行政院國家科學委員會專題研究計畫 期中進度報告

核小體和調節性T細胞在紅斑性狼瘡所扮演的角色(2/3)
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行政院國家科學委員會補助專題研究計畫期中進度報告

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中文摘要

關鍵詞：紅斑狼瘡、調節性 T 細胞、治療

自體免疫疾病在過去幾年來似乎有逐漸增加的趨勢，如何來研究自體免疫疾病的機轉和進一步研發出更新的治療方法，一直是個刻不容緩的課題。這幾年來利用樹突細胞來誘發一個較好的免疫反應，並加以來調節一些特定的免疫疾病，在許多疾病尤其是腫瘤疾病的治療上得到相當不錯的效果。本研究計畫便計畫更進一步利用樹突細胞來研究全身性紅斑狼瘡的 T 細胞抗原決定位，以進一步設計出更有效的免疫療法。而最近的研究更顯示凋亡小體(apoptotic body)可能在全身性紅斑狼瘡的發病機轉中扮演著一個重要的角色， histone、nucleosome 和凋亡小體來當成首要的目標，本研究計畫將分成三年來加以執行：

第一年：由 NZB/W F1 小鼠骨髓的幹細胞培養出樹突細胞，我們將由小鼠的脾臟細胞將 T 細胞分離出來後，再跟自體抗原如凋亡小體、histone 或是 nucleosome 等培養過的樹突細胞一起培養，進一步測定其增殖反應。同時，一但發現這些 T 細胞能夠被刺激後，我們將進一步分析 T 細胞分泌淋巴介質的情形。在第一年的計畫中，我們計畫先找出對小鼠體內自體反應性 T 細胞有最佳刺激反應的抗原。我們將進一步利用合成的 peptides 來分析這些自體反應性 T 細胞的抗原決定位，所以需要合成 onerlapping peptides。同樣的，我們將利用 T 細胞增殖反應及淋巴介質分泌的情形來測定抗原決定位。同時，我們也將培養樹突細胞與這些主要的自體抗原培養，再注射入正常的 DBA-2/NZW F1 小鼠體內，看是否可以誘發自體免疫疾病。

第二年：建立能夠表現 Fas-L 的腺病毒載體，並將這些載體送入樹突細胞內，利用自體抗原呈現的樹突細胞來誘發動物體內自體反應性 T 細胞的死亡。我們有興趣的是此類能夠表現 Fas-L 的樹突細胞是否能夠專一性地誘發抗原特異性 T 細胞的凋亡。如果此種帶有 Fas-L 的樹突細胞具有這樣的功能，是否能夠將已經建立好的過敏動物模式體內的過敏原特異性 T 細胞除掉，而達到治療的目的。一但找到 T 細胞的重要抗原決定位，我們也將利用 peptides 來嘗試治療自體免疫疾病的小鼠。

第三年：在今年度的計畫中，我們將著重在調節性 T 細胞(regulatory T cells)的研究上。主要是因為目前有愈來愈多的證據顯示調節性 T 細胞可能在免疫疾病如自體免疫疾病和器官移植上扮演了一個非常重要的角色。由於我們在前兩年的研究已經顯示 nucleosome 內的 histone 蛋白是重要的 T 細胞抗原，我們將進一步來分離出與 nucleosome 特異性 T 細胞相關的調節性 T 細胞，以期能夠應用到自體免疫疾病治療上的應用。

多年來，研究者認為在全身性紅斑狼瘡的致病機轉中 T 細胞抗原扮演著一個重要的角色，但是對如何來定義出這些抗原決定位卻一直沒有較好的方法，本計畫便是描述一個簡單的方法利用樹突細胞來進行此一工作。同時，我們也可以利用表現 Fas 的樹突細胞及 peptide 療法來抑制體內的自體反應性 T 細胞，也許可以達到一個治療自體免疫疾病的機會。在第三年的計畫中，我們將研究這些自體免疫傾向小鼠體內的調節性 T 細胞活性，並培養出能夠抑制自體反應性 T 細胞的調節性 T 細胞。這些研究的方向和未來的成果都是目前尚未探討過的研究題目，也因此，本計畫在整個紅斑性狼瘡的致病機轉和治療的研發上具有非常重要的意義。

Abstract
Keywoeds: SLE, regulatory T cells, therapy

Systemic lupus erythematosus (SLE) is characterized by persistent production of autoantibodies against DNA, nucleosome and the small nuclear ribonucleoproteins (snRNPs). However, only few studies about the self-T cells have been reported. The information on the self-T epitopes might provide us the key to solve the critical pathogenic mechanisms of lupus. Therefore, in this proposal, we like to use the potent bone marrow-derived dendritic cells (BM-DCs) to define the T cell epitopes of self-reactive T cells in murine lupus. Furthermore, advanced therapeutic approaches such as DCs expressing Fas L or peptides therapy will be used to delete self-reactive T cells and prevent the progress of the lupus. In addition, the role of apoptotic body in the pathogenesis of SLE will be investigated. We also like to investigate the role of regulatory T cells in the regulation of autoimmune response in systemic lupus erythematosus.

First year: *In vitro* culture of dendritic cells isolated from bone marrow stem cells and apoptotic body, histone and nucleosome will also be used as the major self-antigen in the study. DCs pulsed with these self-antigens will be used to characterize the self-reactive T cell response. With this novel approach, we could identify the most critical self-antigen involved in the activation of autoreactive T cells. In addition,synthetic peptides of the self-antigens will be used to further characterize the epitopes recognized by self-reactive T cells. We also like to study if dendritic cells pulsed with apoptotic body could break tolerance in non-autoimmune mice *in vivo*.

Second year: In the second year, we like to explore the possible treatments for the lupus. Establishment of expressing dendritic cells pulsed with the major self-antigen or self peptides will be used to induce apoptosis of self-reactive T cells in murine lupus. Further, the major peptides will also be used to treat the NZB/W F1 mice by following up both the level of autoantibody and life span.

Third year: We like to study the generation of regulatory T cells and for the application of treatment for the lupus. It has been documented that regulatory T cells might play a critical role in the down-regulation of autoimmune process in murine lupus. We plan to study the interaction between regulatory T cells and nucleosome-specific T cells.

In general, we believe the approaches mentioned here will become the pioneer studies on autoimmune diseases for the years to come. In addition, the approaches might be applied for other diseases such as tumor, transplantation and infectious diseases.

前言

此一研究計畫主要是要研究利用樹突細胞當成抗原呈現細胞來定出 T 細胞反應性的抗原決定位，再利用這些抗原決定位來研究是否可能作為治療的方法。我們在之前的研究已經發現利用樹突細胞的確可以在狼瘡小鼠的 T 細胞找到特定的抗原決定位，而將此一抗原決定位的 peptide 利用來注射在小鼠體內，可以有效地降低這些狼瘡小鼠體內的抗 DNA 抗體濃度，而且可以延長狼瘡小鼠的壽命，減輕其腎臟發炎。而在我們第二年的研究計畫中，我們進一步研究樹突細胞應用到表現凋亡的細胞來刺激自體反應性 T 細胞的增生，而且也研究出利用凋亡的細胞可以在小鼠體內誘發出抗 DNA 抗體。而且，我們也發現在狼瘡小鼠體內的調節性 T 細胞數目有較低的情形，如果將其加以抑制，可以觀察到自體抗體的製造增加。

目的

此一研究計畫的主要目的是研究是否能夠在狼瘡小鼠研發出其 T 細胞的抗原決定位，而能夠進一步研究其是否能夠誘發調節性 T 細胞。

Background

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by the production of autoantibodies directed against ubiquitously expressed self-antigens. The hallmark of SLE is the production of autoantibodies directed against nuclear components, including dsDNA, ssDNA, histones, small nuclear ribonucleoproteins (snRNPs) as well as nucleosomes. It is known that during the progression of SLE, the serum level of anti-nucleosome and anti-DNA Abs correlates with the severity of the disease. In addition, nucleosomes have been found in the circulation of patients with SLE; however, the source of autoantigens, such as nucleosomes that can induce pathogenic autoantibodies is still unclear.

Previous studies have shown that autoantigens can be presented on the surface of apoptotic bodies, while some others accumulate inside the apoptotic bodies. For example, during the apoptosis of keratinocyte, the 52kDa ribosomal protein, Ro, accumulates in the ER. In addition, the autoantigens, such as La, Sm, U1-70kDa, PARP, NUMA, and nucleosome can be detected as well in the apoptotic bodies. It has also been suggested that relocation of the cell components occurrs during apoptosis; for example, nuclear RNPs translocate from the nucleus to the cytosol during apoptosis. In addition, previous work has demonstrated that SLE might result from increased apoptotic neutrophils or impaired clearance of apoptotic cells by macrophages. Many studies have shown that defects in the clearance of apoptotic cells may underlie SLE. For example, characteristic autoantibodies and lupus-like pathology arise in mice lacking the complement protein C1q, a protein that binds apoptotic cells. Similarly, mice with deficiencies in the serum amyloid P or mutations in the Mer tyrosine kinase develop autoantibodies to DNA and manifest autoimmune disease. These autoantigens may be captured by dendritic cells (DCs) and may activate autoreactive T cells, which in turn provide help for B cells in recognizing nuclear autoantigens. Therefore, it has been proposed that apoptotic cells may be the major autoantigenic target in lupus.

Several studies have identified the critical autoepitopes of the nucleosome in human lupus or lupus in animal models, such as SWR X NZB F1 (SNF1) or NZB X NZW F1 (BWF1) mice. As described above, nucleosomes can be detected in apoptotic bodies. However, there is no direct evidence demonstrating that apoptotic cells provide self-antigen, such as nucleosome to stimulate autoreactive T cells in lupus. We have previously shown that bone marrow-derived dendritic cells (BMDCs) are able to determine the self-

reactive epitopes in freshly isolated $CD4^+$ T cells from unprimed lupus mice. In this study, BMDCs were used to pulse with apoptotic cells to test for the presence of $CD4^+$ T cells, which would identify apoptotic cells *in vitro*. Furthermore, we also used BMDCs pulsed with apoptotic cells, intravenously injected into non-autoimmune mice, to explore the immune response directed against apoptotic cells induced by DCs.

The results of this study show that BMDCs are capable of processing and presenting apoptotic cells, resulting in the stimulation of autoreactive $CD4^+$ T cells from unprimed BWF1 mice to proliferate *in vitro*. Furthermore, histone peptide–specific T cell lines from BWF1 mice can respond to apoptotic cell-pulsed BMDCs. This suggests that BMDCs can process and present autoantigens, such as nucleosomes, which are contained in apoptotic cells. In addition, apoptotic cell-pulsed BMDCs are able to induce persistent Ab responses to ds and ssDNA in normal mice *in vivo*. Ab (IgG) and complement C3 are deposited in the glomeruli of the kidneys in the immunized mice. The data from our study will help in understanding how pathogenic autoimmune responses develop in spontaneous SLE.

Mice

Female NZB X NZW F1 (BWF1) mice were purchased from Jackson Laboratories (Bar Harbor, ME). DBA-2 X NZW F1 mice are from a non-autoimmune strain with identical major histocompatability complex (MHC) II molecules to BWF1 ($H\text{-}2^{d/u}$) mice. At the age of 6-8 wks, female BWF1 mice and DBA-2 X NZW F1 mice were used as the source of BMDCs. The mice (young BWF1 and DBA-2 X NZW F1 mice) were obtained from and maintained by the Animal Centre of the College of Medicine of National Taiwan University in a pathogen-free facility.

