Bs で6日間刺激後に Foxp3 の発現量(FACS)，培養上清中 IL-2 レベル(ELISA)を検討した。
健常者 PBMC 培養液中に HDACI:TsA, SAHA,VPA を加えた後、Bs 刺激を加えて Foxp3 の発現(FACS)と培養上清中の IL-2 レベルを ELISA で検討した。
3. 健常者 PBMC 培養液中に HDACI:TsA, SAHA, VPA を加えた後、$CD4^+$T 細胞のヒストンのアセチル化を FACS で検討した。

(倫理面への配慮)
対象者にはあらかじめ本研究の目的と方法を十分に説明し同意を得た。

C. 研究結果
I-1.NO 存在下の抗原刺激で Teff は $CD25^+CD122^-$ の $CD4^+$T細胞に分化した(NODT 細胞の誘導)。この NODT 細胞では GATA-3 及び T-bet の mRNA の発現誘導は見られなかったが IFN-γの著明な発現誘導を認めた(RT-PCR)。また、NODT 細胞の培養上清中で IL-2 レベルは上昇、IL-6,-10,-17 レベルは著明に低下した。
I-2. Teff に比して NODT 細胞は有意に CSFE-PBMC の増殖を 92%から 82%へと抑制し、一方 Foxp3 発現を 10%から 26%に増加させた。
I-3. Bs 刺激単独に比して NO は PBMC での Foxp3 の発現を 36.6%から 57.6%へと著明に増加させた。
II-1. Bs 単独刺激で PBMC の Fox3 発現は 6.2%から 36.6%に増加したが、培養上清中の IL-2 レベルの増加は認められなかった。一方、NOドナーを加えた場合では、Bs 単独刺激に比して PBMC の Fox3 発現は著明に増加し、培養上清中の IL-2 レベルは著明に増加した。
II-2. HDACI である TsA, SAHA,VPA は Bs 刺激下でいずれも Foxp3 の発現を著明に増加させたが(Bs 単独:36.6%,TsA:59.7%, SAHA:44.4%, VPA:58.0%)、培養上清中の IL-2 レベルの上昇は認められなかった。
II-3. HDACI である TsA, SAHA, VPA はいずれも単独で $CD4^+$T 細胞のhistoneのアセチル化を増加させた。

D. 考察
1. 1. NO による Teff の分化誘導
NO により Teff は $CD25^+CD122^-$ の NODT 細胞に分化したが、この細胞は主に低親和性の IL-2α受容体を発現していると考えられた。また分化した NODT 細胞では T-bet 発現がごく僅かで IFN-γの発現が亢進していたことから、paradoxical な Th1 polarization を示したことが示唆された。
2. NODT 細胞の免疫抑制作用
CSFE-PBMC の実験から、NODT 細胞は Teff に対して抑制的に作用することが示唆された。しかし、抑制作用の程度から NODT 細胞自体は Treg ではなく、NODT 細胞が共存することにより Treg の分化誘導が促進すると考えられた。従って、NO は NODT 細胞を分化誘導することにより末梢性 Treg の誘導を促進する可能性が示唆された。
3. NO による IL-2 依存性の Foxp3 の発現増加
抗原刺激下で NO は顕著に Foxp3 の発現を増加させた。また培養上清中の IL-2 レベルが著明に増加していたことから、NO はリンパ球が存在する局所環境の IL-2 レベルを保つことにより(つまり IL-2 依存性に)Foxp3 の発現を増加させることが示唆された。この IL-2 レベルの保持作用は、NO 自体が有する免疫抑制効果を考慮すると、IL-2 の産生誘導ではなく Teff による IL-2 消費を抑制することにより行なわれていると考えられた。こうした IL-2 レベルの増加には NODT 細胞の誘導も関与していると考えられた。
4. HDACI による IL-2 非依存性の Foxp3 の発現増加
HDACI による Foxp3 の発現増加は IL-2 レベルが著明に低かったことから、IL-2 非依存性である可能性が示唆された。HDACI は Histone をアセチル化してクロマチン構造のリモデリングを起こし、様々な遺伝子発現を誘導する。転写因子の Foxp3 も誘導される遺伝子のひとつである。HDACI により $CD4^+$T 細胞 Histone のアセチル化の亢進が認められたことから、本実験での HDACI による Foxp3 発現亢進はこのクロマチン構造のリモデリングによることが示唆された。
5. 本実験で確認された Foxp3 の発現増加は、expansion によるものか induction によるものか、あるいは両者なのかは今後検討予定である。
6. 本研究で検討した、末梢性 Foxp3 誘導がどの程度安定な Treg 分化を意味しているのかについて、は、さらに考察を実施する必要がある。現在臨床応用されているステロイド・免疫抑制剤はこうした末梢性 Foxp3 誘導を変化させる可能性があるが、これらの作用機序は IL-2 依存性と IL-2 非依存性の機序を両極において理解されると思われる。今後はステロイド・免

疫抑制剤による Treg 発現変化を IL-2 依存性と IL-2 非依存性の両極で捕らえながら、それがどのように長期的寛解維持に影響するかを解明してゆく必要がある。

E. 結論

ポリクローナルな抗原刺激下で培養液中の NO と HDACI は PBMC 中 $CD4^+$T 細胞の Foxp3 発現を著明に増加させた。

F. 健康危機情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

Ⅲ.　研究成果の刊行に関する一覧表

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IV.　研究成果の刊行物・別刷

Splenic Phagocytes Promote Responses to Nucleosomes in (NZB × NZW) F_1 Mice[1]

Akiko Okamoto,* Keishi Fujio,[2]* Nico van Rooijen,† Nelson H. Tsuno,‡ Koki Takahashi,‡ Hiromichi Tsurui,§ Sachiko Hirose,§ Keith B. Elkon,¶ and Kazuhiko Yamamoto*

