCs show differential secretion of cytokines, we measured the secretion of a number of cytokines using cytometric bead array and a cytokine multiplex kit array. Control, RA, and SLE DCs secrete detectable amounts of IL-6, IL-7, IL-8, IL-10, IL-13, IFN-γ, and TNF-α. The cytokine secreted at the highest levels was the proinflammatory cytokine IL-8, and lupus DCs secreted significantly higher levels of IL-8 than control DCs both before (5459.2 ± 813.7 vs. 2137.9 ± 759.3 pg/ml, respectively, mean ± SEM, p < 0.05) and after TNF-α stimulation (8760.6 ± 1048.6 vs 4817.2 ± 689.4, respectively, mean ± SEM, p < 0.05) (Fig. 7A). Levels of IL-8 secreted by lupus DCs correlated with expression of the maturation marker CD80 (r = 0.56, p = 0.01, Pearson’s correlation), and patients with higher production of IL-8 had higher anti-dsDNA (Fig. 7B) and anti-cardiolipin levels in sera (r = 0.53, p = 0.007 and r = 0.45, p = 0.02, respectively, Pearson’s correlation). When compared with healthy controls, there were no significant increases in IL-8 secretion by DCs from RA patients (Fig. 7B). No significant differences between SLE, RA, and RA patients were observed when the other cytokines were measured in DC supernatants (data not shown). Of interest, IL-8 was also the most abundant cytokine detected in plasma from SLE patients and serum levels were higher than in healthy controls, although no statistical significance was found (57.9 vs 36.3 pg/ml, P = NS).

**Lupus DCs up-regulate T cell proliferation and activation**

Unstimulated and stimulated DCs from lupus patients induced a significant increase in allogeneic T cell proliferation when compared with DCs from healthy controls. As expected, unstimulated DCs from healthy controls were poor stimulators of allogeneic T cell proliferation and, after stimulation with maturation stimuli, their capacity to induce allogeneic T cell proliferation increased significantly. When cocultured with lupus DCs, allogeneic T cells showed a significantly higher percentage of proliferating cells than what was observed with control allogeneic DCs, and the effect was more pronounced in DCs treated with maturation stimuli (LPS and TNF-α) (Fig. 8, A and B). This increase in proliferation required cell-cell interactions, as supernatants from lupus DCs did not induce similar increases in T cell proliferation (data not shown). Similarly, there was a statistically significant increase in the expression of the activation marker CD40L in allogeneic T cells.

**Table II. Expression of maturation and differentiation markers on day 7 monocyte-derived DCs, after in vitro drug treatmenta**

<table>
<thead>
<tr>
<th>CD1a</th>
<th>CD83</th>
<th>CD86</th>
<th>CD80</th>
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<tbody>
<tr>
<td>Un untreated</td>
<td>18.08 ± 6.5</td>
<td>24.86 ± 0.27</td>
<td>34.6 ± 1.67</td>
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<tr>
<td>HC 0.01 μM</td>
<td>19.08 ± 1.76</td>
<td>39.9 ± 9.6</td>
<td>36.7 ± 0.07</td>
</tr>
<tr>
<td>HC 0.1 μM</td>
<td>24 ± 5.01</td>
<td>32.7 ± 1.42</td>
<td>28 ± 10.47</td>
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<tr>
<td>HC 1 μM</td>
<td>31 ± 9</td>
<td>26.06 ± 6.7</td>
<td>26.5 ± 3.2</td>
</tr>
<tr>
<td>6-MP 0.01 μM</td>
<td>10.25 ± 0.2</td>
<td>33.5 ± 2.7</td>
<td>46.7 ± 3.45</td>
</tr>
<tr>
<td>6-MP 0.1 μM</td>
<td>13.3 ± 0.25</td>
<td>27.2 ± 4.2</td>
<td>36.6 ± 0.075</td>
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<tr>
<td>6-MP 1 μM</td>
<td>14.8 ± 0.9</td>
<td>35.4 ± 4.9</td>
<td>47 ± 3.5</td>
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<tr>
<td>INDO 0.01 μg/ml</td>
<td>10.6 ± 5.6</td>
<td>34.4 ± 0.08</td>
<td>33.5 ± 1.7</td>
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<tr>
<td>INDO 0.1 μg/ml</td>
<td>14.6 ± 0.52</td>
<td>35.2 ± 1</td>
<td>35 ± 16.22</td>
</tr>
<tr>
<td>INDO 1 μg/ml</td>
<td>15.5 ± 1.4</td>
<td>35.6 ± 6.5</td>
<td>32.3 ± 16.2</td>
</tr>
<tr>
<td>MMF 0.04 μg/ml</td>
<td>14.8 ± 6.3</td>
<td>30.9 ± 8.3</td>
<td>51 ± 0.5</td>
</tr>
<tr>
<td>MMF 0.4 μg/ml</td>
<td>10.7 ± 5.7</td>
<td>32 ± 11.5</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>MMF 4 μg/ml</td>
<td>18 ± 3.5</td>
<td>37 ± 18.4</td>
<td>41 ± 2.05</td>
</tr>
<tr>
<td>HCQ 0.02 μg/ml</td>
<td>13.5 ± 7.1</td>
<td>28 ± 10.5</td>
<td>51 ± 0.3</td>
</tr>
<tr>
<td>HCQ 0.2 μg/ml</td>
<td>6.9 ± 2.85</td>
<td>31 ± 8.3</td>
<td>57 ± 11.2</td>
</tr>
<tr>
<td>HCQ 2 μg/ml</td>
<td>3.44 ± 1.4</td>
<td>28.7 ± 11.6</td>
<td>51 ± 6</td>
</tr>
</tbody>
</table>

