Nucleosome, the Main Autoantigen in Systemic Lupus Erythematosus, Induces Direct Dendritic Cell Activation via a MyD88-Independent Pathway: Consequences on Inflammation

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Nucleosome, the Main Autoantigen in Systemic Lupus Erythematosus, Induces Direct Dendritic Cell Activation via a MyD88-Independent Pathway: Consequences on Inflammation

Patrice Decker,†* Harpreet Singh-Jasuja,* Sabine Haager,† Ina Kötter,† and Hans-Georg Rammensee*.

Nucleosome is the major autoantigen in systemic lupus erythematosus. It is found as a circulating complex in the sera of patients and seems to play a key role in disease development. In this study, we show for the first time that physiologic concentrations of purified nucleosomes directly induce in vitro dendritic cell (DC) maturation of mouse bone marrow-derived DC, human monocyte-derived DC (MDDC), and purified human myeloid DC as observed by stimulation of allogeneic cells in MLR, cytokine secretion, and CD86 up-regulation. Importantly, nucleosomes act as free complexes without the need for immune complex formation or for the presence of unmethylated CpG DNA motifs, and we thus identified a new mechanism of DC activation by nucleosomes. We have clearly demonstrated that this activation is nucleosome-specific and endotoxin-independent. Particularly, nucleosomes induce MDDC to secrete cytokines known to be detected in high concentrations in the sera of patients. Moreover, activated MDDC secrete IL-8, a neutrophil chemoattractant also detected in patient sera, and thus might favor the inflammation observed in patients. Both normal and lupus MDDC are sensitive to nucleosome-induced activation. Finally, injection of purified nucleosomes to normal mice induces in vivo DC maturation. Altogether, these results strengthen the key role of nucleosomes in systemic lupus erythematosus and might explain how peripheral tolerance is broken in patients. The Journal of Immunology, 2005, 174: 3326–3334.

1 MyD88-Independent Pathway: Consequences on Inflammation

S ystemic lupus erythematosus (SLE) is an inflammatory autoimmune disease of unknown etiology that is characterized by a large amount of circulating autoantibodies with diverse specificities. Among the targets recognized by these autoantibodies is the nucleosome, a complex composed of 180 bp of DNA and the five histone molecules (H1, H2A, H2B, H3, and H4). The nucleosome is the main lupus autoantigen and is believed to play a key role in disease development because it is found as a circulating complex in patient sera (1) and because both autoreactive nucleosome-specific B and Th lymphocytes are detected in patients (2, 3). Moreover, the levels of both anti-nucleosome autoantibodies and circulating nucleosomes have been shown to be associated with disease activity (4–6). Recently, dendritic cells (DC) have been suggested to be involved in lupus initiation by priming autoreactive Th cells (7, 8). DC play a central role in priming naïve T cells (9) as well as in inducing tolerance to self Ag, depending on the maturation state of DC. T cell priming requires mature DC, whereas immature DC can induce T cell tolerance (as explained in Ref. 10). Thus, dysregulation of DC maturation and chronic DC activation may skew self-Ag presentation from tolerance to autoimmunity. Moreover, genomic DNA released by dying cells has been shown to induce maturation of APC (11). However, circulating DNA in SLE is usually not found as free DNA but rather in the form of circulating nucleosomes (1). Thus, we have investigated whether purified nucleosomes might interact with DC and have demonstrated that free nucleosomes induce direct DC maturation. Our results could provide an explanation for the break of peripheral tolerance in SLE patients and link the presence of nucleosomes to DC maturation and T cell activation even in the absence of anti-nucleosome autoantibodies.

Materials and Methods

Animals and cell cultures

BALB/c and C57BL/6 female mice were purchased from Janvier and Charles River Laboratories. MyD88−/− mice on a C57BL/6 background (described in Ref. 12) were obtained from Dr. H. Schild (Institute for Immunology, Johannes Gutenberg-University, Mainz, Germany) who got them from Dr. H. Wagner (Institute of Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany). Bone marrow-derived DC (BMDC) were prepared by culturing bone marrow cells in IMDM (BioWhittaker) containing 10% heat-inactivated FBS and supplemented with 150 U/ml GM-CSF (PeproTech) for 6–8 days as described previously (13). DC were analyzed by flow cytometry for cell surface markers using mAb specific for CD11c, CD14, CD86, and MHC class II (BD Pharmingen) on a FACScalibur apparatus with CellQuest software (Becton Dickinson). For MLR, spleen cells were prepared by passage through a 40-μm strainer. To prepare peritoneal exudate cells (PEC), mice were killed and the peritoneal cavity was washed twice with 5 ml of PBS, 1 mM EDTA. Cells were then harvested and RBC were lysed with 5 ml of hypotonic buffer followed by two washes with PBS. Human PBMC were prepared from buffy coats (Blood Bank) or heparrin blood from normal individuals (altogether >30 normal donors) and 6 lupus patients accordingly to standard procedures. Lupus patients fulfilled the American College of Rheumatology criteria and had the following characteristics: five women/one man; SLE disease activity index (SLEDAI) = 0, 0, 2, 4, 6, and 8; three anti-DNA-positive and three anti-DNA-negative patients; all were anti-nuclear Ab-positive patients; all patients under treatment. Human monocyte-derived DC (MDDC) were obtained from plastic-adherent