Autoantigen presentation to T cells is crucial for the development of autoimmune disease. However, the mechanisms of autoantigen presentation are poorly understood. In this study, we show that splenic phagocytes play an important role in autoantigen presentation in murine lupus. Nucleosomes are major autoantigens in systemic lupus erythematosus. We found that nucleosome-specific T cells were stimulated dominantly in the spleen, compared with lymph nodes, lung, and thymus. Among splenic APCs, F4/80$^+$ macrophages and CD11b$^+$CD11c$^+$ dendritic cells were strong stimulators for nucleosome-specific T cells. When splenic phagocytes were depleted in (NZB × NZW) F_1 (NZB/W F_1) mice, nucleosome presentation in the spleen was dramatically suppressed. Moreover, depletion of splenic phagocytes significantly suppressed anti-nucleosome Ab and anti-dsDNA Ab production. Proteinuria progression was delayed and survival was prolonged in phagocyte-depleted mice. The numbers of autoantibody-secreting cells were decreased in the spleen from phagocyte-depleted mice. Multiple injections of splenic F4/80$^+$ macrophages, not those of splenic CD11c$^+$ dendritic cells, induced autoantibody production and proteinuria progression in NZB/W F_1 mice. These results indicate that autoantigen presentation by splenic phagocytes including macrophages significantly contributes to autoantibody production and disease progression in lupus-prone mice. *The Journal of Immunology,* **2008, 181: 5264–5271.**

Systemic lupus erythematosus (SLE)[3] is an autoimmune disease characterized by autoantibody production and various types of organ damages. Hyperactivation of T (1) and B cells (2) has been observed in human and murine lupus. Several groups have reported that intrinsic abnormalities in APCs are associated with SLE. Dendritic cells (DCs) contribute to the pathogenesis of lupus by producing cytokines or chemokines (3). Monocytosis in BXSB mice (4) and increased macrophages in NZB/W F_1 and MRL/lpr mice have been documented (5). The efficiency of macrophage clearance of apoptotic bodies has been associated with lupus-like disease in mice. For example, MFG-E8$^{-/-}$ (6) mice and *Mer*^{kd} mice (7) produced high titers of autoantibodies. Though the autoantigen load leads to autoimmunity, autoantigen presentation by APCs is poorly understood in lupus-prone mice.

APCs play multiple roles in the immune system: clearance of Ags, cytokine production, and Ag presentation to T cells. DCs are thought to be the most potent cells in Ag presentation, including autoantigens (8). Macrophages produce immunosuppressive and anti-inflammatory cytokines like IL-10 and TGF-β after ingesting apoptotic cells (9–11) Ag presentation by macrophages may induce a tolerogenic response in T cells. In contrast, macrophages are capable of producing proinflammatory cytokines such as TNF-α or type 1 IFN and express costimulatory molecules in response to stimulation of Toll-like receptors by self nucleic acids (12). Thus, activation of macrophages could promote immune responses to self by virtue of inflammatory cytokine production and through its APC function.

Nucleosomes are major immunogens for T cells and are targets for pathogenic autoantibody production in lupus-prone mice (13, 14). Nucleosomes are ubiquitous autoantigens generated by apoptosis of cells (15, 16). Moreover, antinucleosome Ab titers have better specificity and diagnostic confidence than anti-dsDNA Ab titers in human SLE (17). Antinucleosome Abs can be detected earlier than anti-dsDNA Abs in lupus-prone mice (18).

In our previous study, we reconstituted nucleosome-specific T cells and found that nucleosome hyperpresentation in the spleen from prenephritic NZB/W F_1 mice (19).

The purpose of the present study is to determine the pathogenic effect of autoantigen presentation by splenic phagocytes. We demonstrated that nucleosome presentation was dominant in the spleen, and that splenic F4/80$^+$ macrophages presented nucleosomes efficiently. In NZB/W F_1 mice, depletion of splenic phagocytes, including macrophages, suppressed nucleosome presentation in the spleen, autoantibody production, and proteinuria progression. The numbers of autoantibody-secreting cells were decreased in the spleen. Repeated injections of splenic macrophages into prenephritic mice induced autoantibody production and proteinuria progression. These findings demonstrate that autoantigen presentation by splenic phagocytes is immunogenic and contributes to the development of murine lupus.

*Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; †Department of Molecular Cell Biology, Faculty of Medicine, Free University, Amsterdam, The Netherlands; ‡Department of Transfusion Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; §Department of Pathology, Juntendo University School of Medicine, Tokyo, Japan; and ¶Division of Rheumatology, University of Washington, Seattle, WA 98125

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[2] Address correspondence and reprint requests to Dr. Keishi Fujio, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. E-mail address: kfujio-tky@umin.ac.jp

[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; DC, dendritic cell; BM, bone marrow; LN, lymph node; PC, plasma cell; Cl_2MDP, dichloromethylene diphosphonate.

Materials and Methods

Mice

NZB/W F_1, BALB/c, NZB, and NZW mice were obtained from Japan SLC. SWR mice were obtained from The Jackson Laboratory. SNF_1 mice were bred at our laboratory. All animal experiments were conducted in accordance with the institutional and national guidelines.

Plasmid construction

pMXW-AN3-α and pMXW-AN3β were used to generate nucleosome-specific TCR as previously described (19). pMX-DOTAE and pMX-DOTBE were used to generate OVA-specific DO11.10 TCR as previously described (20).

Production of retroviral supernatants and retroviral transductions

Total splenocytes were cultured for 48 h in the presence of Con A (10 μg/ml) and mIL-2 (50 ng/ml) (R&D Systems). Retroviral supernatants were obtained by transfection of pMXW-AN3α, pMXW-AN3β, pMX-DOTAE, or pMX-DOTBE into PLAT-E packaging cell lines using FuGENE 6 transduction reagent (Roche Diagnostic Systems), as previously described (21). Retroviral gene transduction was performed as described (19, 20). In brief, Falcon 24-well plates (BD Biosciences) were coated with the recombinant human fibronectin fragment CH296 (Retronectin; Takara Shuzo). The viral supernatant was preloaded into each well of the CH296-coated plate, and the plate was spun at 2400 rpm for 3 h at 32°C. This procedure was repeated three times. The viral supernatant was washed away, and Con A-stimulated splenocytes were placed into each well (1×10^6 per well). Cells were cultured for 36 h to allow infection to occur.