**Table III. Correlation analysis of specific immunosuppressive/immunomodulator use and expression of differentiation and maturation markers in lupus DCs**

<table>
<thead>
<tr>
<th>Corticosteroids</th>
<th>Antimalarials</th>
<th>Azathioprine</th>
<th>Mycophenolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>p = 0.24 (—0.28)</td>
<td>p = 0.50 (—0.16)</td>
<td>p = 0.63 (0.121)</td>
</tr>
<tr>
<td>CD83</td>
<td>p = 0.77 (0.07)</td>
<td>p = 0.12 (0.37)</td>
<td>p = 0.29 (—0.262)</td>
</tr>
<tr>
<td>CD86</td>
<td>p = 0.14 (—0.31)</td>
<td>p = 0.99 (0.0002)</td>
<td>p = 0.71 (0.08)</td>
</tr>
<tr>
<td>CD80</td>
<td>p = 0.65 (0.11)</td>
<td>p = 0.96 (0.009)</td>
<td>p = 0.47 (—0.18)</td>
</tr>
<tr>
<td>IL-8</td>
<td>p = 0.42 (—0.20)</td>
<td>p = 0.30 (—0.26)</td>
<td>p = 0.30 (0.18)</td>
</tr>
</tbody>
</table>

*p* Results represent *p* value (*r* = value), using Spearman correlation. Negative numbers indicate a negative correlation. All *p* values were > 0.05.
when they were cocultured for 24 h with either unstimulated or stimulated lupus DCs, as compared with DCs from healthy controls (Fig. 8C). An increase in the percentage of cells that coexpress CD25 and CD40L in allogeneic T cells was also observed after these cells were cocultured with either unstimulated or stimulated lupus DCs for 5 days, as compared with unstimulated or stimulated DCs from healthy controls (percent of CD25+CD40L+ T cells: 2.4 ± 1% with unstimulated control DCs were added; 5.8 ± 2.1% when unstimulated lupus DCs were added; 4.8 ± 1.2% when stimulated control DCs were added; 10.8 ± 3% when stimulated lupus DCs were added; \( p < 0.05 \) between control and lupus cells for both conditions; results represent mean ± SEM of independent experiments using five controls and 11 SLE patients). No significant differences were seen for the early T cell activation marker CD69 between control and lupus DCs (data not shown). As expected, DCs treated with maturation stimuli were more effective at inducing allogeneic T cell activation (Fig. 8).