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Nucleosomes, histones, DNA, and mAb

Nucleosomes were prepared from calf thymus as described previously (15) except that all the preparation and purification steps were performed under sterile conditions. DNA and protein contents were checked on a 1.5% agarose gel and an 18% SDS-PAGE, respectively. Without any specific detection in the text, nucleosomes will refer to mononucleosomes. It should be noted in this study that histones are among the most conserved proteins. For instance, there is no difference in protein sequence between histone H4 and H4k, 0.01-2 μg/ml, human IFN-γ (BD Pharmingen; 5000 U/ml). All experiments using animals or human cells have been approved by the local ethics committee (References IM 2/03 and 146/2011V, respectively).

Cell surface nucleosome binding

Nucleosomes induce BMDC maturation

In previous work, we have shown that purified nucleosomes may influence cell behavior. Thus, nucleosomes induced lymphocyte necrosis. On the contrary, DC were relatively resistant (17). We then tested whether purified nucleosomes may favor BMDC maturation. We first evaluated the ability of BMDC cultured in the presence of nucleosomes to stimulate allogenic spleen cells in a mixed leukocyte reaction (MLR) assay. We used a high spleen cells:BMDC ratio (20:1) to minimize the response observed with unstimulated BMDC and to test nucleosome-injected, buffer-injected, and untreated mice were all used for comparison of cpm values and BMDC/MDDC activation by different stimuli.

Results

Nucleosomes induce BMDC maturation

In previous work, we have shown that purified nucleosomes may influence cell behavior. Thus, nucleosomes induced lymphocyte necrosis. On the contrary, DC were relatively resistant (17). We then tested whether purified nucleosomes may favor BMDC maturation. We first evaluated the ability of BMDC cultured in the presence of nucleosomes to stimulate allogenic spleen cells in a MLR assay. We used a high spleen cells:BMDC ratio (20:1) to minimize the response observed with unstimulated BMDC and to get a clear increase with stimulated BMDC. As shown in Fig. 1A, nucleosome-stimulated DC induced proliferation and IL-2 secretion of allogenic spleen cells as compared with DC cultured in medium, whereas only a slight effect was observed in the presence of the purification buffer. The activation was nearly as strong as the activation observed with LPS-stimulated BMDC. Moreover, spleen cell activation was clearly reduced with boiled nucleosomes

First cultured with the LPS inhibitor polymyxin B (Sigma-Aldrich; 50 μg/ml) for 40 min and then the stimuli were added to the cell cultures.

Mixed leukocyte reactions

A total of 10,000 mature DC were cultured with 200,000 allogenic or syngeneic spleen cells. After 24 and 48 h, supernatants were collected and frozen at –80°C until use and replaced by fresh medium. At day 4, cell proliferation was measured by [3H]thymidine (Amersham Biosciences) incorporation (with 1 μCi/well for the last 18 h) using a scintillation counter (Microbeta; Wallac). Supernatants were tested for the presence of IL-2 using the CTL-L2 cell line, the growth of which is dependent on the presence of IL-2. In this case, thymidine incorporation was measured after 18 h in the presence of 10^6 cells/well (with 1.5 μCi/well for the last 8 h).

Cytokine production

Cytokine synthesis was tested in BMDC by intracellular staining with a mAb specific for IL-12p40/70 (BD Pharmingen) following cell fixation and permeabilization according to manufacturer’s recommendations and analyzed by flow cytometry. Cytokine secretion was measured by sandwich ELISA using mAb pairs (BD Pharmingen) and according to manufacturer’s recommendations.

Cell surface nucleosome binding

A total of 10^5 immature DC were incubated with FITC-nucleosomes on ice or at 37°C in 10% FCS-containing PBS for 30 min. Cells were then washed and incubated with PI for flow cytometry analysis. FITC-BSA (Sigma-Aldrich) was used as negative control. In some cases and to distinguish cell surface binding from endocytosis, cells were then incubated with 0.4% of trypsin blue to quench extracellular FITC-fluorescence (18) and immediately analyzed. In this case, dead cells were gated out.