Generation of dendritic cells from Bone marrow cells

BMDCs were prepared as described previously (18). In brief, DCs were generated from bone marrow cell-depleted red cells by treating the cells with ACK lysis buffer and culturing cells for 4-6 days in a medium supplemented with murine rGM-CSF (750 U/ml) and rIL-4 (IL-4) (1000 U/ml) (Pepro Tech Inc. Rocky Hill, NJ). Approximately one million of the cells were placed in 24-well plates in 1 ml of RPMI 1640 medium that was supplemented with 5% of heat-inactivated FCS, 4 mM L-glutamine, 25 mM HEPES (pH 7.2), 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. Every other day, the medium was removed by aspiration to remove the lymphocytes, and fresh medium containing GM-CSF and IL-4 was added. Apoptotic cells were added to the BMDC cultures on day 4 or day 6. After 48 h, non-adherent cells (BMDCs) were collected and washed extensively to remove the free apoptotic cells, and then a MACS method was used to enrich the $CD11c^+$ DCs. $CD11c^+$ DCs were positively selected by anti-CD11c-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of $CD11c^+$ DCs was (> 93%) analysed by flow cytometry, examining the expressions of MHC class II, B7-1, B7-2 and CD11c.

Generation of apoptotic cells

Thymocytes were retrieved from six to 10-wk-old BWF1 or DBA-2 X NZW F1 mice. Apoptotic cells were generated by treating single-cell suspensions of thymocytes in a medium with dexamethasone 1.2×10^{-6} M for 12-15 h. The culture medium was RPMI-1640 medium supplemented with 2% heat-inactivated FCS, 4 mM L-glutamine, 25 mM HEPES (pH 7.2), 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. We used FITC-labeled annexin V (BD PharMingen, San Diego, CA) to evaluate the exposure of phosphatidylserine and DNA-labeled 7-AAD (BD PharMingen) to access plasma membrane integrity. Cells were analyzed by flow cytometry, and the double positive cells were > 82% after treating with dexamethasone for 15 h.

Phagocytosis assay

The phagocytosis of apoptotic cells was quantified by flow cytometry. 7-AAD-labeled apoptotic cells ($2x10^6$ per well) were cocultured with day-4 DCs ($2x10^5$ per well) for 6 h. BMDCs were further purified by anti-CD11c-coated magnetic microbeads (Miltenyi Biotec) according to manufacturer's instructions. Purified BMDCs were stained with FITC-conjugated anti-MHC class I or anti-MHC class II Ab (BD PharMingen). Phagocytosis was quantified by flow cytometry as the percentage of double positive cells (MHC I^+7-AAD^+ or MHC II^+7-AAD^+).

Phagocytosis of apoptotic cells was also visualized by confocal microscopy. CFDASE (Molecular probe, Inc., Eugene, OR) labeled apoptotic cells were incubated with BMDCs for 6 h, and then were prepared on a slide with a cytocentrifuge (Cytospin) and fixed in acetone. The slides were washed with PBS and then stained in PBS with 20 μg/ml I-A^d-PE (BD PharMingen) and 0.5% BSA (19). The slides were washed three times with PBS after staining for 90 min in 37^{o}C, and mounted with 90% glycerol in PBS, observed under a Confocal Spectral Microscope (Leica Microsystems, Wetzlar, Germany).

Cytokine secretion by dendritic cells

For cytokine secretion, day-4 or day-6 BMDCs ($2x10^5$ per well) from BWF1 mice or from DBA-2×NZW F1 mice were cocultured with an increasing number of syngenic apoptotic cells ($1x10^6$, $2x10^6$, $4x10^6$ per well). BMDCs stimulated with LPS (1 μg/ml) were considered as the positive control. The supernatants were collected after 24-48 h and assayed for IL-12p70, IL-12p40, IL10, and TGF- . The concentration of cytokines in culture supernatants was detected by sandwich-ELISA (R&D, Minneapoils, MN).

T cell Proliferation assays

All of the BWF1 mice used in this study developed anti-dsDNA IgG. $CD4^+$ T cells were positively selected from splenocytes by anti-CD4-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. On the other hand, day-4 and day-6 BMDCs from BWF1 mice or DBA-2 X NZW F1 mice were incubated with syngenic or allogenic apoptotic cells for 48 h. $CD11c^+$ DCs were further positively selected by anti-CD11c-coated magnetic microbeads (Miltenyi Biotec). Purified T cells ($1-2\times10^5$ per well) were co-cultured with purified $CD11c^+$ BMDCs (2500-7500 per well) in the presence or absence of anti-I-A^d/E^d (2G9, BD PharMingen) or anti-I-A^k (clone:11-5.2, BD PharMingen) for 4-5 days. During the last 4-6 h of culture, 1 μCi of [^{3}H]thymidine was added to each well. The cells were harvested onto glass fiber filters using an automated multisample harvester. [^{3}H]thymidine incorporation was then measured in a dry scintillation counter (Packard Instrument Co., Meridan, CT). A proliferative response is defined as stimulation index (SI), and SI > 3 indicates a significant proliferative response. SI was evaluated by dividing the mean cpm incorporated in cultures of T cells plus apoptotic cell-pulsed BMDCs by the mean cpm of T cells plus non-antigen-pulsed BMDCs.

Immunization of mice

DBA-2 X NZW F1 mice were intravenously injected with $1.5x10^5$ syngenic BMDCs, which uptaked or not syngenic apoptotic cells, or with PBS at 8 wks of age. Five days later, the mice received an intravenous boost of $2x10^6$ cells /mice, $1x10^7$ cells /mice of apoptotic cells or PBS respectively for the primary (first) and secondary (2nd) response, as indicated in Figure 6. For the tertiary, quaternary response, all groups of mice received an intravenous injection of apoptotic cells or PBS. The mice were treated every 3 wks, and all mice were bled seven days after treatment with apoptotic cells or the PBS boost to

evaluate the titer of anti-DNA Abs. In this study, the mice were sacrified to examine the renal pathology four mo after their initial treatment.

ELISA for anti-DNA Ab production from normal mice

Abs specific for dsDNA and ssDNA were evaluated in serum samples by a standard ELISA assay as previously described (17, 20). Briefly, ELISA plates were coated with 10 μg/ml methylated bovine serum albumin (mBSA; Sigma). Native DNA was prepared by phenol and chloroform extraction from calf thymus DNA (Sigma). Native DNA was denatured by boiling for 20 min and incubated on ice for 20 min to generate ssDNA. DsDNA and ssDNA were coated overnight at 4^{o}C, then washed and blocked with gelatin post-coating solution for 2 h. Serum diluted 100 times for IgG was applied to each well at 37^{o}C for 45 min and then moved to room temperature for 15 min. After washing the plates, HRP-conjugated goat anti-mouse -chain specific Abs (Sigma) was added at 37^{o}C for 45 min. ABTS solution was used as a substrate, and the OD value was evaluated at 405nm. The levels of anti-IgG are presented as ELISA units (EU/ml) compared with mAb 10F10 (17). This mAb is specific to dsDNA or ssDNA. The OD value generated by 37 ng/ml of 10F10 Ab was defined as 1 EU/ml.

Generation of peptide-specific T cell lines

The generation of peptide-specific T cell lines were prepared as described previously (17). In brief, $CD4^{+}$ T cells ($3x10^{6}$/well) from disease-developing BWF1 mice were cultured with histone peptide ($H2B_{81-100}$, $H3_{111-130}$, $H4_{91-110}$) and $U1A_{201-220}$ peptide pulsed syngeneic BMDCs ($1x10^{5}$/well) in serum-free medium (AIM-5 containing TCM) for 2-3 days. On day 2, 50 U/ml rIL-2 was added to each well. On day 3, the culture medium was replaced by RPMI-1640 containing 10% FCS and IL-2 (50 U/ml). After 10-14 days of coculture, T-cell lines were positively selected by anti-Thy1.2-coated magnetic microbeads (Miltenyi Biotec) and then restimulated with apoptotic cells or peptide pulsed BMDCs (2500 per well) for five days. During the last 4-6 h of culture, 1 μCi of [^{3}H]thymidine was added to each well.

Renal deposition of IgG

Histological assessment was performed at 6 mo of age. Kidneys were fixed in formalin for haematoxylin and eosin staining as previously described (21). IgG deposits were studied by embedded kidneys in OCT (Sakura Finetek, Torrance, CA), and 4-μm thick cryostat sections were prepared on poly L-lysine-coated slides (Dako, Kyoto, Japan) at -20^{o}C. Kidney sections for fluorescence microscopy were fixed in an acetone-chloroform (1:1) solution and washed with PBS three times. The slides were stained with 10 μg/ml of fluorescein goat anti-mouse IgG (H+L) (F(ab')2) (Molecular Probes, Inc., Eugene, OR) or fluorescein-conjugated anti-mouse complement C3(F(ab')2) (ICN Pharmaceuticals, Inc., Auroua, Ohio), and then were counterstained with Evan's Blue, mounted with glycergel (Dako, Japan). Images were generated using fluorescence microscopy (Olympus IX 70).

Results

We have published two papers concerning this project in year 2006

Tzeng, T.-C., Suen, J.-L. and **Chiang, B.-L.** Dendritic cells pulsed with apoptotic body activate self-reactive T cells of lupus mice. Rheumatology 2006; 45:1230-1237.*

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Dendritic cells pulsed with apoptotic cells activate self-reactive T-cells of lupus mice both *in vitro* and *in vivo*

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Objectives. **Systemic lupus erythematosus (SLE) is characterized by the presence of autoantibodies (autoAbs) directed against the nuclear structure. Previous studies have demonstrated that dendritic cells (DCs) can process and present self-antigens (Ags) from apoptotic cells (ACs) in lupus. However, there is no direct evidence demonstrating that ACs provide self-Ags, such as histones, to stimulate autoreactive T-cells in lupus.**

Methods. **AC-pulsed bone marrow-derived DCs (AC-BMDCs) were used to stimulate autoreactive T-cells *in vitro* and *in vivo*.**

Results. **In our study, we found that AC-BMDCs could induce the proliferation of $CD4^+$ T-cells from unprimed NZB × NZW F1 (BWF1) mice, which spontaneously develop SLE, but not $CD4^+$ T-cells, from non-autoimmune DBA-2 × NZW F1 (DWF1) mice. In addition, AC-BMDCs could induce significant proliferative responses to certain histone peptide-specific T-cells. Furthermore, these AC-BMDCs could induce a considerable anti-DNA Ab response *in vivo* after adoptive transfer into DWF1 mice, suggesting that AC-BMDCs can break tolerance in normal mice and initiate an autoimmune response.**

Conclusion. **Our study provides a direct link between self-epitopes from ACs presented by DCs and autoreactive T-cell activation, and demonstrates that ACs are critical for the induction of autoimmunity *in vivo*.**

KEY WORDS: Apoptosis, Autoantigens, $CD4^+$ T-cell, Dendritic cell, SLE.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by the production of autoantibodies (autoAbs) directed against ubiquitously expressed self-antigens (Ags). The hallmark of SLE is the production of autoAbs directed against nuclear components, such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) and histones as well as nucleosomes. It is known that during the progression of SLE, the serum level of anti-nucleosome and anti-DNA Abs correlates with the severity of the disease [1]. In addition, nucleosomes have been found in the circulation of patients with SLE [2]. Previous studies have shown that such autoAgs can be presented on the surface of apoptotic bodies, while others accumulate inside apoptotic bodies [3]. In addition, the previous work suggested that SLE might be a result of increased apoptotic neutrophils or impaired clearance of apoptotic cells (ACs) by macrophages [4]. Many studies have shown that defects in the clearance of ACs may underlie SLE. For example, characteristic autoAbs and lupus-like pathology arise in mice lacking the complement protein C1q, which binds ACs [5]. Similarly, mice with deficiencies in the serum amyloid P or mutations in the Mer tyrosine kinase develop autoAbs to DNA and exhibit symptoms of autoimmune diseases [6, 7]. Therefore, ACs may be the major reservoir of autoAgs in lupus.