Cell purification

A $CD4^+$ T cell population was prepared by negative selection with MACS using anti-CD19 mAb, anti-CD11c mAb, and anti-CD8 mAb. $CD11c^+$ DCs were prepared as previously described (19). In brief, spleen cells were digested with collagenase type IV (Sigma Aldrich) and DNase I, and the $CD11c^+$ DCs were selected twice by positive selection with MACS CD11c microbeads and magnetic separation columns. The purity of the isolated $CD11c^+$ cells was consistently >90%. $F4/80^+$ macrophages were isolated by incubating single cell suspensions with anti-F4/80-biotin mAb (Caltag Laboratories) and anti-CD11c-conjugated MicroBeads. After negative selection for CD11c positive cells, negative fraction was additionally incubated with streptavidin-conjugated microbeads, and $F4/80^+$ macrophages were isolated with two rounds of positive selection using magnetic separation columns (Miltenyi Biotec). The purity of the isolated $F4/80^+$ cells was consistently >90%, and the contamination was <0.5% for $CD11c^+$ cells and 3.5% for $CD19^+$ cells. Cell viability was always >97%, as detected by trypan blue exclusion. $CD11b^+$ $CD11c^+$ DCs and $CD8^+$ $CD11c^+$ DCs were prepared by positive selection with MACS using the indicated Abs and a FITC MultiSort Kit (Miltenyi Biotec).

Transfer experiments

The indicated number of cells suspended in PBS was i.v. injected into mice. Cell viability was always >97%, as detected by trypan blue exclusion.

FACS analysis

For analyzing MHC class II expression levels of splenic DCs or macrophages, splenocytes were stained with anti-CD11c-FITC mAb, anti-F4/80-PE mAb, and anti-I-A/I-E-biotin mAb followed by streptavidin-allophycocyanin. Plasma cells (PCs) were stained with anti-CD138-PE mAb (BD Pharmingen) as previously described (22). For analyzing CFSE-labeled AN3-transduced T cells in the spleen, splenocytes isolated from recipient mice were stained with anti-CD69-PE mAb, anti-Vβ4-biotin mAb followed by streptavidin-allophycocyanin, and anti-CD4-allophycocyanin-Cy7 mAb. Cytometric analysis was performed using a FACSVantage cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

Proliferation assay

At 24 h post infection, purified $CD4^+$ T cells were cultured at 1×10^4 cells/well, with 1×10^4 cells/well of irradiated APCs or 5×10^5 cells/well of irradiated splenocytes in 96-well flat-bottom plates in volumes of 100 μl of complete medium.

In the experiment of OVA presentation, indicated number of purified DO11.10 TCR-transduced $CD4^+$ T cells were cultured with 1×10^4 cells/well of irradiated APCs in 96-well flat-bottom plates in volumes of 100 μl of complete medium with 3 μM chicken $OVA_{323-339}$ peptide. [^{3}H] thymidine incorporation was determined as previously described (19).

In vivo depletion of splenic phagocytes

Splenic phagocytes were depleted in vivo using dichloromethylene diphosphonate (Cl_2MDP) encapsulated in liposomes. Cl_2MDP-liposomes and PBS-liposomes were prepared as described (23). Cl_2MDP was a gift of Roche Diagnostics. Cl_2MDP-liposomes or PBS-liposomes (0.1 ml/10g body weight) were i.v. injected into female NZB/W F_1 mice at 20 and 22 wk of age.

In the experiment of OVA presentation, purified OVA-reactive $CD4^+$ T cells were cultured at 1×10^5 cells/well, with 5×10^5 cells/well of irradiated splenocytes from Cl_2MDP- or PBS-liposome-treated mice in the presence of 100 μg/ml whole OVA protein in 96-well flat-bottom plates in volumes of 100 μl of complete medium.

Immunohistochemistry

Cryopreserved sections of spleen were incubated with a rat Alexa488-labeled mAb to CD4, Alexa546-labeled mAb to F4/80 or CD11c, and Cy5-labeled mAb to B220 (Vector Laboratories). To detect the deposition of immune complexes at glomeruli, we incubated sections with FITC-labeled goat Abs to mouse IgG or to C3 (ICN Pharmaceuticals).

Evaluation of nephritis

Kidneys were fixed in 10% formalin for 24 h at 4°C. Paraffinized sections of kidneys were stained with H&E, and periodic acid-Schiff reagents. Histopathologic findings in glomerular lesions were graded on a scale of 0–3, where 0 = normal, 1 = mild (cell proliferation and/or cell infiltration), 2 = moderate (cell proliferation and/or cell infiltration with membrane proliferation), and 3 = severe (cell proliferation and/or cell infiltration, membrane proliferation, and crescent formation and/or hyalinosis). The glomerular lesion index was calculated from the sum of the scores for 40 random glomeruli per kidney as previously described (24–26).

ELISA

IgG anti-DNA Abs were measured using ELISA plate coated with λ-phage-derived purified dsDNA (MESACUP DNA-II TEST; Medical & Biological Laboratories). The DNA-bindings activities were expressed in units, referring to a standard curve obtained by serial dilutions of a standard serum pool from 7- to 9-mo-old NZB/W F_1 mice, containing 1000 U/ml (19). IgG anti-nucleosome Abs were quantified using ELISA plates coated with human nucleosome (Orgentec).