**Discussion**

This study establishes that myeloid DCs from individuals with SLE display distinct phenotypic and functional differences characteristic of proinflammatory DCs and promote abnormal T cell responses. These phenotypic changes are characterized by accelerated differentiation from the monocyte to the myeloid DC stage (as measured by increased expression of the DC differentiation marker CD1a), up-regulation of different costimulatory molecules which are involved in T cell priming, even in the absence of exogenous maturation stimuli; and increased production of the proinflammatory cytokine IL-8. In addition, myeloid DCs from SLE patients display a selective down-regulation of the maturation marker CD83. These abnormalities were seen not only on in vitro-derived DCs, but also in DCs obtained from peripheral blood, indicating that this phenomenon is not the consequence of added cytokines in vitro or a serum withdrawal effect but likely represent true abnormalities on the differentiation/maturation pathways of myeloid DCs in vivo.

These phenotypic differences correlate with specific clinical and serologic manifestations of the disease. Because the population studied was fairly well-controlled, with an SLEDAI of 4.5 ± 3 (mean ± SEM), the results of this study suggest that the abnormalities observed in SLE DCs are not merely the result of active disease but might have pathogenic significance. Further, these phenotypic differences in lupus DCs likely have functional relevance, as both unstimulated and stimulated myeloid DCs from lupus patients induce significant increases in allogeneic T cell proliferation and activation, when compared with healthy controls. Immature DCs are known to have a low T cell activation potential (20) and this promotion of T cell proliferation and activation is likely the consequence of an increased mature phenotype. Accelerated differentiation from the monocyte (or potentially other myeloid precursors) to the DC stage, and up-regulation of maturation markers could promote and enhance the capabilities of lupus DCs to prime and activate T cells in the spleen and other lymphoid organs, further contributing to the T cell hyperresponsiveness and enhanced activation described in SLE. Supporting this idea, a recent study reported up-regulation of CD11b+CD11c+ DCs in the thymus and spleen in aged BWF, lupus-prone mice (56), suggesting accelerated migration to these organs. Studies in humans have also shown that myeloid and plasmacytoid DCs are markedly decreased in SLE blood and it has been speculated, although not proven, that this might reflect an accelerated migration of these cells from the blood into tissues (22, 57). Certainly, the up-regulation of CD86 and other maturation markers in DCs would support the possibility that a mature DC phenotype would be associated to increased migration to lymphoid organs of SLE DCs and stimulation and priming of T cells in these organs. DCs can induce either T cell tolerance or strong innate and adaptive immunity to specific Ag. In general, tolerance is initiated when DCs are immature, whereas the initiation of immunity requires an effective DC maturation signal. Therefore, accelerated DC maturation in SLE in the absence of extrinsic danger signals suggests that these cells can become very
efficient autoantigen-presenting cells and drive autoimmune responses. In addition, the role of costimulatory molecules is well documented in murine models of lupus, including the observation that treatment with a combination of blocking mAbs to CD80 and CD86, before the onset of murine lupus, significantly improves survival and severity of the disease (58, 59).

Given that mature myeloid DCs are able to break tolerance and induce lupus autoantibodies in normal hosts (60), the increase in DC maturation in SLE suggests that these abnormalities might be very relevant in the induction and perpetuation of autoimmunity in SLE. Increased secretion of IL-8 by lupus DCs could potentially contribute to tissue damage, as this cytokine has been proposed to play an important role in the development of lupus nephritis (61–64) and has been found to be elevated in lupus patients with CNS involvement (65). IL-8 is produced by numerous cell types, including monocytes/macrophages and DCs (64, 66, 67). IL-8 is mainly active on neutrophils, promoting their recruitment and also their strong activation which triggers the leukotriene pathway, induces the release of their granular content, elastase and lactoferrin and increases their adherence to endothelial cells (68–71). In fact, migration of neutrophils is influenced by DCs primarily by IL-8 (64). IL-8 is also a chemotractant for other cell types including T cells (72). In addition, we found a significant association between IL-8 secretion and serum levels of anti-dsDNA. Anti-dsDNA can enhance the release of proinflammatory cytokines, including IL-8 and TNF-α from mononuclear cells to augment inflammatory reactions and can polarize the immune reaction toward the Th2 pathway (72–74). Therefore, an increase in the production of this cytokine by DCs might enhance the ability to recruit cells in the glomerulus and enhance neutrophil adhesion to vascular endothelium which in turn may contribute to renal and vascular damage. Interestingly, a recent study has shown that the IL-8 gene and its receptor CXCR-2 are up-regulated in PBMCs from SLE patients using microarrays (75).