Blanco *et al.* [8] have demonstrated that serum interferon-alpha (IFN-α) can induce the differentiation of monocytes to dendritic cells (DCs) in SLE patients. Such DCs might be able to efficiently uptake ACs and nucleosomes present in the blood of SLE patients. Such self-Ag presented by DCs could subsequently activate autoreactive T-cells, which in turn provide help for B-cells in recognizing nuclear autoAgs. A previous study from our laboratory has shown that nucleosome-pulsed DCs can present histone epitopes to stimulate autoreactive $CD4^+$ T-cells from NZB × NZW F1 (BWF1) mice [9]. However, there is no direct evidence demonstrating that these self-Ags derived from ACs could stimulate autoreactive T-cells in lupus. To address this question in this study, we used bone marrow-derived DCs (BMDCs) pulsed with ACs to test for the presence of $CD4^+$ T-cells capable of recognizing ACs *in vitro*. We also intravenously injected BMDCs pulsed with ACs into non-autoimmune mice to further evaluate the DC-induced immune response directed against ACs.

The results of this study show that BMDCs are capable of processing and presenting ACs, resulting in the stimulation and proliferation of histone peptide-specific $CD4^+$ T-cells from unprimed BWF1 mice *in vitro*. In addition, AC-pulsed BMDCs (AC-BMDCs) are able to induce persistent Ab responses to ds and ssDNA in normal mice *in vivo*. Both Ab (IgG) and complement C3 were deposited in the glomeruli of the kidneys in mice immunized with AC-BMDCs. Results from our study will help in understanding how pathogenic autoimmune responses develop in spontaneous SLE.

Materials and methods

Mice

Female BWF1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). DBA-2 x NZW F1 (DWF1) mice are a

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non-autoimmune strain with identical major histocompatibility complex (MHC) class II molecules to BWF1 (H-$2^{d/u}$) mice. At the age of 6–8 weeks, female BWF1 mice and DWF1 mice were used as the source of BMDCs. The young mice were obtained from and maintained by the Animal Center of National Taiwan University in a pathogen-free facility. Studies were performed in accordance with the institutional animal research committee guidelines.

Generation of DCs from bone marrow cells

BMDCs were prepared as described previously [10]. Briefly, DCs were generated from bone marrow cells cultured with murine recombinant granulocyte monoctye-colony-stimulating factor (GM-CSF, 750 U/ml) and interleukin-4 (IL-4, 1000 U/ml) (Pepro Tech Inc. Rocky Hill, NJ) for 4 or 6 days. ACs were added to the BMDC cultures on day 4 or day 6. After 48 h, non-adherent cells were collected and washed extensively to remove the free apoptotic bodies. $CD11c^+$ DCs were positively selected by anti-CD11c-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA). The purity of $CD11c^+$ DCs was (>93%) analysed by flow cytometry, examining the expression of MHC class II, B7-1, B7-2 and CD11c.

Generation of ACs

Thymocytes were retrieved from 6- to 10-week-old mice. ACs were generated by treating single-cell suspensions of thymocytes in a medium with dexamethasone 1.2×10^{-6} M for 12–15 h. We used fluorescein isothiocyanate (FITC)-labelled annexin V (BD PharMingen, San Diego, CA) to evaluate the exposure of phosphatidylserine and DNA-labelled 7-amino-actinomycin D (7-AAD) (BD PharMingen) to assess plasma membrane integrity. Cells were analysed by flow cytometry, and the double-positive cells were >82% after treating with dexamethasone for 12–15 h. For the phagocytosis assay, thymocytes were stained with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular probe, Inc., Eugene, OR) or 7-AAD before treatment with dexamethasone.

Phagocytosis assay

The phagocytosis of ACs was quantified by flow cytometry. CFSE or 7-AAD-labelled ACs (2×10^6 per well) were co-cultured with day-4 or day-6 DCs (2×10^5 per well) for 6 h. In the CSFE-labelled experiment (Fig. 1A), the cells were harvested and stained with 7-AAD to exclude the contamination of dead cells and AC-bound BMDCs. In another experiment, BMDCs were further purified by anti-CD11c-coated magnetic microbeads (Miltenyi Biotec). Purified BMDCs were stained with fluorescence-conjugated anti-MHC class I or anti-MHC class II Ab (BD PharMingen), as indicated in Fig. 1B. Phagocytosis was quantified by flow cytometry as the percentage of double-positive cells.

Phagocytosis of ACs was also visualized by confocal microscopy. CFSE-labelled ACs were incubated with BMDCs for 6 h, prepared on a slide with a cytocentrifuge (Cytospin), and fixed in acetone. The slides were stained with 20 μg/ml of phycoerythrin (PE)—anti-I-A^d (BD PharMingen) for 90 min at 37°C, and mounted with 90% glycerol in phosphate-buffered saline (PBS), and observed under a Confocal Spectral Microscope (Leica Microsystems, Wetzlar, Germany).

Cytokine secretion by BMDCs

For cytokine secretion, day-4 or day-6 BMDCs (2×10^5 per well) were co-cultured with an increasing number of syngeneic ACs (1×10^6, 2×10^6, 4×10^6 per well). BMDCs stimulated with lipopolysaccharide (LPS) (1 μg/ml) were used as the positive control. The supernatants were collected after 24 and 48 h and assayed for IL-12p70, IL-12p40, IL-10 and transforming growth factor-beta (TGF-β). The concentration of cytokines in culture supernatant was detected by ELISA (R&D, Minneapoils, MN).

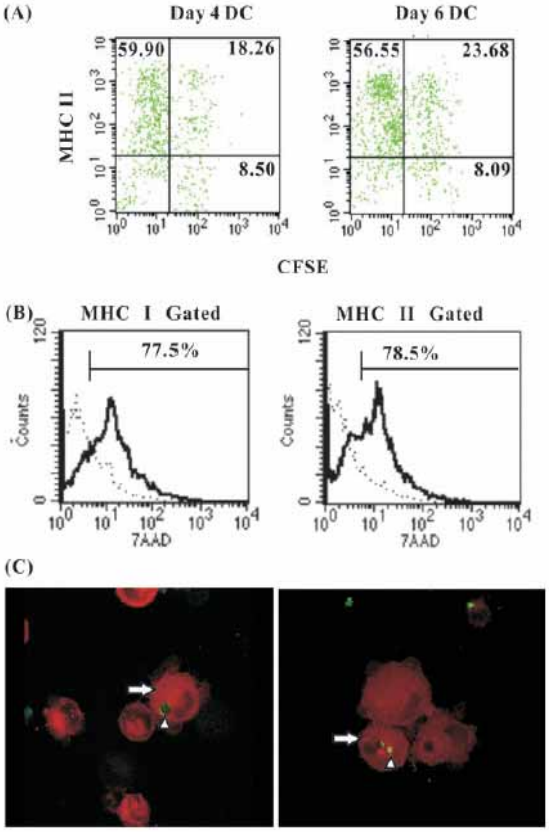

FIG. 1. Phagocytosis of ACs by BMDCs. (**A**) CFSE-labelled ACs were co-cultured with day-4 or day-6 BMDCs for 6 h at a ratio of 20:1. Cells were harvested and stained with PE-anti-I-A^d monoclonal Ab and 7-AAD. Shown here are 7-AAD negative cells analysed for PE-anti-I-A^d and CFSE staining. (**B**) Day-4 BMDCs were co-cultured with 7-AAD-labelled ACs for 6 h. $CD11c^+$ BMDCs were further purified and stained with FITC-conjugated anti-MHC class I or class II monoclonal Ab. The MHC class I (left) or MHC class II (right) positive cells gated from AC-BMDCs were used to analyse the ratio of BMDCs engulfing ACs (solid line). The dotted line indicates the BMDCs pulsed with ACs without 7-AAD staining. (**C**) Confocal microscopy analysis of AC-BMDCs. ACs (arrowhead) were stained with CFSE (green colour), and day-4 BMDCs (arrow) were stained with anti-MHC class II Ab conjugated to PE (red colour).

T-cell proliferation assays

All of the BWF1 mice used in this experiment developed anti-dsDNA IgG. $CD4^+$ T-cells were positively selected from splenocytes by anti-CD4-coated magnetic microbeads (Miltenyi Biotec). Day-4 and day-6 BMDCs from young BWF1 mice or DWF1 mice were incubated with syngeneic ACs at different

ratios for 48 h. Purified $CD4^+$ T-cells ($1–2 \times 10^5$ per well) were co-cultured with purified $CD11c^+$ BMDCs (2500–7500 per well) in the presence or absence of anti-I-A^d/E^d (2G9, BD PharMingen) or anti-I-A^k (clone:11-5.2, BD PharMingen) monoclonal Ab for 4–5 days. During the last 4–6 h of culture, 1 μCi of [^{3}H]thymidine was added to each well. The cells were harvested onto glass fibre filters using an automated multisample harvester. [^{3}H]Thymidine incorporation was then measured in a dry scintillation counter (Packard Instrument Co., Meridan, CT). A proliferative response is defined as stimulation index (SI), and SI $\geq$3 indicates a significant proliferative response. SI was evaluated by dividing the mean counts per minute (cpm) incorporated in cultures of T-cells plus AC-BMDCs by the mean cpm of T-cells plus non-Ag-pulsed BMDCs. The proliferative response of $CD4^+$ T-cells stimulated by LPS-treated BMDCs was used as a positive control.

Immunization of mice

Day-4 BMDCs from DWF1 mice were pulsed with or without ACs for 48 h. BMDCs were further purified using anti-CD11c microbeads. Naïve DWF1 mice (8 weeks old) were intravenously injected with PBS or 1.5×10^5 syngeneic BMDCs, which either had or had not been pulsed with syngeneic ACs. Five days later, for the primary response, the mice that had received PBS or untreated DCs were given an intravenous boost of PBS, whereas mice that had received AC-BMDCs were given an injection of 2×10^6 cells/mice or 1×10^7 cells/mice of ACs. This treatment of BMDC followed by AC was repeated for secondary response after 3 weeks as indicated in Fig. 2A. For the tertiary and quaternary response, the mice received an intravenous injection of PBS or ACs after 3 weeks, respectively. All mice were bled 7 days after treatment with ACs or the PBS boost to evaluate the titre of anti-DNA Abs. In this study, the mice were sacrificed to examine the renal pathology 4 months after their initial treatment.

ELISA for anti-DNA Ab production

Abs specific for dsDNA and ssDNA were evaluated in serum samples by a standard ELISA assay as previously described [11]. Briefly, ELISA plates were coated with 10 μg/ml methylated bovine serum albumin (mBSA; Sigma). dsDNA and ssDNA were coated overnight at 4°C, then washed and blocked with gelatin post-coating solution for 2 h. Serum diluted 100-fold for IgG was applied to each well at 37°C for 45 min. After washing the plates, horseradish peroxidase (HRP)-conjugated goat anti-mouse γ-chain-specific Abs (Sigma) were added. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution was used as a substrate, and the optical density (OD) value was evaluated at 405 nm. The levels of anti-IgG are presented as ELISA units (EU/ml) compared with monoclonal Ab 10F10 [11]. This monoclonal Ab is specific to dsDNA or ssDNA. The OD value generated by 37 ng/ml of 10F10 Ab was defined as 1 EU/ml.