Quantification of Ab-secreting cells by ELISPOT

Single cell suspensions of the spleen and bone marrow (BM) were filtered through a Falcon cell strainer (70 mm), washed, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Anti-dsDNA Ab-secreting cells and anti-nucleosome Ab-secreting cells were analyzed using a modified ELISPOT technique as previously described (22, 27). ELISA plates coated with λ-phage-derived purified dsDNA (Medical & Biological Laboratories) or human nucleosome (Orgentec Diagnostika) were blocked with PBS containing 3% BSA for 1 h at room temperature. The phage-derived purified dsDNA gave an OD260/280 of 1.8. No contamination of proteins was seen by agarose gel electrophoresis and silver staining in the phage-derived purified dsDNA. The purity of human nucleosome was 88%. Subsequently, splenocytes or BM cells were added to each well (5×10^6 cells/well) and incubated for 16 h at 37°C in a humid atmosphere with 5% CO_2. Then, the cells were analyzed for DNA-specific or nucleosome-specific Ab-secreting cells. The absolute numbers of Ab-secreting cells in total BM were calculated as previously described (27).

Repeated injections of splenic phagocytes

Five $\times 10^5$ splenic $F4/80^+$ macrophages or $CD11c^+$ DCs were transferred in NZB/W F_1 mice at the age of 8, 12, 16, and 20 wk. Proteinuria progression and serum Ab levels were assessed.

Statistical analysis

Statistical analyses were performed using the Student's t test, the Mann-Whitney U test for nonparametric data, or one-way ANOVA followed by Bonferroni correction. Proteinuria and survival data were analyzed using Kaplan-Meier curves and the log-rank test. A p value of <0.05 was considered to be significant.

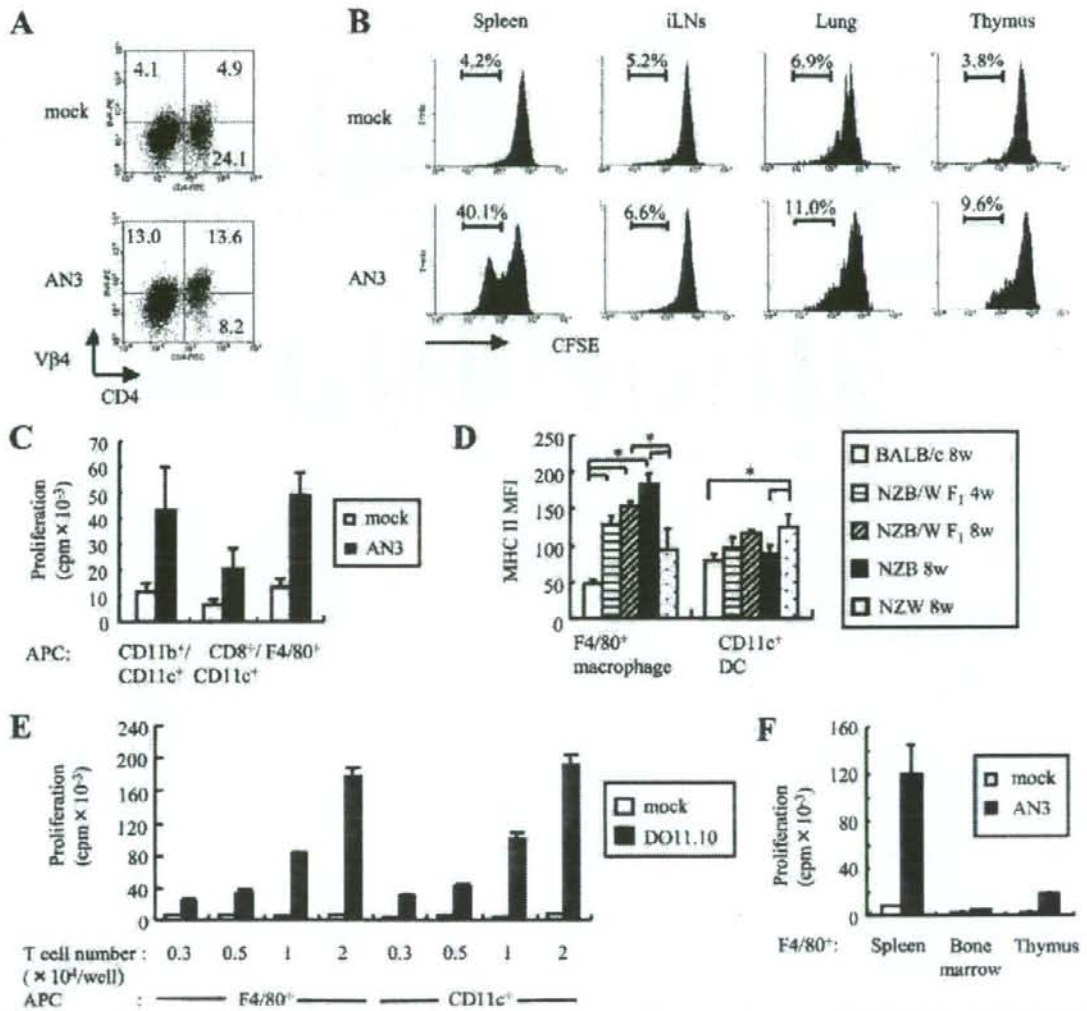

FIGURE 1. Splenic F4/80$^+$ macrophages are potent activators of nucleosome responsive T cells. Ten-wk-old NZB/W F_1 mice were used for the experiments. *A*, Anti-CD4 and anti-Vβ4 staining of pMXW (mock)- or AN3-transduced splenocytes. *B*, CFSE-labeled AN3- or mock-transduced CD4$^+$ T cells were i.v. transferred into 10-wk-old NZB/W F_1 mice. Forty-eight h later, single-cell suspensions of spleen, inguinal LNs (iLNs), lung or thymus from recipient mice were examined for CFSE$^+$Vβ4$^+$CD4$^+$-gated cells. *C*, Proliferation of mock- or AN3-transduced CD4$^+$ T cells to splenic APC subsets. Results represent means ± SD of triplicate wells. *D*, The MHC class II expression of splenic F4/80$^+$ macrophages and CD11c$^+$ DCs from BALB/c, NZB/W F_1, NZB, or NZW mice. *, Significant difference ($p < 0.05$) compared with BALB/c mice or NZW mice. *E*, Proliferation of mock- or DO11.10-transduced CD4$^+$ T cells to splenic F4/80$^+$ macrophages or CD11c$^+$ DCs with 3 μM $OVA_{323-339}$ peptide. *F*, Proliferation of mock- or AN3-transduced CD4$^+$ T cells to F4/80$^+$ macrophages from indicated organs. Results represent means ± SD of triplicate wells. Data shown are representative of three independent experiments with similar results.