In vivo DC maturation

BALB/c mice were first bled to prepare sera. Mice were then left untreated or received injections of 300 μg of purified nucleosomes or the same volume of purified buffer or 100 μg LPS in 10% sucrose buffer. Four, 8, 24, and 72 h later, mice were bled again to prepare sera and PEC were collected after 72 h as described above. It should be noted that i.p. injections of bacterial products have already been shown to induce in vivo activation of peritoneal DC (19). Cells were then incubated with an FcR-blocking mAb (BD Pharmingen), stained with mAb specific for CD11c and CD86 (BD Pharmingen; 10 ng/ml), PGE2 (Sigma-Aldrich; 1 μg/ml), human TNF-α (BD Pharmingen; 200 μg/ml), LPS (Sigma-Aldrich; from Salmonella typhimurium, 0.01–2 μg/ml), human IFN-γ (BD Pharmingen; 5000 U/ml), and incubated with PI for flow cytometry analysis. A total of 100,000 events were acquired for each sample. Cells were gated on living PEC (7-AAD/BDF Pharmingen) to exclude dead cells, and analyzed by flow cytometry. A total of 100,000 events were acquired for each sample. Cells were gated on living PEC (7-AAD/BDF Pharmingen) to exclude dead cells, and analyzed by flow cytometry. A total of 100,000 events were acquired for each sample. Cells were gated on living PEC (7-AAD/BDF Pharmingen) to exclude dead cells, and analyzed by flow cytometry.
(70 and 55% reduction of cell proliferation and IL-2 secretion of allogenic cells, respectively) or when spleen cells were cultured in the presence of nucleosomes but without BMDC indicating that activation is due to nucleosome-induced BMDC maturation and not to direct nucleosome-induced spleen cell activation. Importantly, purified nucleosomes were used in the absence of anti-nucleosome Ab, indicating that nucleosomes act in the form of free complexes and are not in the form of immune complexes.

We then tested nucleosome-induced cytokine production by BMDC. IL-12p40/70 synthesis was induced by nucleosomes (Table I) but not by the purification buffer, as measured by intracellular staining followed by flow cytometry. This synthesis could be significantly inhibited by heating nucleosomes at 95°C, suggesting that LPS contamination was not responsible for BMDC activation because LPS is known to be resistant to heating. It also indicates that DC were not unspecifically activated by aggregates or large molecular complexes. Moreover, cytokine secretion was also tested by ELISA. As shown in Fig. 1B, IL-6 and IL-12p40/70 secretion were induced in the presence of purified nucleosomes in a dose-dependent manner but not with the purification buffer. As described above, heated nucleosomes failed to induce IL-12 secretion. More importantly, we have shown that nucleosome-induced cytokine secretion could be partly inhibited by nucleosome depletion using culture plates coated with LG2-2 mAb that recognizes nucleosomes in solution, proving that DC maturation was nucleosome-specific and not due to any contaminant (Fig. 1B). No inhibition was observed with the LG2-2 mAb alone or with an isotype-matched mAb (data not shown).

Endotoxin contamination is not responsible for nucleosome-induced BMDC maturation

In a first series of experiments, all the stimuli used were tested for their endotoxin content by a LAL assay. BMDC were then cultured in the presence of the corresponding stimuli and activation was tested by cytokine secretion as determined by ELISA. The results were then normalized to the endotoxin level measured in the nucleosome preparation, meaning that all the stimuli analyzed have similar and low endotoxin potencies. In these conditions, only nucleosomes were able to induce a strong IL-12p40/70 secretion, but not LPS or the buffer (Fig. 2A). More importantly, we compared the behavior of BMDC prepared from MyD88−/− and wild-type (WT) mice. MyD88 is associated with various TLR including TLR4. LPS-induced cytokine production by BMDC from MyD88−/− mice is known to be abolished in comparison to WT mice (20). As shown in Fig. 2B, nucleosomes induced IL-6 secretion from both WT and MyD88−/− BMDC (although moderately in the latter), whereas LPS only induced strong IL-6 secretion in WT BMDC, indicating that

![Image](image-url)

**FIGURE 1.** Nucleosomes activate BMDC. A, BMDC were cultured in the presence of different stimuli and used as stimulator cells in MLR assays with allogenic or syngenic spleen cells (with a spleen cells:BMDC ratio of 20:1). After 24 h, supernatants were harvested and tested for the presence of IL-2 using the CTLL-2 cell line and cell proliferation was estimated at day 4. The differences observed between cpm values with buffer-treated BMDC and nucleosome-activated BMDC were statistically significant in all cases as determined using a Student’s t test (p ≤ 0.002). B, BMDC were cultured as in A and supernatants were harvested after 24 h and tested for the presence of IL-6 and IL-12p40/70 by ELISA. The supernatants used to measure IL-6 or IL-12 were from two independent experiments in which different stimuli were tested. The differences between buffer- and Nuc 100-induced cytokine secretion were statistically significant (p ≤ 0.04). Concentrations of stimuli are in micrograms per milliliter. NT, Not tested. Nuc boiled and Nuc/LG2-2 were performed with 100 μg/ml Nuc. Shown is one representative experiment of three. SDs are included.

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>IL-12p40/70</th>
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<tr>
<td>Medium</td>
<td>0.3</td>
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<tr>
<td>Buffer</td>
<td>0.2</td>
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<tr>
<td>Nuc 50 μg/ml</td>
<td>10.7</td>
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<tr>
<td>Nuc 50 μg/ml, 95°C</td>
<td>3.0</td>
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<td>LPS 1 μg/ml</td>
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*BMDC were incubated for 6–8 h with the indicated stimuli in the presence of mOmson. Following fixation/permeabilization, cytokine synthesis was measured by intracellular staining using a mAb specific for IL-12p40/70 and analyzed by flow cytometry. Results are expressed as percentages of IL-12-producing BMDC. Shown is one representative experiment of four independent experiments.