Generation of peptide-specific T-cell lines

The generation of peptide-specific T-cell lines was prepared as described previously [11]. In brief, $CD4^+$ T-cells (3×10^6/well) from disease-developing BWF1 mice were cultured with histone peptide- ($H2B_{81–100}$, $H3_{111–130}$, $H4_{91–110}$) and $U1A_{201–220}$ peptide-pulsed syngeneic BMDCs (1×10^5/well) in serum-free medium (AIM-5, Invitrogen, Carlsbad, CA) containing serum replacement TCM (piotide pharmaceuticals, Inc, Paul, MN) for 2–3 days. On day 2, 50 U/ml human recombinant IL-2 was added to each well. After 10–14 days of co-culture, T-cell lines were positively selected by anti-Thy1.2-coated magnetic microbeads (Miltenyi Biotec) and then restimulated with ACs or peptide-pulsed BMDCs (2500 per well) for 5 days. During the last 4–6 h of culture, 1 μCi of [^{3}H]thymidine was added to each well.

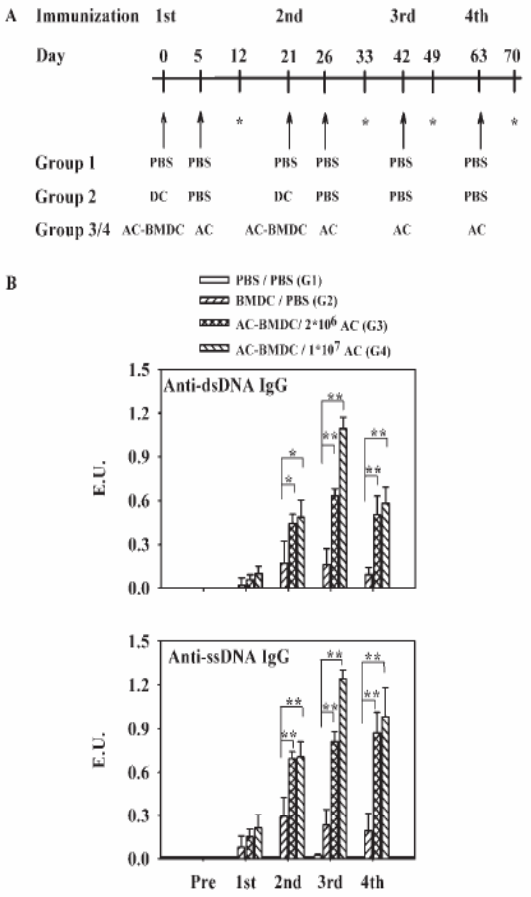

FIG. 2. Induction of anti-DNA Abs in normal mice immunized with AC-BMDCs. (**A**) Schematic depiction of immunization protocol, as described in 'Materials and methods' section. Briefly, DWF1 mice were intravenously injected with BMDC alone (group 2), or AC-pulsed day-4 BMDCs (groups 3 and 4) followed by boosts of PBS (group 2) or different doses of AC (group 3 or 4), respectively, at the primary and secondary immunizations. The mice were challenged with ACs (2×10^6 or 1×10^7/mice) 7 days after adoptive transfer with AC-BMDCs. At the third and forth immunizations, the mice were only challenged with PBS (group 1 and group 2) or ACs (groups 3 and 4) without BMDCs. Bleeding times are denoted by an asterisk (*). Control mice (group 1) were mice were immunized with PBS only. Each time, 7 days after challenging the mice, anti-dsDNA IgG (**B**, upper panel) or anti-ssDNA IgG (**B**, lower panel) in the serum was detected by ELISA. Serum obtained prior to immunization is indicated as pre (pre-immune serum). Results are presented as the mean EU ($\pm$SD) in six mice per group at each bleeding time. G, group. $^*P < 0.05$; $^{**}P < 0.01$.

Renal deposition of IgG

Histological assessment was performed at 6 months of age. Kidneys were fixed in formalin for haematoxylin and eosin staining

was carried out as previously described [12]. Kidney sections for fluorescence microscopy were fixed in an acetone–chloroform (1:1) solution. The slides were stained with 10 μg/ml of fluorescein goat anti-mouse IgG (H+L) (F(ab,)2) (Molecular Probes, Inc., Eugene, OR) or fluorescein-conjugated anti-mouse complement C3(F(ab,)2) (ICN Pharmaceuticals, Inc., Auroua, Ohio), and then counterstained with Evan's Blue, mounted with glycergel (Dako, Japan). Images were generated using fluorescence microscopy (Olympus IX 70).

Statistical analysis

The Mann-Whitney U-test was used to calculate the statistical significance for the difference in the titre of anti-DNA IgG between different groups. A P-value of < 0.05 was considered to be statistically significant.

Results

Phagocytosis of ACs by BMDCs

To examine the ability of BMDCs to capture ACs, syngeneic thymocytes were treated with dexamethasone for 12–15 h. Staining with annexin V and 7-AAD showed that approximately 82% of the thymocytes were apoptotic after treatment (Supplementary Fig. 1). CFSE-labelled ACs were co-cultured with day-4 or day-6 BMDCs for 6 h at a ratio of 5:1, 10:1 or 20:1. Uptake of ACs by BMDCs increased with the ratio of ACs to BMDCs (data for the 20:1 ratio shown in Fig. 1A). About 23 and 30% of day-4 and day-6 BMDCs (double-positive cells/total I-A^{d+} cells) uptook ACs, respectively.

To exclude the contamination of dead cells and non-BMDCs, BMDCs were enriched by anti-CD11c$^+$ microbeads, and the phagocytosis of ACs (7-AAD$^+$) by BMDCs (MHC class I$^+$ or class II$^+$) was determined by flow cytometry. Results showed that more than 75% of MHC I or MHC II positive cells were also 7-AAD positive (Fig. 1B). In addition, the uptake of ACs was also confirmed by confocal microscopy. Effective phagocytosis was indicated by the localization of ACs or bodies (green colour) within the MHC I or MHC II-positive BMDCs (red colour) (Fig. 1C).

ACs can induce the production of IL-12p40 by immature BMDCs

It remains unclear whether ACs can regulate the immune response in lupus mice by promoting or repressing DC maturation. Therefore, in this study, we also investigated the effect of ACs on the maturation of BMDCs. First, we investigated the modulation of surface marker expression known to be upregulated upon DC maturation. The surface expression of molecules such as B7.1, B7.2, MHC class I, MHC class II, CD11c, DEC205 and 33D1 was not altered after either day-4 or day-6 BMDCs from BWF1 or DWF1 mice were pulsed with syngeneic ACs for 48 h (three experiments; Supplementary Fig. 2). We next analysed cytokine production from AC-BMDCs. The IL-12p40 production increased in a dose-dependent manner with the DC AC rate for both day-4 (Fig. 3A), and day-6 (Fig. 3B) BMDCs from BWF1 mice. The amount of IL-12p40 from BWF1 mice was significantly higher than that from DWF1 mice. ACs were not able to induce IL-12p70, IL-10 and TGF-β in these two strains of mice (data not shown). Together, these data suggest that the presence or the phagocytosis of ACs from thymocytes does not induce features of BMDC maturation, with the exception of IL-12p40 production in BWF1 mice.

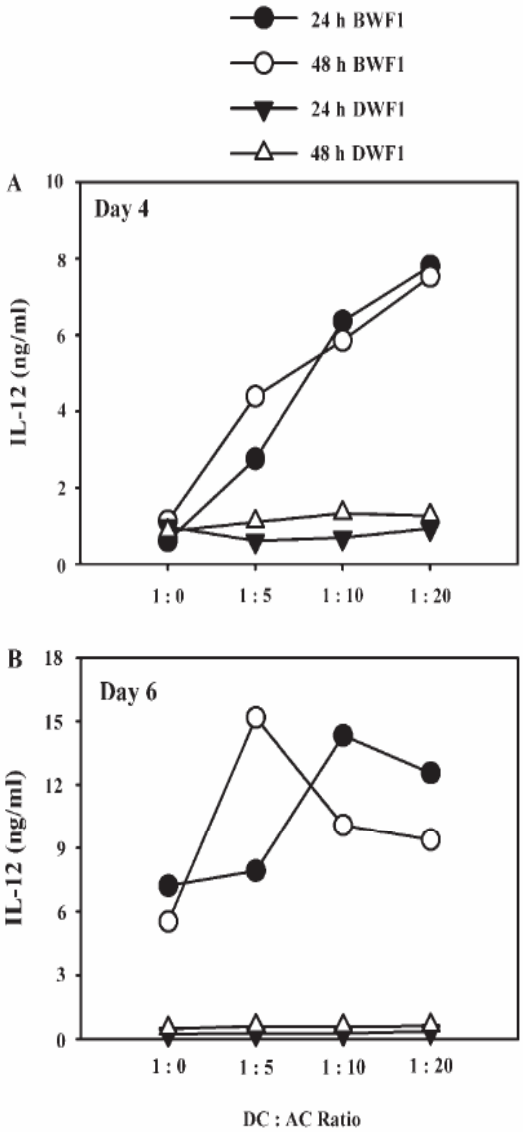

FIG. 3. IL-12p40 production of BMDCs treated with different numbers of ACs. The day-4 (**A**) or day-6 (**B**) BMDCs from BWF1 mice or DWF1 mice were co-cultured with syngeneic ACs at a ratio of 1:5, 1:10 and 1:20. Culture supernatant was collected at 24 and 48 h. As positive controls, the IL-12p40 concentration of LPS (1 μg/ml)-treated BMDCs was 20 ng/ml for day-4 BMDCs and 19 ng/ml for day-6 BMDCs from BWF1 mice, and 6 ng/ml for day-4 BMDCs and 13 ng/ml for day-6 BMDCs from DWF1 mice. There was no obvious difference in IL-12p40 production between 24 and 48 h LPS treatment. The concentration of IL-12p40 was measured using a standard ELISA. The data shown is the result of one of the two repeated experiments.

BMDCs can uptake and present autoAgs from ACs to stimulate CD4$^+$ T-cells from BWF1, but not normal mice

To examine whether CD4$^+$ T-cells from BWF1 mice can recognize autoAgs from ACs presented by BMDCs, day-4 and day-6 BMDCs pulsed with syngeneic ACs were used to detect the proliferative response of freshly isolated splenic CD4$^+$ T-cells

from disease-developing BWF1 mice or age-matched normal mice (Fig. 4A). A significant response was defined as an SI $\geq$3.0. $CD4^+$ T-cells from BWF1 mice responded to day-4 BMDCs pulsed with syngeneic ACs in a dose-dependent manner, but not to similarly pulsed day-6 BMDCs. As expected, purified T-cells from DWF1 mice did not exhibit a proliferative response to BMDCs pulsed with syngeneic ACs. In addition, necrotic cell-pulsed BMDCs did not elicit a proliferative response of $CD4^+$ T-cells from BWF1 mice (data not shown).

To ensure that the proliferative response stimulated by AC-BMDCs was MHC class II-dependent, a blocking Ab (2G9, specific for $I\text{-}A^d/I\text{-}E^d$ molecules) was used to block the interaction between the TCR and MHC class II molecules. As shown in Fig. 4B, the $CD4^+$ T-cell proliferative response was inhibited by 50% by 2G9 monoclonal Ab, but not by the control monoclonal Ab (anti-$I\text{-}A^k$). The proliferative response cannot be completely inhibited because $I\text{-}A^u/I\text{-}E^u$ molecules may also contribute to the presentation of self-Ags.

To address whether the difference in T-cell proliferative responses between BWF1 and DWF1 mice was due to the abnormality of BMDCs and ACs from BWF1 mice, crossover experiments were performed (Supplementary Fig. 3). Different sources of MHC-matched BMDCs pulsed with syngeneic ACs (Supplementary Fig. 3A) and different sources of ACs pulsed with BMDCs from DWF1 mice (Supplementary Fig. 3B) were used to stimulate $CD4^+$ T-cells from BWF1 mice. The data demonstrates that BMDCs can process and present self-Ags provided by ACs and then stimulate autoreactive $CD4^+$ T-cells from lupus mice, no matter the source of MHC-matched BMDCs and ACs.