Results

Splenic phagocytes presented nucleosome spontaneously in lupus-prone mice

We have previously reported a reconstitution of nucleosome specificity in NZB/W F_1 CD4$^+$ T cells by TCR gene transfer (19, 28). Retroviral vectors with nucleosome-specific AN3 TCR (pMXW-AN3α and pMXW-AN3β) were used for the gene transfer. T cells expressing AN3 TCR recognize nucleosomes in the context of I-A^d (13, 14, 19, 29). Because the TCR β-chain of AN3 belongs to Vβ4 subfamily, retroviral infection of the AN3 TCR genes into NZB/W F_1 splenocytes resulted in a 40–45% increase of the Vβ4$^+$ population in CD4$^+$ T cells compared with pMXW-infected splenocytes (Fig. 1*A*). The efficacy of the Vβ4 introduction into the CD4$^+$Vβ4$^-$ population was calculated to be 50–60%. Thus, the proportion of clonotypic AN3-TCR-expressing cells was estimated to be 25–36% in CD4$^+$ T cells. These cells were referred to as AN3 CD4$^+$ T cells, and pMXW-infected CD4$^+$ T cells were referred to as mock CD4$^+$ T cells. When CFSE-labeled AN3 or mock CD4$^+$ T cells were transferred to NZB/W F_1 mice, extensive proliferation of AN3 CD4$^+$ T cells was observed in the spleen whereas only slight proliferation was observed in the inguinal lymph nodes (LNs), lung or thymus (Fig. 1*B*). Because AN3 TCR

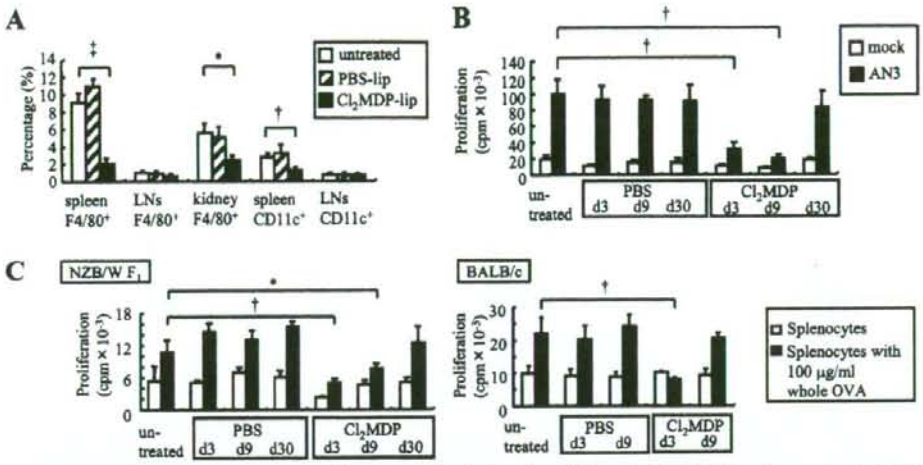

FIGURE 2. Depletion of phagocytes suppresses autoantigen presentation in the spleen. *A*, Ten-wk-old NZB/W F_1 mice were treated with i.v. injection of Cl_2MDP- or PBS-liposomes. Three days later, single-cell suspensions from the spleen, inguinal LNs, and kidneys were prepared and analyzed by FACS. The percentages of $CD11c^+$ DCs and $F4/80^+$ macrophages in the indicated organ are shown. Results represent means ± SD of nine mice/group. *B*, Proliferation of nucleosome-specific T cells to splenocytes harvested 3, 9, and 30 days after liposome treatment. *C*, Proliferation of OVA-primed $CD4^+$ T cells to splenocytes from liposome-treated mice and untreated mice in the presence or absence of whole OVA. In *B* and *C*, results represent means ± SD of triplicate wells. Data shown are representative of three independent experiments with similar results. *, Significant difference ($p < 0.05$) compared with untreated mice. †, Significant difference ($p < 0.01$) compared with untreated mice. ‡, Significant difference ($p < 0.001$) compared with untreated mice.

was originally derived from (SWR × NZB) F_1 (SNF_1) mice, proliferation of AN3 or mock $CD4^+$ T cells was also examined in SNF_1 mice. As with NZB/W F_1 mice, AN3 $CD4^+$ T cells proliferated predominantly in the spleen of SNF_1 mice (data not shown). Together, these results indicate that nucleosome-specific $CD4^+$ T cells are mostly activated in the spleens of lupus-prone mice.