**FIGURE 2.** BMDC activation is not due to endotoxin contamination. A, BMDC were cultured in the presence of medium, 100 μg/ml nucleosome, or 0.1 μg/ml LPS, and supernatants were harvested after 24 h. In parallel, the extent of endotoxin contamination present in 100 μg/ml nucleosomes, 0.1 μg/ml LPS and in the same volume of sucrose buffer was assessed in a LAL assay. Supernatants were then tested by ELISA for the presence of IL-12p40/70 and cytokine secretion was normalized according to the level of endotoxin present in 100 μg/ml nucleosomes. B, BMDC were prepared from MyD88 KO as well as WT mice and cultured with different stimuli. After 24 h, supernatants were harvested and IL-6 secretion was determined by ELISA. Shown is one representative experiment of three. SDs are included. In all cases, differences between buffer- and nucleosome-induced cytokine secretion were statistically significant (p ≤ 0.03).
nucleosome-induced BMDC activation is not due to endotoxin contamination. Several other controls are used in the next experiments described below and in agreement with nucleosome-induced DC activation in the absence of endotoxins. For example, DNase I-treated nucleosomes as well as LG2-2-treated nucleosomes do not induce DC activation (Figs. 3 and 4), whereas very low endotoxin-containing nucleosomes do activate DC (Fig. 5).

**Nucleosomes induce MDDC maturation**

We next analyzed human DC maturation in the presence of purified nucleosomes. As shown in Fig. 3, nucleosomes induced CD86 up-regulation on MDDC as observed by flow cytometry. Both the percentage of CD86-positive cells as well as the level of CD86 expression (Fig. 3A and Table II) were increased in the presence of nucleosomes (starting at 25 μg/ml, data not shown). Moreover, both CD1a-negative and CD1a-positive DC (obtained with or without human serum, respectively) were activated by nucleosomes (data not shown). More importantly, CD86 up-regulation was not detected in the presence of an unrelated purified protein (BSA) or protein aggregates in solution in the purification buffer and containing a similar level of endotoxin contamination as tested by a LAL assay. Likewise, no up-regulation was observed in the presence of DNA purified from the corresponding nucleosome preparations. Importantly, we observed a 69% inhibition of CD86 up-regulation when MDDC were cultured in plates coated with LG2-2 mAb as described above for BMDC (Fig. 3B) and a 86% inhibition when nucleosomes were first DNase I-treated (Fig. 3C). No inhibition was observed with the LG2-2 mAb alone or with an isotype-matched mAb (data not shown). As described for BMDC, purified nucleosomes were used in the absence of anti-nucleosome Ab, indicating that nucleosomes act in the form of free complexes and are not in the form of immune complexes. Nevertheless, CD83 was not clearly up-regulated.

MDDC activation was also analyzed at the level of cytokine secretion. TNF-α as well as IL-6, IL-10, IL-8, and IL-12p40, but not IL-12p70, were secreted in the presence of purified nucleosomes (starting at 25 μg/ml, data not shown), but not with the purification buffer (Fig. 4A). It should be noted that CD1a+ MDDC were more sensitive to activation than CD1a− MDDC and that nucleosome-induced IL-10 secretion was stronger with the CD1a+ population. MDDC preparations were always checked for CD14 expression and were in all cases negative for this marker, suggesting that these cytokines were not produced by contaminating monocytes. Moreover, IL-8 secretion by DC in the presence of nucleosomes has been checked by intracellular staining followed by flow cytometry analysis using a gate excluding monocytes (data not shown). Moreover, we compared nucleosome-induced cytokine secretion of MDDC as well as lymphocyte-rich and monocyte-rich fractions from the same blood donor and cultured in the same conditions. As shown in Fig. 4B, MDDC were the main cytokine producers following nucleosome exposure, whereas all cell types were strongly activated in response to LPS and IFN-γ. Because monocytes are highly sensitive to LPS, this result is also in favor of a low endotoxin contamination of nucleosomes. As described for CD86 up-regulation, purified BSA, protein aggregates, DNA purified from nucleosomes, as well as commercially available histones failed to induce IL-6 secretion (Fig. 4C). However, histones induced an intermediate IL-8 secretion. Nucleosome-induced cytokine secretion could be completely inhibited when MDDC were cultured in LG2-2-coated plates (Fig. 4D) or when nucleosomes were first treated with DNase I, suggesting that nucleosome integrity is essential for DC activation (Fig. 4E). No inhibition of cytokine secretion was observed with the LG2-2 mAb alone or with an isotype-matched mAb (data not shown).