BMDCs can uptake ACs and present epitopes of core histones

In our previous study, we identified several potential auto-T-cell epitopes of core histones [9] and U1A protein [11] from BWF1 mice. These include peptides derived from H3 (residue 111–130), H4 (residue 91–110) and U1A (residue 201–220). In order to examine whether ACs can provide such nuclear Ags to BMDCs, $CD4^+$ T-cells from disease-developing BWF1 mice were purified and co-cultured with peptide ($H3_{111-130}$, $H4_{91-110}$ or $U1A_{201-220}$)-pulsed BMDCs for 10–14 days to generate peptide-specific T-cell lines as described in the 'Materials and methods' section. T-cell lines were then re-stimulated by AC-BMDCs. As a negative control, we used an H2B peptide (residues 81–100), which is not an auto-T-cell epitope in BWF1 mice [9]. As shown in Fig. 5, the specificity of T-cell lines was confirmed by stimulating these T-cells with BMDCs pulsed with the corresponding peptide. Most of the T-cell lines were able to respond to the corresponding peptide-pulsed BMDCs, with the exception of the $H2B_{81-100}$-specific T-cell line. In addition, $H3_{111-130}$ and $H4_{91-110}$ but not $U1A_{201-220}$ or $H2B_{81-100}$ specific T-cell lines were able to respond to AC-BMDCs. This indicates that BMDCs can process ACs and present certain histone epitopes to autoreactive $CD4^+$ T-cells.

Normal mice injected with AC-BMDCs develop anti-DNA autoAbs and renal deposition of immune complexes

We further investigated whether AC-BMDCs can induce anti-DNA Ab production in normal mice *in vivo*. In our study, ACs did not alter the phenotype of BMDCs, and the majority of both non-Ag-pulsed and AC-pulsed $CD11c^+$ BMDCs exhibited a mature phenotype (Supplementary Fig. 2). In order to break down the tolerance of $CD4^+$ T- and B-cells, naïve DWF1 mice were intravenously injected AC-BMDCs to activate autoreactive T-cells, followed by a boost of ACs after 5-days to activate autoreactive T-cells, and ACs were subsequent to activate

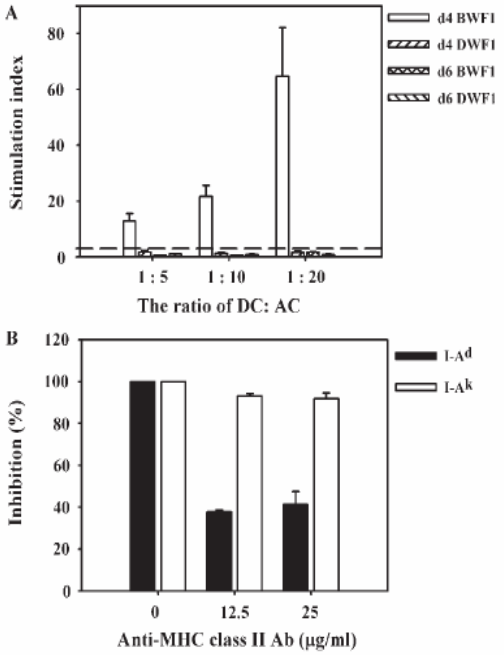

FIG. 4. $CD4^+$ T-cells from BWF1, but not normal, mice can respond to syngeneic BMDCs pulsed with ACs. (**A**) Day-4 or day-6 BMDCs (2×10^5 per well) were incubated with syngeneic ACs at a ratio of 1:5, 1:10 and 1:20. $CD11c^+$ BMDCs (5000 cells per well) were co-cultured with syngeneic purified $CD4^+$ T-cells from disease-developing BWF1 mice or normal mice for 4 days. Results are shown as SI (mean $\pm$ SD, n=6–8). Background (T-cells plus non-Ag-pulsed BMDCs) cpm for day-4 or day-6 BMDCs from BWF1 mice were 223 and 845, respectively. Background cpm for day-4 or day-6 BMDCs from DWF1 mice were 2090 and 4581, respectively. The SI of positive control (T-cells stimulated with LPS-treated BMDCs) varied from 12–80 in this experiment. The horizontal line indicates an SI of 3.0 (d4, day 4; d6, day 6), (**B**) AC-pulsed day-4 BMDCs (the ratio of BMDCs: ACs = 1:20) were pretreated for 30 min with various concentrations of anti-MHC class II ($I\text{-}A^d$ or $I\text{-}A^k$) Abs and then co-cultured with $CD4^+$ T-cells from BWF1 mice. The proliferation of $CD4^+$ T-cells without anti-MHC class II Ab treatment was set at 100% (mean cpm = 2127). The data presented are the averages from three mice in each group.

autoreactive B-cells. As shown in Fig. 2, normal mice injected with non-Ag pulsed-BMDCs developed detectable anti-DNA Abs with time compared with mice injected with PBS. However, AC-BMDCs induced statistically significantly higher titres of anti-DNA IgG than did non-Ag-pulsed BMDCs or PBS injected into DWF1 mice.

Due to the high level of autoAb production in mice immunized with ACs, we sought to further characterize their pathogenic effect, since anti-dsDNA IgGs closely correlate with the development of glomerulonephritis. Glomerular samples taken from mice immunized with BMDCs or AC-BMDCs were examined by immunofluorescence. IgG and complement C3 were not detected in the glomeruli of mice immunized with PBS (Fig. 6C and D) or BMDCs (Fig. 6E and F), but IgG and C3 deposition were detected in the glomeruli of mice immunized with AC-BMDCs (Fig. 6G and H) and 8-month-old BWF1 mice (Fig. 6A and B). However, these mice did not develop proteinuria at any point in their lives. This suggests that other susceptibility factors may contribute to the manifestation of renal disease.

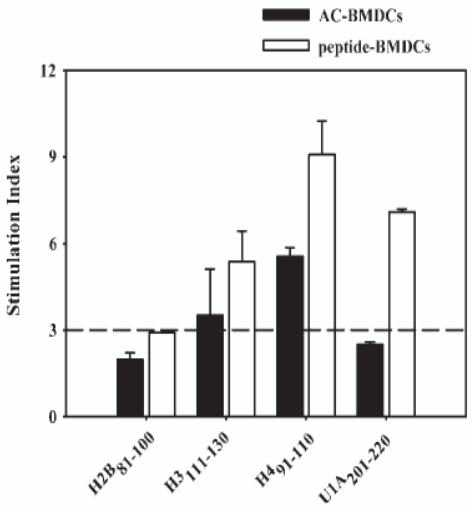

FIG. 5. Histone peptide-specific T-cell lines can respond to AC-BMDCs. T-cell lines were generated by co-culture of $CD4^+$ T-cells from 7-month-old BWF1 mice and histone peptide- or U1A peptide-pulsed syngeneic BMDCs. After 10 days, T-cell lines were harvested and re-stimulated with ACs (the ratio of DCs to ACs was 1:10) or corresponding peptide (10 μg/ml)-pulsed BMDCs. The mean background cpm varied from 293 to 569. The data here is one representative result of two repeated experiments. Results are expressed as the mean SI ± SD from triplicate wells. The dotted line indicates an SI of 3.0.

Discussion

Recent studies have indicated that ACs may be a potential reservoir of autoAgs that can initiate and drive systemic autoimmunity. This is the first report demonstrating the existence of histone-specific $CD4^+$ T-cells that can recognize ACs presented by BMDCs in unmanipulated BWF1 mice but not in normal mice. Furthermore, the AC-BMDCs in this study could break tolerance and induce lupus autoAbs in normal mice.

It remains controversial whether ACs can regulate the immune response in SLE by promoting or repressing DC maturation. A previous study [13] suggested that the uptake of ACs, not necrotic cells, allows DCs to induce tolerance to autoAgs. However, it has also been reported that the ingestion of ACs resulted in a reduction of LPS-induced DC maturation, such as by decreasing IL-12 production and CD86 expression [14]. These divergent results might be due to the use of different numbers of ACs to pulse DCs and the different treatment modalities to induce DC maturation. Large amounts of excess ACs can enhance *in vitro* DC maturation and efficient Ag presentation, whereas fewer numbers of ACs influence the maturation of DCs [15, 16]. Moreover, DC maturation is repressed by the uptake of early ACs but promoted by late ACs [17, 18]. Late ACs have also been shown to be more immunogenic than the early ACs [18]. In our study, immature BMDCs have been cultured with large amounts of late-stage ACs in the *in vitro* culture, thus allowing autoreactive T-cells from unprimed BWF1 mice to be efficiently stimulated by AC-BMDCs.

Recently, Chang and colleagues [19] demonstrated that oxidants produced during apoptosis can modify protein and lipid moieties on membrane lipid to form neoself-epitopes. Thus, late-stage ACs induced by dexamethasone are immunogenic and pro-inflammatory. However, the surface makers of the $CD11c^+$ BMDCs from BWF1 or control mice, including B7.1, B7.2, MHC II and CD40, did not change significantly after pulsing ACs (Fig. 3). Bioactive IL-12 is a heterodimeric cytokine of 70 kDa comprising of covalently linked p40 and p35 [20]. In our study, ACs could induce IL-12p40 (Fig. 3A), but not p70 secretion of BMDCs in BWF1 mice. Free p40 can form homodimers or be present as free monomers in mice, which have been proposed as natural inhibitors of IL-12 [21]. This suggests that the overproduction of p40 from BMDCs in response to ACs may disturb the function of IL-12 in BWF1 mice. In addition, the role of IL-23, which shares p40 with IL-12 as one of its subunits, needs to be further examined in lupus. It has been found that IL-23 plays an important role in the progression of organ-specific autoimmunity [22, 23].

There have been three histone peptides ($H2B_{10-33}$, $H4_{16-39}$ and $H4_{71-94}$) that have been reported as epitopes of autoreactive Th cells in (SWR × NZB) lupus mice and have been identified in patients screened with overlapping histone peptides covering the four core histones H2A, H2B, H3 and H4 [24]. These peptides trigger the pathogenic Th cells of SNF1 mice *in vivo* to induce the development of severe lupus nephritis [25, 26]. In addition, our group recently identified the epitopes of the core nucleosome recognized by autoreactive T-cells from BWF1 mice [9]. The epitopes include the amino acid residues 111–130 of H3 and 91–110 of H4. As shown in Fig. 5, T-cell lines could be generated by non-stimulatory peptide-pulsed BMDCs (residue 81–100 of H2B) [11]. One possible explanation for this result is that the BMDCs could also present self-peptides by MHC molecules by uptaking bystander ACs, even though there were no other Ags or non-stimulatory peptide added during the *in vitro* culture. Thus, the T-cell line generated by $H2B_{81-100}$-pulsed BMDCs may contain a broad range of T-cell specificities. This may explain why no significant proliferative response was detected when '$H2B_{81-100}$-specific' T-cell lines were restimulated by the corresponding peptide-pulsed BMDCs.

Naturally occurring autoreactive T-cells derived from unprimed BWF1 mice encompass a far broader range of specificities against self AGs [27] than do T-cell lines enriched for reactivity to specific self-peptides. Thus, the response from unprimed T-cells represents the sum of the responses from each subset, each with its own specificity. In contrast, T-cell lines contain a few TCR specificities, and the chance of a particular TCR binding to its cognate peptide-bound MHC on AC-BMDCs is much lower. In addition, differences in T-cell response may be due to *in vitro* culture conditions, as the T-cell lines were cultured *in vitro* for 2 weeks, *vs* the unprimed $CD4^{\pm}$ T-cells, which were stimulated with BMDCs immediately after purification from the spleen. The explanation described above may contribute to the differential proliferative response between unprimed $CD4^+$ T-cells and enriched T-cell lines.