The functional relevance of decreased levels of CD83 in SLE is unclear at this point. CD83 is a cell surface membrane glycoprotein whose surface expression is largely restricted to DCs (55). The precise functions of this molecule remain unknown (76, 77), but CD83 may serve important roles during intercellular interactions (77–79), as membrane-bound CD83 increases the stimulatory capacity of DCs (79). Further, previous studies suggest that CD83 mediates adhesion to monocytes and CD8+ T cells (80). CD83-Ig enhances T cell proliferation and increases the proportion of CD8+ T cells (80), and engagement of CD83 delivers a significant signal

FIGURE 5. Responses of monocyte-derived DCs to maturation stimuli are abnormal in lupus DCs. A, Bar graphs display mean fluorescence intensity of CD86 in DCs from 10 healthy controls, 5 RA patients, and 20 SLE patients before and after stimulation with TNF-α. B, Representative histograms of CD86 expression on DCs from 1 healthy control, 1 patient with SLE, and 1 patient with RA before and after stimulation with LPS and TNF-α. C, Bar graphs display mRNA expression by real-time RT-PCR of differentiation and maturation markers normalized to housekeeping genes in DCs, after stimulation with LPS and TNF-α in 6 healthy controls and 17 SLE patients. Results are reported as the fold change when compared with unstimulated day 7 DCs. *, p < 0.05 when compared before and after stimulation; results are presented as mean ± SEM.

FIGURE 6. Aberrant expression of differentiation and maturation markers in lupus myeloid DCs obtained from peripheral blood. A, Bar graphs display expression of DC differentiation and maturation markers in freshly isolated CD11c+ cells from peripheral blood, in 11 healthy controls and 24 SLE patients. B, Bar graphs display correlation of anti-dsDNA Abs with CD1a+ DCs in peripheral blood. *, p < 0.05, results represent the mean ± SEM of independent experiments.
specifically supporting the expansion, survival and function of newly primed naïve CD8+ T cells (81). CD8+ T cells in lupus-prone mice are impaired in expansion, acquisition of memory, secretion of cytokine, and suppression of autoimmunity (8, 82) and because CD83 appears to have a role in CD8+ function, it is possible that down-regulation of the former could contribute to abnormal CD8+ function in SLE. In addition, CD4+ T cells that develop in a CD83 mutant animal fail to respond normally following allogeneic stimulation (83), at least in part due to an altered cytokine expression pattern characterized by an increased production of IL-4 and IL-10 and diminished IL-2 production, findings typically seen in SLE. Thus, absence or decrease of CD83 in SLE DCs may result in the generation of T cells with an altered activation and cytokine profile. Future studies will address these possibilities. The factors inducing down-regulation of CD83 in lupus DCs remain to be determined. Although we detected that autologous serum down-regulates the percentage of DCs that express CD83, this phenomenon was observed for both lupus and control serum, and therefore cannot explain the differences in expression of this molecule observed between the two groups. Similarly, treatment with IFN-α did not alter CD83 expression.

DCs from SLE patients responded to extrinsic maturation stimuli by up-regulating the expression of maturation markers, further increasing IL-8 secretion and enhancing T cell proliferative and activation responses. However, the up-regulation of some of the membrane-bound maturation markers was blunted when compared with the degree of up-regulation seen in healthy controls. It is possible that the preactivation status of lupus DCs, as confirmed by the overexpression of maturation markers even before maturation stimuli, makes the lupus cells more refractory to further up-regulation of cell surface maturation markers. Lupus DCs treated with maturation stimuli did become more efficient at inducing allogeneic T cell proliferation, T cell activation and cytokine secretion, than untreated lupus DCs.

Although we cannot entirely exclude the possibility that immunosuppressive drugs could play a role in the phenotypic and functional differences of lupus myeloid DCs, correlation analysis did not show an association between expression of specific differentiation and maturation markers and secreted cytokines with different medications used by SLE patients. Further, when we treated monocytes with drugs commonly used to treat SLE and differentiated them into DCs, we found no differences in the maturation and differentiation patterns seen in untreated or treated cells. Also, while RA patients are also exposed to immunosuppressive medications, DCs from patients with this disease did not show any significant differences in the expression of maturation and differentiation markers and cytokine profile when compared with healthy controls. These observations suggest that our findings are not related to the use of medications in these patients. In addition, an effect of corticosteroid treatment on DC function can reasonably be excluded, as these agents have a short biological half-life of 12–36 h (84) and therefore can only have a marginal effect on maturation of DCs after a 7-day culture. Further supporting that our results are not related to medication use, a recent report in a novel lupus-prone mouse strain, B6.Sle3 (not subject to any type of immunosuppressive treatment), indicates that its DCs are hyperstimulatory, more mature and proinflammatory, overexpress CD80 and CD86 among other costimulatory molecules, and induce T cell hyperactivity (85), similar to our findings in human lupus DCs.