To exclude again the possibility that contaminating endotoxins might be responsible for MDDC maturation, a series of control experiments were performed. First, IL-12p70 secretion was measured following DC exposure to different stimuli. Both nucleosomes and LPS were unable to induce IL-12p70 secretion. Secretion of IL-12p70 was only observed with LPS in combination with IFN-γ. Nevertheless, no IL-12p70 secretion was induced when nucleosomes were combined with IFN-γ (Fig. 5A), suggesting that the amount of endotoxins in the nucleosome preparation is low. As a control, all the stimuli induced TNF-α secretion. We next used very low endotoxin-containing nucleosome preparations (~1 IU/mg) to activate MDDC and we compared the results to MDDC activation induced with the LPS concentration containing a similar level of endotoxins and having thus a similar potency, namely 10 ng/ml (see the table in Fig. 5B). As shown in Fig. 5B, this nucleosome preparation was still able to induce both CD86 up-regulation as well as IL-8 secretion by MDDC, whereas 10 ng/ml LPS failed to induce activation. Actually, 500 ng/ml LPS were required to get a cytokine secretion similar to the secretion observed with nucleosomes. Regarding CD86 expression, LPS alone (500 ng/ml) was not a strong stimulus and needed to be combined with IFN-γ and is therefore not included in the figure. Moreover, nucleosome-induced MDDC maturation was not inhibited in the presence of polymyxin B, an LPS inhibitor, in contrast to LPS-induced activation. In agreement with a role of nucleosome independently of endotoxin, Bausinger et al. (21) have shown that “very low endotoxin”-contaminated heat shock protein 70 (containing 9.5 IU/mg endotoxins) does not induce cytokine secretion in MDDC. In this study, we have shown that nucleosomes containing as little as 1 IU/mg endotoxins still activate MDDC.

**FIGURE 3.** Nucleosomes activate MDDC. A, MDDC were cultured in the presence of various stimuli and CD86 expression was measured at day 3 by flow cytometry. The resulting CD86 expression is shown in terms of percentage of positive cells. CD86 up-regulation was inhibited in the presence of a nucleosome-specific mAb (B, expressed in term of molar excess) or when nucleosomes were DNase I-treated (C). Shown is one representative experiment. In B and C, Nuc corresponds to 100 μg/ml nucleosomes. TNF-α with PGE2, or LPS with IFN-γ were used as positive controls for DC activation.
Nucleosomes activate enriched MDDC, freshly isolated blood DC, as well as lupus MDDC

To show that nucleosomes directly interact with DC and that DC maturation is not a secondary effect of nucleosomes on contaminating cells, MDDC were prepared from monocytes isolated by negative selection. Such MDDC preparations were at least 90% pure (data not shown). As described above, nucleosomes induced CD86 up-regulation as well as cytokine secretion (Table II). Moreover, we did not observe any correlation between the extent of contaminating cells and DC maturation.

Although monocytes have been shown recently to differentiate to DC in vivo (22), we have analyzed activation of freshly isolated mDC and pDC obtained by negative selection. As shown in Table II, both mDC and pDC up-regulated CD86 in the presence of nucleosomes. The CD86 staining was indeed lower in pDC than in mDC but this was observed with all culture conditions and not only in the presence of nucleosomes. Thus, the increase of the CD86 staining induced by nucleosomes was comparable for both mDC and pDC. Moreover, CD83 up-regulation by mDC and pDC was clearly observed in the presence of nucleosomes in contrast to MDDC. Therefore, we can exclude TLR2 and TLR4 as being involved in signaling and thus exclude LPS as being responsible for DC activation. Regarding cytokine secretion, only IL-10 was induced in mDC by nucleosomes. IL-10 is an anti-inflammatory cytokine that can acquire proinflammatory activity during immune response. Thus, IFN-α priming of APC results in a gain of proinflammatory function of IL-10 (23). Because serum levels of IFN-α are increased in lupus patients, IL-10 could act as a proinflammatory cytokine in SLE, meaning that nucleosome-activated mDC probably do not act as regulatory or anti-inflammatory DC. In contrast, no cytokine secretion was induced in pDC. On the contrary, a slight inhibition in IL-6 secretion was observed, partly due to the buffer itself, whereas the cells responded to the stimulation by CpG 2006. Nevertheless, IFN-α has not been tested. MDDC being rather related to mDC than to pDC, these results are in agreement with the observations described above.

To assay to any intrinsic difference between normal and lupus DC, MDDC were prepared from patient blood and cultured in the presence of purified nucleosomes. As observed with normal MDDC, nucleosomes induced a clear CD86 up-regulation and a higher percentage of CD86-positive cells in lupus MDDC (Table II). Likewise, nucleosome-induced activation of lupus MDDC results in a higher TNF-α, IL-6, and IL-8 secretion as compared with DC incubated with medium or buffer-containing medium and to a level similar to normal MDDC incubated with nucleosomes (Table II and data not shown). Thus, the effect of nucleosomes on lupus DC is similar to the one observed with normal DC, supporting the existence of such an activation mechanism in vivo in patients. Importantly, nucleosome-induced DC activation was observed with DC from patients with SLEDAI = 0 as well as with elevated SLEDAI scores.