An elegant study by Georgiev and colleagues [28] has demonstrated that mature DCs can break tolerance and induce high titre of autoAbs in normal hosts but do not lead to clinical manifestations of disease. This study showed that mice injected with DCs without feeding dying cells developed autoAbs at levels similar to those in mice receiving DCs that had been pulsed with ACs. The discrepancies between our studies may be explained by differences in several factors, as follows: (1) different MHC haplotype of mice were used ($H\text{-}2^b$ *vs* $H\text{-}2^{d/u}$); (2) different DC culture conditions (GM-CSF *vs* GM-CSF plus IL-4) and different time point of AC-BMDCs were used in the adoptive transfer experiments (day-6 *vs* day-4 DCs). In our investigation, day-4 and day-6 DCs had a similar ability to engulf ACs (Fig. 1A). However, day-4 DCs could more efficiently stimulate the proliferative responses of autoreactive T-cells than day-6 DCs (Fig. 4A); (3) different numbers of DCs were used for the *in vivo* experiment (8×10^5 *vs* 1.5×10^5 cells per mice); and (4) the stage of dying thymocytes fed to DCs was different (early *vs* late apoptosis). The factors important in DC-induced autoimmunity need to be further elucidated.

Through the phenotypes detected via congenic dissection, disease development of lupus can be organized into three

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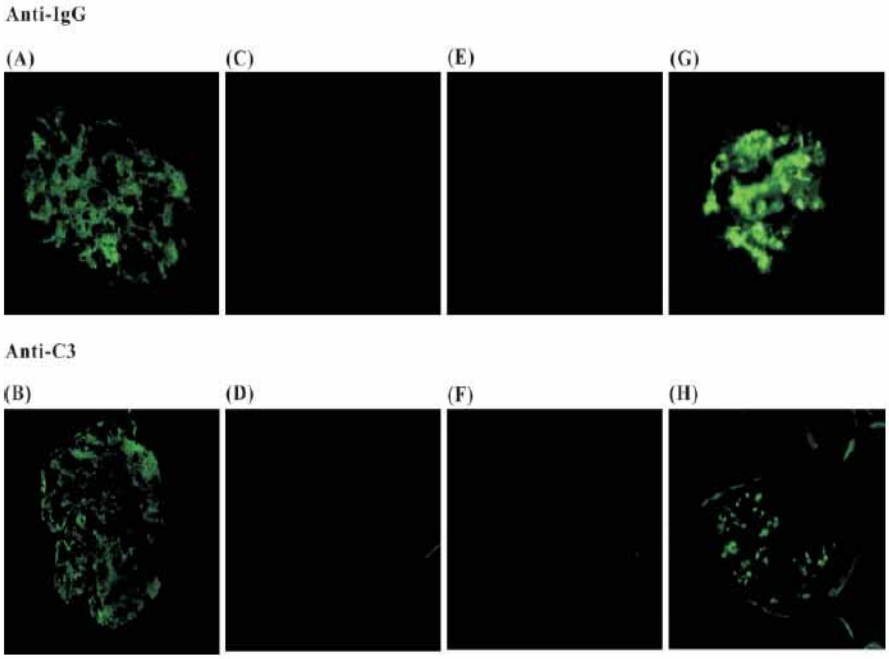

FIG. 6. Immune complex and complement C3 deposition in non-autoimmune mice treated with AC-BMDCs. Kidney sections were stained with fluorescein-conjugated anti-mouse IgG (**A**, **C**, **E**, **G**) or fluorescein-conjugated anti-mouse complement C3 (F(ab,)2) (**B**,**D**,**F**,**H**). Representative renal glomeruli of 8-month-old BWF1 mice (**A**, **B** positive control), and BWF1 mice i.v injected with PBS (**C**, **D**), non-Ag-pulsed BMDCs (**E**, **F**), or AC-BMDCs (**G**, **H**) at 200× magnification. The exposure time for each group was 10 s.

hypothetical pathways [29]. The first pathway requires tolerance to nuclear Ags [30, 31]. Dysregulation of the immune response is also important for pathogenesis to occur. Studies with congenic mice suggest that the break in tolerance to nuclear Ags does not lead to disease unless there is an amplification of the autoimmune response, such as T-cell and B-cell hyperproliferation [32, 33]. The third pathway is the development of end-organ damage [34]. In the present study, IgGs found in the kidneys may likely have been directed against DNA, because a high titre of anti-ssDNA and anti-dsDNA IgG was present in the non-autoimmune mice immunized with AC-BMDCs (Fig. 2). However, no proteinuria was detected in the mice immunized with AC-BMDCs. It has been demonstrated that the pathogenic potential of autoAbs is determined by the IgG subclass [35, 36]. In addition, the receptor involved in complement-fixing also influences the development of severe nephritis [37]. We successfully broke tolerance by inducing anti-DNA Abs in normal mice, although there may be other key factors, such as regulatory T-cells, involved in the mechanism of end-stage disease. It has been reported that the number of regulatory T-cells ($CD4^+CD25^+$ cells) significantly decrease in the peripheral blood of SLE patients compared with normal people [38, 39].

The findings in this study suggest an important role for ACs in the initiation and progression of SLE. However, the detailed mechanisms by which ACs regulate DC activity need to be further characterized.

Rheumatology	Key message
	• Apoptotic cell-pulsed bone marrow-derived dendritic cells (AC-BMDCs) can present epitopes of histone proteins to stimulate autoreactive $CD4^+$ T-cells from unprimed BWF1 mice.

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The authors have declared no conflict of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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The role of $CD4^+CD25^+$ T cells in autoantibody production in murine lupus

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Summary

Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease characterized by the loss of tolerance to self-antigen. Because it is currently not known if regulatory T (T_{reg}) cells are involved in the pathogenesis, we determined the frequency of $CD4^+CD25^+$ T cells and assayed the related gene expression levels in $CD4^+CD25^+$ T cells isolated from both lupus mice (NZB/NZW F_1) and normal control mice (DBA2/NZW F_1). The results showed that the frequency of $CD4^+CD25^+$ T cells in lupus mice was lower than that of normal mice. Except for the high expression level of interleukin (IL)-10 mRNA, $CD4^+CD25^+$ T cells from lupus mice expressed normal forkhead box P3 (Foxp3) and transforming growth factor (TGF)-β mRNA, and exerted suppressive functions. Furthermore, we depleted $CD25^+$ T_{reg} cells of non-autoimmune mice with anti-CD25 antibody and broke their tolerance with apoptotic cell-pulsed dendritic cells for the follow-up of autoantibody levels. The mice in the $CD25^+$ cell-depleted group had higher titres of anti-double-strand/single-strand DNA antibodies than those of the isotype control antibody-treated group. These findings indicated that $CD4^+CD25^+$ T cells might be involved in the regulatory mechanism of autoantibody production.

Keywords: autoantibodies, lupus/ systemic lupus erythematosus, regulatory T cells

Introduction

The primary mechanism that sustains self-tolerance is thymic deletion of autoreactive T cells, but this process might be incomplete. During development, self-reactive T cells that escape thymic deletion and exit into the peripheral circulation are controlled by mechanisms responsible for peripheral tolerance. These mechanisms include T cell anergy, ignorance, phenotypic skewing and apoptosis [1]. Recent studies have suggested that $CD4^+CD25^+$ regulatory T (T_{reg}) cells exhibit immune suppressive activity and also play a critical role in the maintenance of self-tolerance [2–5]. Nude mice transferred with $CD4^+$ T cells depleted of $CD25^+$ cells developed various organ-specific autoimmune diseases. Reconstitution of $CD4^+CD25^+$ cells within a limited period after transfer of $CD4^+CD25^-$ cells prevented the onset of these diseases [6]. The *forkhead box P3* (*Foxp3*) gene is shown to be central in the development and function of $CD25^+$ T_{reg} cells [7–9]. Although there is a marked contrast with regard to the importance of immunosuppressive cytokines *in vivo* compared to $CD25^+$ T_{reg} cell suppression *in vitro*, several cytokines have been implicated as mediators of inhibition, such as interleukin (IL)-10 and transforming growth factor (TGF)-β [10].

Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease characterized by the loss of tolerance to self-antigen and the production of autoantibodies against components of the cell nucleus [11]. Many factors are involved in the pathogenesis of SLE, including genetic factors, environmental factors, hormone, hyperactivated B cells and T cells, abnormal phagocytic functions and abnormal immunoregulation [12–14]. Female NZB × NZW F_1 (BWF_1) mice develop lupus resembling human SLE spontaneously, with polyclonal B cell activation leading to the production of autoantibodies and immune complex-mediated glomerulonephritis. To examine if $CD4^+CD25^+$ T_{reg} cells are also involved in the pathogenesis of SLE, recent studies have revealed a deficiency of T_{reg} cell numbers in two murine models, BWF_1 and SNF_1 [15] and in human patients [16–18].

Moreover, to determine if there are any defects in $CD4^+CD25^+$ T cells of BWF_1 mice, we compared their frequencies and functions with $CD4^+CD25^+$ T cells of DBA-$2 \times$ NZW F_1 ($D2WF_1$) mice. We attempted to investigate whether a deficiency of $CD4^+CD25^+$ T cell numbers could cause lupus-like diseases in normal mice. In our previous studies, we have successfully broken the self-tolerance of normal mice with apoptotic cells. $D2WF_1$ mice produced high titres of anti-DNA antibodies after immunization with apoptotic cell-pulsed dendritic cells (DCs) and then received a boost of apoptotic cells [19]. The approach of this study was the depletion of $CD25^+$ T cells before breaking the tolerance of normal mice. We found that mice lacking $CD4^+CD25^+$ T cells had more severe autoimmune responses than mice with normal T_{reg} cell numbers.

Materials and methods

Mice

Female NZB $\times$ NZW F_1 (BWF_1) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Female DBA-$2 \times$ NZW F_1 ($D2WF_1$) mice of non-autoimmune strain were obtained from and maintained by the Animal Centre of the College of Medicine of the National Taiwan University in a pathogen-free facility. At 6–8 weeks of age, female $D2WF_1$ mice were used as the source of bone marrow-derived dendritic cells (BMDCs). Mice of both strains have identical major histocompatibility complex class II ($H\text{-}2^{d/u}$). The experimental protocol was approved by the Animal Committee of the College of Medicine of the National Taiwan University.

Isolation of cells

The $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified with auto-magnetic affinity cell sorting (MACS) using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec, Sunnyvale, CA, USA). First, $CD4^+$ T cells were enriched from splenocytes stained with $CD4^+CD25^+$ regulatory T cell biotin–antibody cocktail and anti-biotin microbeads. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell magnetic separations were performed by staining with CD25-phycoerythrin (PE), followed by staining with anti-PE microbeads. Antigen-presenting cells (APC) were selected negatively from splenocytes by depleting Thy-1·2$^+$ T cells. DCs were positively selected with anti-CD11c-coated magnetic microbeads (Miltenyi Biotec).

Flow cytometry

After the red blood cells (RBC) were depleted using ACK lysis buffer (0·15M NH_4Cl, 10mM $KHCO_3$, 0·1mM Na_2 EDTA, pH7·2), splenocytes or peripheral blood were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 monoclonal antibody (MoAb) (145–2C11; eBioscience, San Diego, CA, USA), PE-conjugated anti-CD25 MoAb (PC61; eBioscience), cychrome-conjugated anti-CD4 Moab (L3T4; eBioscience) or relevant isotype controls (eBR2a; eBioscience) for 30 min at 4°C. The cells were then washed with fluorescence activated cell sorter (FACScan) buffer and analysed on FACScan (BD Biosciences) using CellQuest software.