We next determined the specific APC subsets that presented nucleosomes in the spleen. In prenephritic NZB/W F_1 and SNF_1 mice, $F4/80^+$ macrophages as well as $CD11b^+CD11c^+$ DCs stimulated AN3 $CD4^+$ T cells (Fig. 1*C* and data not shown). As we reported previously, (19), $CD19^+$ B cells did not stimulate AN3 $CD4^+$ T cells (data not shown). When we examined phenotype of $F4/80^+$ macrophages and $CD11c^+$ DCs in NZB/W F_1 and control BALB/c mice, the MHC class II expression levels of NZB/W $F4/80^+$ macrophages were ~3 times as high as those of BALB/c $F4/80^+$ macrophages. NZB/W and NZB $F4/80^+$ macrophages exhibited significantly increased expression levels of MHC class II compared with BALB/c and NZW $F4/80^+$ macrophages (Fig. 1*D*). The expression levels of CD40 and CD86 showed no significant elevation in NZB/W $F4/80^+$ macrophages (data not shown). In contrast, MHC class II expression levels of NZB/W $CD11c^+$ DCs were comparable to those of BALB/c $CD11c^+$ DCs. The MHC class II expression levels of NZB/W $F4/80^+$ macrophages were similar in 4 wk old and 8 wk old NZB/W F_1 mice and were comparable to those of NZB/W $CD11c^+$ DCs (Fig. 1*D*). With regard to exogenous Ag presentation, the OVA peptide presentation of $F4/80^+$ macrophages to DO11.10 TCR-transduced $CD4^+$ T cells (DO11.10 $CD4^+$ T cells) was essentially similar to that of $CD11c^+$ DCs in NZB/W F_1 mice (Fig. 1*E*).

Because $F4/80^+$ macrophages showed increased expression of costimulatory molecule and the number of $F4/80^+$ macrophages was ~3 to 5 times as large as that of $CD11c^+$ DCs in the spleen (Fig. 2*A* and data not shown), we examined nucleosome presentation of $F4/80^+$ macrophages derived from other organs where apoptosis occurred spontaneously. $F4/80^+$ macrophages directly isolated from the BM hardly stimulated AN3 T cells. $F4/80^+$ macrophages from the thymus stimulated AN3 T cells significantly more weakly than those from the spleen (Fig. 1*F*). We also checked exogenous Ag presentation using DO11.10 $CD4^+$ T cells. $F4/80^+$ macrophages from the spleen, BM, and thymus stimulated DO11.10 $CD4^+$ T cells similarly in the presence of $OVA_{323-339}$ peptide (data not shown). These data indicate that nucleosome presentation by splenic $F4/80^+$ macrophages is a relatively specific phenomenon.

In vivo depletion of splenic phagocytes reduced autoantigen presentation

To determine the relevance of these in vitro observations in vivo, NZB/W F_1 mice were treated with Cl_2MDP (dichloromethylene diphosphonate)-liposomes. As described in previous publications, Cl_2MDP-liposomes are artificial lipid vesicles that encapsulate Cl_2MDP solution (23). Splenic macrophages are depleted dominantly by i.v. administration of Cl_2MDP-liposomes (23). Three days after Cl_2MDP-liposome injection to NZB/W F_1 mice, splenic $F4/80^+$ macrophages showed an 80% decrease in number (Fig. 2*A*). In contrast, splenic $CD11c^+$ DCs decreased in number by <50%. Intravenous injection of Cl_2MDP-liposome produced an only marginal effect on $F4/80^+$ macrophages and $CD11c^+$ DCs in the LNs.

We then examined nucleosome presentation by splenocytes after phagocyte depletion. Compared with PBS-liposome treatment, Cl_2MDP-liposome treatment resulted in a highly significant reduction of AN3 T cell proliferation at days 3 and 9, but the T cell response recovered at day 30 (Fig. 2*B*). Because depletion and recovery of phagocytes reflected the nucleosome presentation capacity, it was concluded that phagocytes were responsible for nucleosome presentation in the spleen.

To confirm that Ag uptake of splenocytes was reduced by Cl_2MDP-liposome treatment, we cultured OVA-reactive T cells with splenocytes from PBS- or Cl_2MDP-liposome-treated mice in the presence of whole OVA protein. In NZB/W F_1 mice, splenocytes harvested at 3 or 9 days after Cl_2MDP-liposome treatment