Nucleosomes are engulfed by DC via macropinocytosis

To elucidate by which mechanism nucleosomes induce DC maturation, we analyzed the ability of FITC-nucleosomes to bind to
the cell surface of immature DC by flow cytometry. Whereas a clear signal was observed at 37°C for living MDDC (Fig. 6A, PI-negative cells), low binding was observed at 4°C (the same result was obtained with BMDC, data not shown). Moreover, the signal observed with living cells at 37°C was poorly inhibited in the presence of trypan blue (Fig. 6B), indicating that most nucleosomes are not localized at the cell surface but have been endocytosed by the DC. It was indeed shown that trypan blue only quenches the extracellular FITC fluorescence of living cells (18). As a control, the signal detected with a FITC-conjugated anti-HLA-DR mAb was strongly inhibited, indicating that the mAb is mostly bound at the cell surface. These results rather suggest that nucleosomes do not bind specifically to a cell surface receptor present on DC but enter DC by macropinocytosis. A similar pattern was observed with FITC-BSA and DC (data not shown). It should be noted that using the same protocol, we have already shown that FITC-nucleosomes specifically bind to the cell surface of B lymphocytes at 4°C (17). The signal observed with FITC-nucleosomes on PI-positive cells is not due to cell killing by nucleosomes. We could indeed never observe cell killing after such a short incubation (30 min).

In vivo nucleosome-induced DC maturation

To strengthen the role of circulating nucleosomes in DC maturation, we have tested in vivo DC maturation in mice. For this purpose, purified nucleosomes were i.p. injected and the phenotype of PEC was analyzed at day 3. PEC have indeed already been shown to contain DC and i.p. injections (of bacterial products) have been shown to induce in vivo activation of peritoneal DC as well as DC recruitment (19). As shown in Fig. 7, the percentage of living CD11c+/CD86+ double-positive cells was 2.9- to 4.7-fold increased in nucleosome-injected mice as compared with noninjected mice (p = 0.002) and with buffer-injected mice (p = 0.009), and these differences were statistically significant as evidenced using a two-tailed Student’s t test. Only slight differences were observed between noninjected and buffer-injected mice. It should be noted that a fourth group of mice was planned as a positive control, consisting in LPS injection. However, we observed that LPS, in contrast to nucleosomes, induced CD11c down-regulation in part of the mice (data not shown) and thus these mice could not been included in this histogram. Nevertheless, LPS-induced maturation of DC could be achieved in vivo because we observed a clear CD86 up-regulation (data not shown). This also suggests that the nucleosome effect is unrelated to LPS contamination. Finally, mouse sera were also tested by ELISA for determination of the concentrations of several circulating cytokines (see Materials and Methods). Nevertheless, no difference could be observed in cytokine serum concentrations between nucleosome-injected and noninjected mice (data not shown). On the contrary and as expected, a clear increase was observed for several cytokine concentrations after LPS injection (data not shown), which also confirms that our purified nucleosomes contain only low levels of LPS. Due to the low number of activated DC in PEC, it was not surprising to observe no increase in cytokine secretion after nucleosome injections. As a comparison, LPS activated other cell types than DC that represent higher cell numbers among PEC. Moreover, LPS is probably a more potent activator than nucleosomes.

Discussion

Nucleosomes are known as major lupus autoantigens and are found as circulating complexes in patients. In this study, we show for the first time that purified nucleosomes directly induce DC maturation. Importantly, this activity was found for nucleosomes in the form of free complexes without the need of being engaged in immune complexes and independently of the presence of unmethylated CpG DNA motifs. Thus, we identified a new mechanism of DC activation by nucleosomes. Indeed, chromatin-containing immune complexes have been recently shown to induce BMDC activation (24). In that paper, the authors showed that nucleosome-containing immune complexes, but not free nucleosomes, induce TNF-α secretion and CD86 up-regulation in BMDC. Nevertheless, MDDC were not tested in that study. On the contrary, we have found that free nucleosomes also activate DC. We essentially detected IL-6 and IL-12 secretion by nucleosome-activated BMDC, whereas TNF-α was in most cases not induced by nucleosomes or at low levels. Moreover, we only detected nucleosome-induced CD86 up-regulation in MDDC but not in BMDC. Thus our results are not in contradiction with those described by Boulé et al. (24). The observed difference comes from the origin of the cells used, as well as the cytokines tested to measure DC activation. Moreover, it is possible that nucleosome-containing immune complexes induce a stronger DC activation than free nucleosomes. Nevertheless, our result is particularly important because it points to a role of free nucleosomes in lupus and might partly explain how peripheral tolerance is broken. It should be noted that the involvement of DC in SLE has been recently suggested by other studies (reviewed in Ref.7). In accordance with our present results, the scenario would be as follows. Circulating nucleosomes, perhaps rendered immunogenic by modifications or by large excess, are engulfed by DC, processed, and presented to autoreactive Th cells, furnishing signal 1 for T cell priming. In the same time, DC are induced to mature, leading to up-regulation of
costimulatory molecules and secretion of cytokines, furnishing signal 2 and signal 3 for efficient T cell priming. In this scenario, autoreactive nucleosome-specific Th cells are now activated and can help autoreactive and nucleosome-specific B cells to produce autoantibodies. Other cytokines induced by nucleosomes, such as IL-8, might recruit neutrophils and favor inflammation and tissue damages as observed in patients. It should be reminded in this study that SLE is classified as an inflammatory disease.