Analysis of gene expression

Total cellular mRNA was extracted from 1×10^6 isolated cells using GenesStrip™ hybridization tubes (RNATure, West Irvine, CA, USA) and reverse transcribed into cDNA by using SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primer (RNATure). Gene expression was quantified by real-time polymerase chain reaction (PCR) using Assays-on-Demand™ gene expression products [forkhead box P3 (Foxp3), IL-10, TGF-β, IL-4 and hypoxanthine phosphoribosyltransferase (HPRT)], Assays-on-Design™ Gene Expression Products (IFN-γ) and the ABI/PRISM 7700 sequence detection system (Applied Biosystems Inc., Foster City, CA, USA). Samples were run in triplicate. The amount of gene expression was calculated from the standard curve (a serial dilution of $D2WF_1$ $CD4^+CD25^+$ cDNA) and the relative expression of the target gene was determined by dividing the target gene value by the HPRT value.

Suppression assay

Isolated $CD4^+CD25^-$ T cells (1×10^5 cells/well) were stimulated in triplicate with autologous T-depleted, irradiated splenocytes (1×10^5 cells/well) in the presence of 1 μg/ml anti-CD3 MoAb (145–2C11; eBioscience). Isolated $CD4^+CD25^+$ T cells were used as T_{reg} cells and added to the wells at a different ratio ($CD4^+CD25^-$ T cells: $CD4^+CD25^+$ T cells = 1 : 0·25, 1 : 0·5, 1 : 1). Cells were co-cultured in a final volume of 200 μl of RPMI-1640 supplemented with 10% fetal calf serum (FCS) in 96-well round-bottomed plates for 3 days. The wells were pulsed with 1 μCi of [^{3}H]-thymidine 18 h before harvesting and the cells were collected onto glass fibre filters using an automated multi-sample harvester, and the amount of incorporated [^{3}H]-thymidine was then measured with a dry scintillation counter (Packard, Meridan, CT, USA).

Generation of dendritic cells and apoptotic cells

BMDCs were prepared as described previously [20]. In brief, erythrocytes-depleted bone marrow cells were cultured in a medium supplemented with recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF) (750 U/ml) and IL-4 (1000 U/ml) (Pepro Tech Inc. Rocky Hill, NJ, USA). Apoptotic cells were generated by treating single-cell suspensions of thymocytes with $1{\cdot}2 \times 10^{-6}$ M

dexamethasone for 15 h. On day 4 of culture, apoptotic cells were added to BMDC cultures at the ratio of 10 : 1. After 48 h, cells were collected and DCs were enriched by anti-CD11c-coated magnetic microbeads.

Immunization of mice

$D2WF_1$ mice were divided into three groups and given an intravenous injection of phosphate-buffered saline (PBS), isotype control antibody (250 μg/mouse) (A110-1, PharMingen, San Diego, CA, USA) or anti-CD25 antibody (250 μg/mouse) (PC61, PharMingen) on day 0. The mice were then immunized with PBS or dendritic cells pulsed with apoptotic cells ($1{\cdot}5 \times 10^5$ DCs/mouse) on day 4 and boosted with PBS or apoptotic cells (1×10^7 cells/mouse) on day 9. All the mice were bled at indicated time-points. Sera were collected for autoantibody detection.

Enzyme-linked immunosorbent assay

Antibodies to double-strand (ds) DNA and single-strand (ss) DNA in the serum samples were measured by enzyme-linked immunosorbent assay (ELISA). The level of anti-DNA IgG was presented as ELISA units (EU/ml) compared with the MoAb 10F10, which is specific for either dsDNA or ssDNA. The absorbance value generated by 74 ng/ml of 10F10 antibody was defined as 1 EU/ml.

Statistical analysis

The significant differences between the experimental and control groups were analysed with the Mann–Whitney *U*-test by using GraphPad Prism version 4·00 (GraphPad Software, San Diego, CA, USA). A *P*-value of $< 0{\cdot}05$ was considered to be statistically significant.

Results

Percentage of $CD4^+CD25^+$ T cells in BWF_1 and $D2WF_1$ mice

In order to examine whether lupus mice have a deficiency in $CD4^+CD25^+$ T cells, we determined the percentage of these cells in the splenocytes of lupus and normal mice. Because $D2WF_1$ mice have identical major histocompatibility complex class II ($H\text{-}2^{d/u}$) with BWF_1, we used $D2WF_1$ mice as the normal control mice. The percentage of $CD4^+CD25^+$ T cells in splenic $CD4^+$ T cells of BWF_1 mice was significantly lower than that of $D2WF_1$ mice, and the mean percentages of BWF_1 mice and $D2WF_1$ mice were 13·0% and 15·2%, respectively (Fig. 1). We also measured the levels of peripheral $CD4^+CD25^+$ T cells in autoimmune and normal mice. However, the frequency of peripheral $CD4^+CD25^+$ T cells of BWF_1 mice was similar to that of $D2WF_1$ mice (data not show).

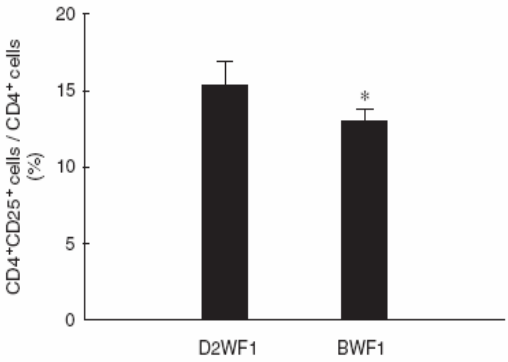

Fig. 1. The relative percentage of splenic $CD4^+CD25^+$ T cell in DBA-$2 \times$ NZW F_1 ($D2WF_1$) mice and NZB $\times$ NZW F_1 (BWF_1) mice. The $CD3^+$ T cells were gated and the percentage of $CD25^+$ cells in $CD4^+$ T cells was shown above. Bars show mean ± s.d. $^*P < 0{\cdot}05$ ($D2WF_1$, $n = 4$; BWF_1, $n = 5$).

Gene expression profile of $CD4^+CD25^+$ T cells in $D2WF_1$ and BWF_1 mice

We further analysed the expression of some specific genes which are relative to the development and the function of T_{reg} cells, such as Foxp3, IL-10 and TGF-β. Cells were isolated from splenocytes of both mice at different ages. The purity of $CD4^+CD25^+$ T cells was greater than 90%, and that of $CD4^+CD25^-$ T cells was greater than 95% (data not shown). The mRNA expression of freshly isolated cells was quantified by real-time PCR. We found that the mRNA expression of Foxp3 was predominant in $CD4^+CD25^+$ T cells. Foxp3 expression in BWF_1 $CD4^+CD25^+$ T cells was similar to that in $D2WF_1$ $CD4^+CD25^+$ T cells. The expression level of Foxp3 in $CD4^+CD25^-$ T cells was used as a negative control. However, we found that Foxp3 expression in BWF_1 $CD4^+CD25^-$ T cells was higher than that in $D2WF_1$ $CD4^+CD25^-$ T cells (Fig. 2a).

Moreover, TGF-β expression in BWF_1 $CD4^+CD25^+$ T cells was also normal (Fig. 2c). Both BWF_1 $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells expressed higher levels of IL-10 than $D2WF_1$ $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, and this phenomenon was more significant in old BWF_1 mice at 6–11 months of age (Fig. 2b).

The suppressive function of $CD4^+CD25^+$ T cells from $D2WF_1$ and BWF_1 mice

To determine the suppressive function of $CD4^+CD25^+$ T cells isolated from both mice, different numbers of $D2WF_1$ $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were added to the culture of $CD4^+CD25^-$ T cells to suppress their proliferation. It was found that $CD4^+CD25^+$ T cells from both $D2WF_1$ (Fig. 3a) and BWF_1 mice (Fig. 3b) inhibited the proliferation of autologous $CD4^+CD25^-$ T cells at a ratio of 0·25 : 1, but $CD4^+CD25^-$ T cells did not. The 1 : 1 ratio of $CD25^+$ cells to $CD25^-$ cells was then used to compare the activities of

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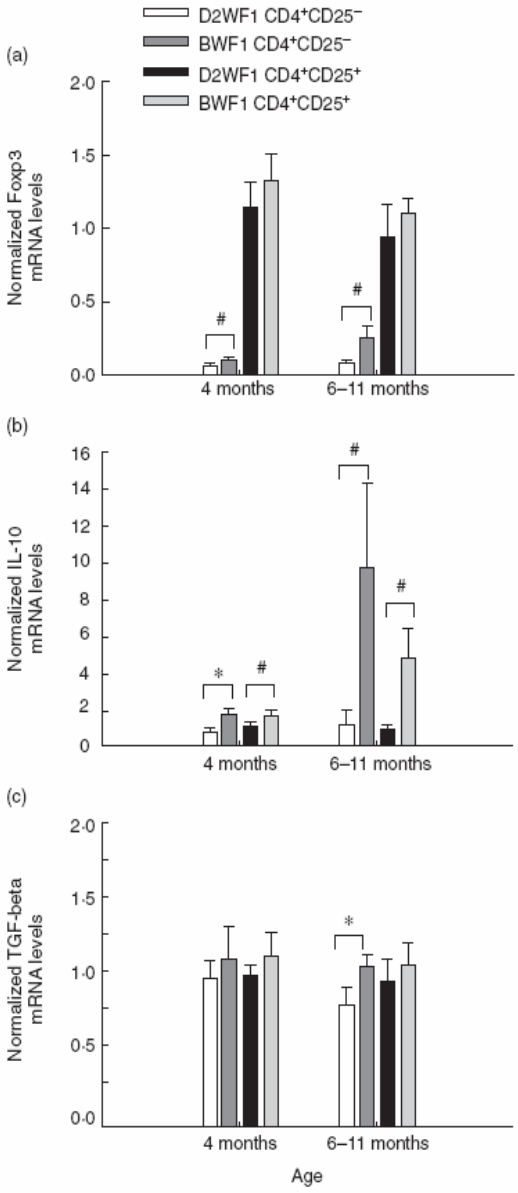

Fig. 2. Gene expression profiles of $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells isolated from DBA-2 × NZW F_1 ($D2WF_1$) mice or NZB × NZW F_1 (BWF_1) mice at different ages. The relative mRNA expression of forkhead box P3 (Foxp3) (a), interleukin (IL)-10 (b) and transforming growth factor (TGF)-β (c) was determined by normalizing the expression of each target gene to hypoxanthine phosphoribosyltransferase (HPRT). Bars show mean ± s.d. $^*P < 0{\cdot}05$, $\#P < 0{\cdot}01$ ($n = 5$).

$D2WF_1$ $CD4^+CD25^+$ T cells with BWF_1 $CD4^+CD25^+$ T cells. Data showed that $CD4^+CD25^+$ T cells have similar suppressive capability and the mean inhibition of $D2WF_1$ and BWF_1 mice were 50·8% and 54·8%, respectively (Fig. 3c).

$CD4^+CD25^+$ T cells play a role in controlling autoantibody production

The data above indicate that $CD4^+CD25^+$ T cells in BWF_1 mice had a deficiency in cell numbers, but had a normal expression level of Foxp3 and normal suppressive functions. To investigate further if the deficiency of T_{reg} cell numbers in BWF_1 mice causes the development of lupus-like disease, we depleted $CD25^+$ cells in normal mice and then broke down their tolerance. The depleted efficiency in mouse peripheral blood was followed after mice received an intravenous injection of anti-CD25 MoAb. Data showed that the percentage of $CD4^+CD25^+$ T cells in peripheral $CD4^+$ T cells decreased from 4% to less than 1% and was maintained for more than 20 days (Fig. 4).