Our results on the differentiation and maturation status of myeloid derived DCs differ from the ones previously described by Koller et al. (54), where no significant increases in the expression of maturation markers in lupus DCs and a decrease in T cell proliferation by lupus DCs were found. We believe that these discrepancies might be secondary to differences in methodology. The number of patients studied by Koller et al. (54) was significantly smaller and disease activity also appeared to be lower, although a different scoring system was used. That group used positive selection to isolate monocytes, which could contribute to activation of these cells, as has been reported for other PBMCs (86). In addition, their cells were cultured for longer periods of time and under conditions that were not serum-free. It has been demonstrated that serum contains growth factors that could affect DC development, differentiation and maturation in vitro (87, 88) and that serum-free conditions like the ones used in our study lead to more optimal DC harvest that non-serum free conditions (89). Furthermore, Koller et al. (54) used irradiated DCs for their mixed-lymphocyte reaction studies, while we used nonirradiated DCs. Irradiated DCs can undergo apoptosis (90) and posttranslational modifications of proteins during apoptosis can potentially modify T cell activation and proliferation (90). Our results do confirm a previous observation that CD83 expression on lupus DCs is not up-regulated after maturation stimuli (91), are supported by similar ex vivo findings in DCs isolated from peripheral blood and, as mentioned, are reminiscent of what has been reported in lupus animal models (85).

Our observations that DCs from RA patients do not display the phenotypic abnormalities seen in SLE are in consensus what has been previously been reported in the RA literature in monocyte-derived DCs (92, 93). Our observations may not necessarily indicate...
that all RA DCs have normal phenotype and function. Indeed, monocyte-derived DCs might not reflect changes occurring in the inflamed synovium and there is evidence that DCs in the joint of RA patients show a distinct phenotype, with differential expression of specific TLRs (94). DCs are abundant both in synovial tissue and in synovial fluid of RA patients (95) and it has been proposed that RA synovitis may be a delayed-type hypersensitivity reaction generated by the interaction of synovial DCs and T cells (96). Furthermore, the cytokine profile in RA and SLE is known to be distinctly different (35, 93, 97) and there is evidence that the mechanisms leading to autoimmunity in SLE and RA are probably quite distinct (98, 99). Therefore, the lack of hyperactivated DCs in RA patients does not imply that the abnormal DC phenotype observed in SLE is not important in maintaining autoimmunity, and this statement is supported by findings in lupus animal models (56, 85).

Immune complexes consisting of DNA and anti-dsDNA Abs isolated from the sera of patients with SLE can induce plasmacytoid DCs to produce high levels of IFN-α (100). Increased IFN-α production in SLE causes increased monocyte differentiation into DCs (22). These cells are able to capture apoptotic cells and present their Ags to autologous T cells and induce potent MLRs (22). We propose that, in addition to the IFN-α, there are endogenous abnormalities in myeloid DC differentiation and maturation, as a serum effect and exogenous IFN-α could not account for, or abrogate, the differences in phenotype and function seen in lupus monocyte-derived DCs. Purified nucleosomes directly induce in vitro DC maturation of mouse bone marrow-derived DC, human monocyte-derived DC and purified human myeloid DCs as observed by stimulation of allogenic cells in MLR, IL-8 secretion, and CD86 up-regulation (101), findings similar to the ones we report in this study. Further, nucleosomes complexed with antinucleosome Abs can activate DCs (102). Therefore, autoantigens and immune complexes could play an important role in vivo by inducing accelerated differentiation and maturation of lupus DCs.

Taken together, our data suggest that monocyte-derived DC differentiation and maturation are altered in SLE and contribute to enhanced T cell proliferation and activation and to an increase in the secretion of proinflammatory cytokines. We hypothesize that these events could help to initiate and maintain the autoimmune response in lupus.
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References

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