We have clearly shown that physiologic nucleosome concentrations (as explained in Ref. 17) induce DC maturation. This phenomenon was observed with BMDC as well as MDDC, either CD11c+ or CD11c-. Importantly, freshly isolated blood mDC were also sensitive to nucleosome-induced maturation. Regarding pDC, only CD83 and CD86 up-regulation were observed, whereas IL-6 secretion was rather inhibited. In agreement, MDDC are more closely related to mDC than to pDC according to the literature. DC maturation was also observed with highly pure MDDC, suggesting that nucleosomes directly interact with DC and that maturation is not indirectly induced by interaction with contaminating cells. It also suggests that cytokines detected in supernatants are secreted by mature DC and not by contaminating cells. Moreover, to highlight the role of nucleosomes in SLE and to exclude any intrinsic difference between normal and lupus DC, we have shown that lupus MDDC also mature in the presence of nucleosomes. More importantly, nucleosomes induce in vivo DC maturation in normal mice, suggesting altogether that circulating nucleosomes might behave in a similar way in patients. Nevertheless, we did not detect any increase in serum cytokine levels, possibly due to the low DC number. It is likely that in an autoimmune context the effect caused by circulating nucleosomes would be more severe because lupus patients have a decreased phagocytic activity and an impaired DNase I activity in serum. These alterations appear to be in favor of a higher half-life of nucleosomes in serum and a potential higher activity. Moreover, the observed phenomenon might be induced following a local and transient increase of the nucleosome concentration without any systemic dissemination. Thus, nucleosome-induced activation results in the development of probably highly efficient DC, as observed by CD86 up-regulation and cytokine secretion. We did not detect a clear CD83 or CCR7 up-regulation on MDDC in response to nucleosomes. On the contrary, CD83 up-regulation was clearly observed with mDC and pDC. MDDC might functionally differ from mDC and pDC. Nevertheless, it is possible that nucleosome-induced maturation in combination with another stimulus, such as infection, lowers the threshold required for full DC maturation and T cell priming. In agreement with this, bacterial and viral infections have been suggested as potential SLE triggers.

As mentioned above, CD11c was down-regulated on peritoneal DC following LPS injection, in contrast to nucleosome injection. LPS is known to induce CCR7 expression on BMDC and it is accepted that CCR7 expression is necessary for DC migration. Thus, upon stimulation with a strong stimulus like LPS, it is possible that peritoneal DC migrate to lymph nodes, resulting in an apparent CD11c down-regulation. Moreover, we know that BMDC are differentially activated in vitro by nucleosomes and LPS, resulting in a different CD11c expression (data not shown).

Importantly, we could clearly exclude that endotoxin contamination was responsible for DC maturation. First, nucleosomes treated at 95°C failed to induce strong DC maturation, whereas LPS is known to be resistant to heat treatment. Second, nucleosome-induced DC maturation was not inhibited in the presence of polymyxin B. Third, no activation was observed with buffer-containing medium or unrelated purified proteins in solution in the buffer, whereas they all contained similar levels of endotoxins as compared with nucleosomes as measured by LAL assay. Fourth, DNase I-treated nucleosomes showed an impaired activity. Fifth, depletion of nucleosomes with a specific Ab was able to disrupt nucleosome-mediated DC maturation. And sixth, nucleosomes induced cytokine secretion of BMDC from MyD88 knockout (KO) mice, proving that nucleosomes can act independently of LPS. Even if nucleosome-induced activation was reduced in the absence of MyD88 as compared with WT BMDC, the results differed clearly from those obtained with LPS. Whereas IL-6 secretion was
Reduced 4 times between WT and KO BMDC after nucleosome exposure, the secretion was reduced 115 times in the case of LPS. Thus, although endotoxin contamination might be partly involved in BMDC activation, we demonstrated that nucleosomes per se activated BMDC. Nevertheless, it is also possible that BMDC from MyD88 KO mice are less sensitive to nucleosome-induced activation or that MyD88 is partly involved in nucleosome signaling, which would mean that nucleosomes activate BMDC by two different pathways. It should be noted in this study that we did not use MyD88 KO mice to clearly identify the signaling pathway induced by nucleosomes but to prove that endotoxin contamination of nucleosome preparations is not responsible for BMDC activation. Regarding MDDC, we have also shown that very low endotoxin-containing nucleosome preparations still induced activation, whereas low LPS concentrations containing similar levels of endotoxins did not. Moreover, BMDC are usually more sensitive to endotoxins than MDDC, which strengthens a nucleosome effect independently of LPS in the human system. Importantly, because nucleosomes activated both mDC and pDC, we could exclude TLR2 and TLR4 as being involved in signaling and thus exclude LPS as being responsible for DC activation. pDC indeed do not express TLR2 or TLR4 and are not sensitive to LPS (25–27). Because TLR9 is not expressed on MDDC, we can also exclude this receptor in the signaling pathway induced by nucleosomes.