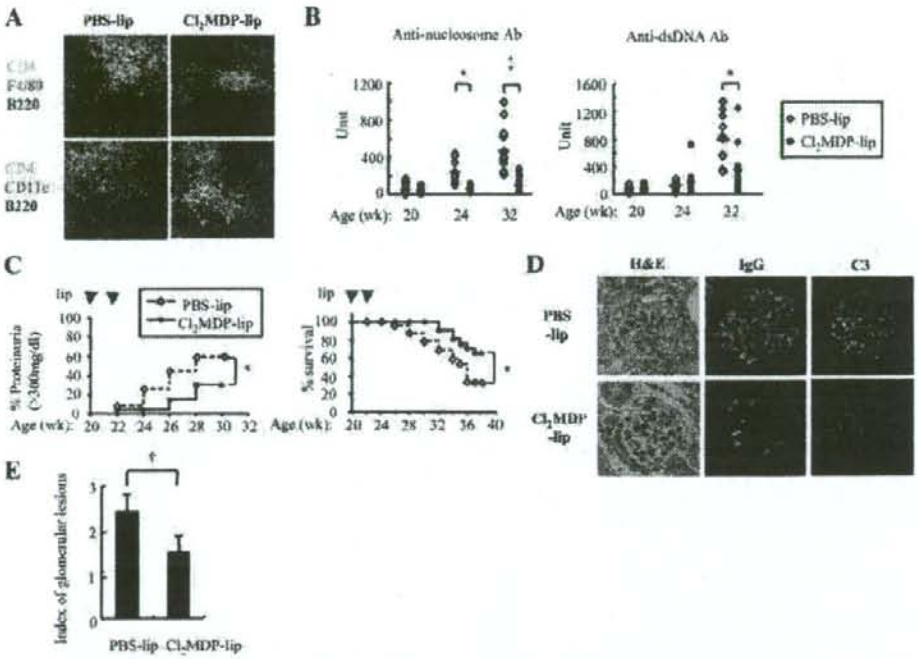

FIGURE 3. Splenic phagocytes contribute to autoantibody production and disease progression. NZB/W F_1 mice were treated with Cl_2MDP- or PBS-liposome at 20 and 22 wk of age. *A*, At 28 wk of age, spleen sections from PBS-liposome-treated and Cl_2MDP-liposome-treated mice were stained with Alexa546-labeled anti-F4/80 or anti-CD11c (red), Alexa488-labeled anti-CD4 (green), and Cy5-labeled anti-B220 (blue). *B*, Serum autoantibody levels of NZB/W F_1 mice treated with Cl_2MDP- or PBS-liposome (n = 12/group). *C*, Kaplan-Meier plots of proteinuria progression and overall survival of mice treated with liposomes (n = 19/group). *D*, At 36 wk of age, kidney sections from PBS- and Cl_2MDP-liposome-treated mice were stained with H&E, anti-IgG, or anti-C3. *E*, Evaluation of glomerulonephritis in PBS- and Cl_2MDP-liposome-treated mice at 36 wk of age. The degree of tissue damage was graded as described in *Materials and Methods*. Values are the mean and SD index of glomerular lesions (1GL: 40 random glomeruli per kidney) (n = 6/group). Images of *A* and *D* are representative of three mice per group. *, Significant difference ($p < 0.05$) compared with PBS-liposome-treated mice. †, Significant difference ($p < 0.01$) compared with PBS-liposome-treated mice. ‡, Significant difference ($p < 0.001$) compared with PBS-liposome-treated mice.

stimulated OVA-reactive T cells more weakly than those from PBS-liposome-treated mice. In NZB/W F_1 mice, splenocytes regained their OVA presentation capacity at 30 days after Cl_2MDP-liposome treatment as well as their nucleosome presentation capacity (Fig. 2*C*, *left panel*). These results verified that Cl_2MDP-liposomes resulted in a transient reduction in APC function in the spleen. However, the course of recovery was different between NZB/W F_1 and BALB/c mice. In BALB/c mice, splenocytes recovered their OVA presentation capacity 9 days after Cl_2MDP-liposome treatment (Fig. 2*C*, *right panel*). This result suggests that NZB/W F_1 mice have a reduced turnover of phagocytes.

Depletion of splenic phagocytes suppresses autoantibody production and proteinuria progression

We next investigated whether depletion of splenic phagocytes altered autoantibody titers. We i.v. injected Cl_2MDP-liposomes to NZB/W F_1 mice only twice at 20 and 22 wk of age. Though the number of splenic $CD11c^+$ DCs was almost comparable between PBS-liposome- and Cl_2MDP-liposome-treated mice at 28 wk of age, splenic $F4/80^+$ macrophages of Cl_2MDP-liposome-treated mice were markedly reduced (Fig. 3*A*). Interestingly, Cl_2MDP-liposome treatment suppressed IgG anti-nucleosome Ab titers at 24 and 32 wk of age (Fig. 3*B*). IgG anti-dsDNA Ab titers were also suppressed at 32 wk of age. Serum total IgG levels were not affected in Cl_2MDP-liposome-treated mice (data not

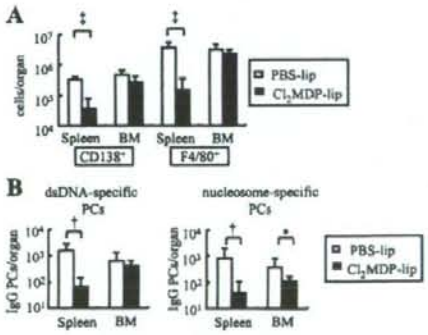

FIGURE 4. Splenic plasma cells were decreased by Cl_2MDP-liposome treatment. NZB/W F_1 mice were treated with Cl_2MDP- or PBS-liposomes at 20 and 22 wk of age (n = 8/group). At 24 wk of age, single-cell suspensions from the spleen or BM were prepared and analyzed. *A*, The numbers of $CD138^+$ plasma cells and $F4/80^+$ macrophages in the spleen and BM are shown. *B*, The numbers of autoantibody-secreting cells in the spleen and BM are shown. Ag-specific IgG secreting cells were detected by ELISPOT and quantified from individual organs. Results represent means ± SD. Data shown are representative of three independent experiments with similar results. *, Significant difference ($p < 0.05$) compared with PBS-liposome-treated mice. †, Significant difference ($p < 0.01$) compared with PBS-liposome-treated mice. ‡, Significant difference ($p < 0.001$) compared with PBS-liposome-treated mice.

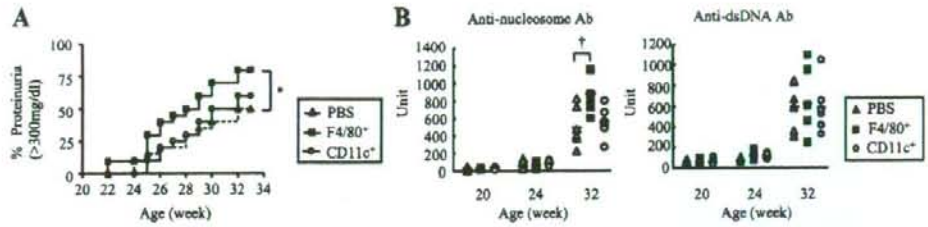

FIGURE 5. Splenic F4/80$^+$ macrophages contribute to disease progression and autoantibody production. *A*, Five × 10^5 splenic macrophages or DCs were transferred in NZB/W F_1 mice at 8, 12, 16, and 20 wk of age. Kaplan-Meier plots of proteinuria progression are shown (n = 20/group). *, Significant difference ($p < 0.05$) compared with PBS-injected mice. *B*, Serum autoantibody levels in mice treated as described in *A* (n = 6/group). The horizontal lines represent the mean value of each group. †, Significant difference ($p < 0.01$) compared with PBS-injected mice.

shown). Moreover, Cl_2MDP-liposome treatment at 20 and 22 wk of age significantly suppressed proteinuria progression and prolonged survival in NZB/W F_1 mice (Fig. 3*C*). In immunofluorescense analysis, though PBS-liposome-treated mice showed severe glomerulonephritis with thickening of the capillary walls with marked deposition of IgG and complement, Cl_2MDP-liposome-treated mice showed mild glomerular lesions and deposition of IgG and complement was only restricted to the mesangial area (Fig. 3*D*). The histological index of glomerular lesion was significantly reduced by Cl_2MDP-liposome treatment (Fig. 3*E*). These results strongly suggest that nucleosome presentation by splenic phagocytes promotes pathogenic anti-nucleosome autoantibodies responsible for nephritis.