Our previous experiments showed that after adoptive transfer of apoptotic cell-pulsed DCs into $D2WF_1$ mice, it initiated an autoimmune response in normal mice, such as the production of anti-dsDNA antibody and anti-ssDNA antibody [19]. The effect of depletion of $CD25^+$ cells 4 days before $D2WF_1$ mice were immunized with PBS or apoptotic cell-pulsed DCs was studied. Mice further received a boost of PBS or apoptotic cells to enhance their immune response on day 9 and were bled at indicated time-points, as shown in Fig. 5a. The ELISA data of mouse serum showed that mice treated with apoptotic cell-pulsed DCs and a boost of apoptotic cells produced anti-ssDNA and anti-dsDNA antibodies (Fig. 5b,c). In addition, mice in the $CD25^+$ cell-depleted group developed a more severe autoimmune response than mice in the isotype control antibody-treated groups.

Discussion

SLE is a prototype of systemic autoimmune disease. Multiple defects in the immune system of SLE patients have been described, including B and T cell hyperactivity and aberrant cytokine production. Some studies concerning the role of $CD4^+CD25^+$ T cells in lupus reveal decreased frequencies of these cells in SLE patients [16–18] and lupus mice [15]. However, it remains unclear if the suppressive function of T_{reg} cells in SLE is impaired. From our findings, splenic T_{reg} cells in lupus mice show deceased frequency, but T_{reg} cells of lupus mice have no significant defect in their suppressive activity. Similar results have been noted in lupus-prone MRL/Mp mice. Their $CD4^+CD25^+$ T cells have normal frequencies and display only subtle abnormalities of function [21].

Foxp3 is a critical molecule for the development and function of $CD4^+CD25^+$ T cells [7–9]. At both mRNA and protein levels, Foxp3 expression in peripheral $CD4^+$ T cells was predominant in the $CD4^+CD25^+$ subset. Scurfy mice with spontaneous Foxp3 mutation have a fatal lymphoproliferative disease associated with multi-organ infiltration and early death at 3–4 weeks of age in hemizygous (sf/Y) males [22]. Moreover, mutations in human FOXP3 have also been iden-

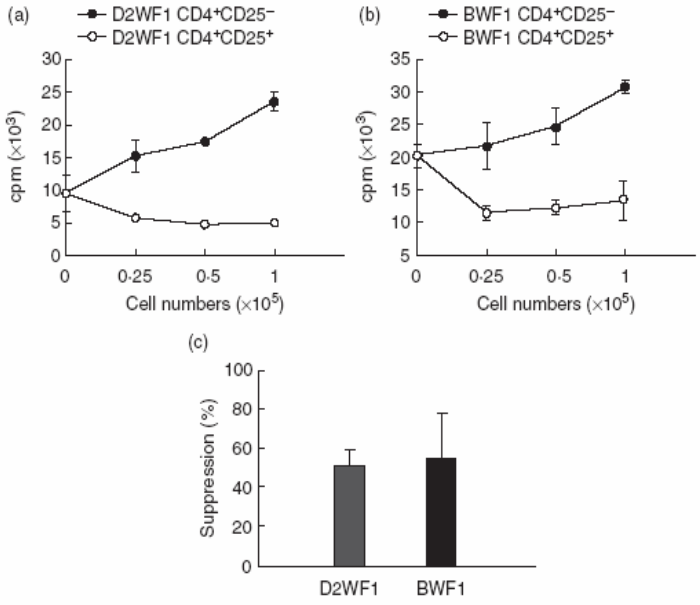

Fig. 3. The suppressive function of $CD4^+CD25^+$ cells isolated from DBA-2 × NZW F_1 ($D2WF_1$) mice and NZB × NZW F_1 (BWF_1) mice. Different numbers of $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ cells from $D2WF_1$ mice (a) or BWF_1 mice (b) were added to the culture of $CD4^+CD25^-$ T cells stimulated with autologous T-depleted splenocytes and anti-CD3 monoclonal antibody. (c) $CD4^+CD25^+$ T cells from $D2WF_1F_1$ mice and BWF_1 mice suppressed the proliferation of autologous $CD4^+CD25^-$ cells at the ratio of 1 : 1. Bars show mean ± s.d. ($n = 3$).

tified in patients with a severe fatal autoimmune/allergic syndrome, known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X–linked syndrome) [23–25].

An indispensable study for Foxp3 in T_{reg} cell development showed directly that $CD4^+CD25^+$ T cells in the periphery of scurfy or Foxp3-deficient mice were neither anergic nor suppressive *in vitro* [8]. In BWF_1 mice, Foxp3 expression seemed normal in $CD4^+CD25^+$ T cells and these cells had normal suppressive function. We therefore excluded the possibility of Foxp3 deficiency in BWF_1 mice.

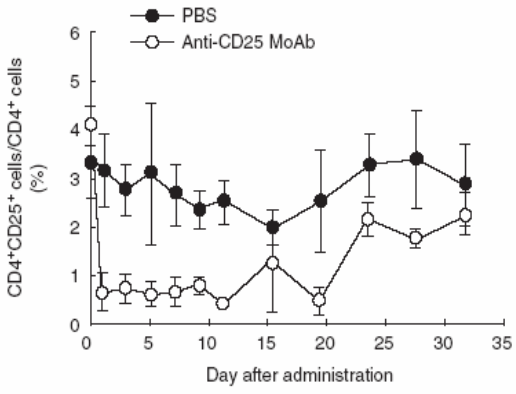

Fig. 4. Depletion of $CD4^+CD25^+$ cells in DBA-2 × NZW F_1 ($D2WF_1$) mice by administration of anti-CD25 monoclonal antibody (MoAb). $D2WF_1$ mice received an intravenously injection of phosphate-buffered saline or anti-CD25 antibody (250 μg/mouse) on day 0. All mice were bled before immunization and at each time-point. The $CD3^+$ T cells were gated and the relative percentage of $CD4^+CD25^+$ T cell in $CD4^+$ T cells was determined. Values are mean ± s.d. ($n = 4$).

The $CD4^+CD25^+$ T cells have diverse T cell receptor (TCR) repertoires [26,27], suggesting that they are capable of responding to a wide spectrum of antigens. Some studies of human $CD4^+CD25^+$ T cells have shown that they suppress proliferation and cytokine production to both self- and foreign antigens. In our study, although the suppressive function of lupus $CD4^+CD25^+$ T cells to TCR stimulation (anti-CD3) is normal, we do not know whether they fail to suppress the response to self-antigen. Recent findings have indicated that $CD25^+$ T cells from New Zealand mixed 2328 mice had defects in suppressing lupus glomerulonephritis and sialoadenitis [28]. Defective antigen-specific T_{reg} cells may thus contribute to the pathogenesis of lupus. Therefore, the antigen-specific suppression of lupus T_{reg} cells merits being investigated further.

IL-10 is an important immunoregulatory cytokine, mainly by inhibiting the production of proinflammatory cytokines and by regulating the differentiation and proliferation of several immune cells, such as T cells, B cells and APC [29]. In some experimental systems, the effect of T_{reg} cells is also mediated by IL-10 [30]. However, high amounts of IL-10 have been found in SLE patients and most of the IL-10 have been secreted by monocytes and B lymphocytes, with a small contribution from T lymphocytes [31].

In our study, both $CD4^+CD25^+$ T and $CD4^+CD25^-$ T cells isolated from BWF_1 mice with severe lupus disease produced higher levels of IL-10 mRNA than those of normal mice. It is still unknown whether this represents the $CD4^+CD25^+$ T cells exerting their suppressive function

(a)

Immunization	1st	2nd	3rd
Group A	PBS	PBS	PBS
Group B	IC	DC-apop. cell	Apop. cell
Group C	anti-CD25	DC-apop. cell	Apop. cell

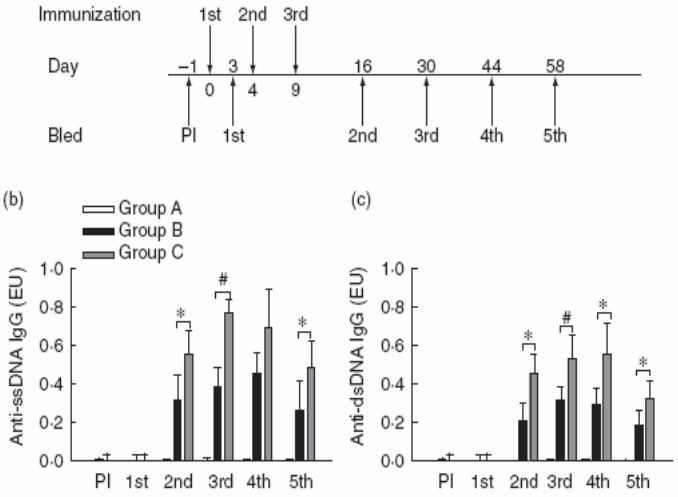

Fig. 5. IgG autoantibodies levels in the serum of DBA-2 × NZW F_1 ($D2WF_1$) mice. (a) Schematic depiction of immunization protocol as described in Materials and methods. $D2WF_1$ mice were divided into three groups and received an intravenously injection of phosphate-buffered saline (PBS) (group A), 250 μg isotype control antibody (group B) or 250 μg anti-CD25 antibody (group C) on day 0. Then, the mice were immunized with PBS (group A) or $1{\cdot}5 \times 10^5$ dendritic cells pulsed with apoptotic cells (groups B, C) on day 4 and boosted with PBS (group A) or 1×10^7 apoptotic cells (groups B, C) on day 9. All mice were bled 1 day (PI) before and 3 (1st), 16 (2nd), 30 (3rd), 44 (4th) and 58 (5th) days after first injection. Serums were collected for anti-ssDNA antibody (b) and anti-dsDNA antibody (c) detection. Bars show the mean ± s.d. $*P < 0{\cdot}05$, $\#P < 0{\cdot}01$ ($n = 5$ for each group).

through IL-10, or if lupus mice have an unusual genetic transcription of IL-10.

The major source of autoantigens in SLE might be the apoptotic cells. Our previous study indicated that DCs treated with apoptotic cells could initiate the response of autoreactive T cells and the production of autoantibodies [19]. DCs presenting self-antigen can initiate autoantibody response, indicating that autoreactive cells exist in the normal body under the control of peripheral tolerance. When the mechanism of peripheral tolerance is broken, such as depletion of T_{reg} cells, autoreactive cells would cause autoimmune diseases. Recent data also show that $CD4^+CD25^+$ T cells down-regulate the maturation and function of the DCs [32] and inhibit the maturation, rather than initiation, of autoantibody responses [33]. In our experiment, the depletion of T_{reg} cells combined with accumulated autoantigens caused severe autoimmune responses in normal animal model.

A recent study demonstrated that eliminating $CD4^+CD25^+$ T cells induced an increase in anti-nuclear antibodies and accelerated the development of glomerulonephritis during the pre-active phase in BWF_1 mice [34]. In our *in vivo* experiment, $D2WF_1$ mice whose $CD25^+$ cells were depleted produced higher titres of autoantibodies after being immunized with apoptotic cells-pulsed DCs. However, the production of autoantibodies was inhibited further after day 44 (Fig. 5). The reason may be that the haematopoietic system could reconstitute $CD4^+CD25^+$ T cells to control autoimmune response in normal mice.

Moreover, high titres of anti-DNA antibodies were not accompanied by the development of kidney disease in our animal experiment. Other key factors may be involved in the mechanism of end-organ damage. In lupus-prone NZM2328 mice, a locus *Cgnz1* on chromosome 1 was linked to chronic glomerulonephritis and severe proteinuria in females. The study indicated that breaking tolerance to dsDNA and chromatin is not required for the pathogenesis of lupus nephritis [35].

The pathogenesis of SLE is complex. Several studies suggest that the causes of SLE were defective clearance of autoantigens and abnormal immunoregulation. Our study suggested that the defect of T_{reg} cells in BWF_1 mice may also contribute to ineffective inhibition of autoantibody production and subsequent pathological damage.

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