Control experiments have shown that DC maturation was specifically induced by nucleosomes because this was inhibited in the presence of the LG2-2 mAb. Moreover, it appeared that nucleosome integrity was essential for DC maturation because heat-treated nucleosomes as well as DNase I-treated nucleosomes were poor activators of DC. It is important to recall in this study that SLE patients have an impaired DNase I activity (28). Now, it is known that DNase I is the major serum DNase and impaired DNase I activity has been suggested to be responsible for higher nucleosome concentrations in sera from SLE patients. Moreover, we have shown that purified DNA and, to a lower extent, purified histones, did not induce DC maturation in contrast to nucleosomes. In the case of histones, we could only check absence of cytokine secretion and not CD86 expression because histones became toxic with time.

We could not show binding to the cell surface of DC of FITC-nucleosomes. In contrast, B lymphocytes do show cell surface staining with FITC-nucleosomes (17). We clearly showed that nucleosomes are engulfed by immature DC, probably by macropinocytosis. Our results suggest that nucleosomes activate an intracellular receptor. Likewise, CD11c+ blood DC activation via TLR3 occurs inside the cell and probably requires internalization of its ligand, namely dsRNA (29). We thus could not confirm the hypothesis that a ubiquitous nucleosome-specific receptor exists on the cell surface. Nevertheless, to our knowledge, nucleosome binding onto DC has never been tested so far. In contrast, our results are still in agreement with the existence of a ubiquitous nucleosome receptor, but which is not necessary on the cell surface depending on the cell type.

For both BMDC and MDDC, we have demonstrated that nucleosome-induced activation results in cytokine secretion. Regarding human DC, TNF-α as well as IL-6, IL-8, IL-10, and IL-12p40 were induced following nucleosome exposure. Importantly, all these cytokines have been reported to be present at higher concentrations in patient sera as compared with normal individuals and in some cases their concentrations were positively correlated with disease activity (30), as it is for nucleosome concentrations. In contrast, we could not detect any increase in IL-12p70. Interestingly, this cytokine has been shown to be present at lower concentrations in patient sera as compared with normal individuals and its serum concentration negatively correlates with disease activity (31). Regarding blood mDC, we could only detect IL-10 induction. Nevertheless, blood DC are described as CD1aDC and as mentioned above, CD1aDC are more difficult to activate.

As described above, IL-8 secretion was induced following human DC exposure to nucleosomes. IL-8 is a chemoattractant for neutrophils and serum IL-8 levels have been shown to be elevated in lupus patients and to correlate with disease activity (32). Moreover, Via et al. (33) have shown that sera from lupus patients in active phase contain an activity responsible for neutrophil activation. It should be noted that neutrophils have been suggested to link innate and adaptive immunity. Thus, nucleosome-induced IL-8 secretion might result in neutrophil recruitment, inflammation, and activation of the autoimmune response.

In agreement with our results, mature DC have been shown to be enriched in rheumatoid synovium (34). Moreover, Yu et al. (35) have found nucleosomes in synovial fluid of rheumatoid arthritis patients and have suggested that the presence of nucleosomes characterizes inflammation.
The effect of purified mammalian DNA on immune cells, especially monocytes and B lymphocytes, has already been analyzed in previous works. Ishii et al. (11) have shown that genomic DNA induced the maturation of APC. Moreover, the release of uric acid (which is produced during the catabolism of purines) by dying cells has been shown to induce DC maturation (36). Nevertheless, it is now well accepted that DNA is not found free in the sera of patients but in the form of nucleosomes. Moreover, the effect of purified nucleosomes on DC, the only APC responsible for T cell priming, has not been examined so far. We could not observe DC maturation in the presence of purified DNA. Nevertheless, we have only tested DNA on MDDC and not on BMDC as described in Ref. 11, and it is possible that BMDC are more sensitive to DNA than MDDC. Moreover, most experiments in the latter study were performed with the RAW 264.7 mouse macrophage cell line. When the authors used BMDC, only a slight effect was observed in the presence of DNA (10% of CD40/CD11c double-positive cells after treatment vs 2% in the presence of medium). Only DNA transfection induced a strong BMDC maturation.

In conclusion, we have shown that purified nucleosomes are engulfed by DC and induce DC activation, giving rise to highly potent APC. Nucleosome-induced IL-8 secretion may induce neutrophil recruitment, inflammation, and tissue damage. It should also be mentioned that we have previously shown that nucleosomes induced lymphocyte necrosis (17) and that necrosis is known to induce inflammation. Altogether, these results highlight the key role of nucleosomes in SLE development and suggest that nucleosomes may link innate and adaptive immunity in SLE and may have adjuvant-like activity. They also suggest that an increase in the concentration of circulating nucleosomes might result in T cell priming and might thus partly explain how peripheral tolerance might be broken in lupus patients. The consequences of such DC activation on neutrophil recruitment and inflammation as well as the adjuvant-like effect of nucleosomes are currently under investigation.

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Disclosures
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