To determine whether Ag-priming capacity of s.c. LNs was preserved after Cl_2MDP-liposome treatment, we immunized NZB/W F_1 mice with OVA in the footpad 3 days after liposome treatment. When analyzed at day 21, Cl_2MDP-liposome-treated, PBS-liposome-treated and untreated mice had equivalent IgG anti-OVA Ab titers (data not shown). This preservation of the immune response to peripheral immunization is consistent with the marginal effects of Cl_2MDP-liposomes on the APCs in the draining LN (Fig. 2*A*). Therefore, depletion of splenic phagocytes had a relative specific effect on pathogenic autoantibodies in NZB/W F_1 mice.

Abundant PCs have been detected in the spleen and kidneys of NZB/W F_1 mice (27) and both long- and short-lived PCs contain anti-DNA Ab-secreting cells (22). We therefore examined whether phagocyte depletion affected autoantibody-secreting PCs. After two injections of Cl_2MDP-liposomes, CD138-positive PCs were decreased in the spleen (Fig. 4*A*). In contrast, neither PC nor F4/80$^+$ macrophage was significantly affected by Cl_2MDP-liposome treatment in the BM. Consistent with the decrease of PCs, the ELISPOT assay revealed that both anti-dsDNA Ab-secreting cells and nucleosome-specific PCs were decreased in the spleen after Cl_2MDP-liposome treatment (Fig. 4*B*). In the BM, Cl_2MDP-liposome treatment affected nucleosome-specific PCs more strongly than dsDNA-specific PCs. These results suggest that phagocytes either directly or indirectly contribute to the survival of autoantigen-specific PCs in the spleen.

Because Cl_2MDP-liposome treatment depleted both F4/80$^+$ macrophages and CD11c$^+$ DCs in the spleen, we next evaluated the pathogenic effect of the adoptive transfer of splenic F4/80$^+$ macrophages or CD11c$^+$ DCs. We injected splenic F4/80$^+$ macrophages or CD11c$^+$ DCs repeatedly to prenephritic NZB/W F_1 mice. Injections of splenic macrophages significantly induced proteinuria progression, compared with PBS or splenic DCs (Fig. 5*A*). Serum anti-nucleosome Ab levels were increased in F4/80$^+$ macrophage-injected mice, but serum anti-dsDNA Ab levels were not increased (Fig. 5*B*).

In addition, we investigated the effect of Cl_2MDP-liposome treatment on other lupus mice strains. In MRL/lpr mice, Cl_2MDP-liposome treatment also suppressed anti-nucleosome Ab titers (data not shown) 2 wk after Cl_2MDP-liposome treatment.

Nucleosome-specific T cell retention in the splenic red pulp

Many of the lymphocytes entering the spleen are released in the marginal zone. Some of these cells pass to the outer region of the marginal zone and then to the red pulp or directly into venous sinuses. A fraction of the lymphocytes take a different route to appear within the B and T cell areas of the white pulp (30). We next investigated whether nucleosome-specific T cells migrate to the red pulp, where most of splenic F4/80$^+$ macrophages reside. AN3-transduced CD4$^+$ T cells were CFSE labeled and i.v. transferred to 10-wk-old NZB/W F_1 mice. Because about half of AN3-transduced CD4$^+$ T cells were Vβ4 negative (Fig. 1*A*), we

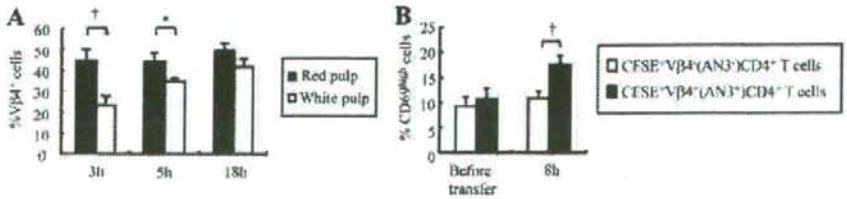

FIGURE 6. Nucleosome-specific T cells are stimulated in the splenic red pulp. CFSE-labeled AN3-transduced T cells were i.v. transferred into 10-wk-old NZB/W F_1 mice. *A*, Percentage of CFSE$^+$Vβ4$^+$CD4$^+$ T cells (AN3 CD4$^+$ T cells) among CFSE$^+$ CD4$^+$ T cells was shown. Three, 5 and 18 h later, spleen sections from recipient mice were stained with Alexa546-labeled anti-F4/80 (red) and biotin-labeled anti-Vβ4 followed by streptavidin-Cy5 (blue). *, Significant difference ($p < 0.05$) compared with white pulp. †, Significant difference ($p < 0.01$) compared with white pulp. *B*, Expression of CD69 on CFSE$^+$Vβ4$^+$CD4$^+$ T cells (AN3$^+$CD4$^+$ T cells) and CFSE$^+$Vβ4$^-$CD4$^+$ T cells (AN3$^-$CD4$^+$ T cells) were examined before and eight hours after the transfer. Percentages of CD69^{high}CD4$^+$ T cells among CFSE$^-$Vβ4$^+$CD4$^+$ T cells and CFSE$^+$Vβ4$^-$CD4$^+$ T cells were shown. Data shown are representative of three independent experiments with similar results. †, Significant difference ($p < 0.01$) compared with Vβ4$^-$CD4$^+$ T cells.