Blunting Autoantigen-induced FOXO3a Protein Phosphorylation and Degradation Is a Novel Pathway of Glucocorticoids for the Treatment of Systemic Lupus Erythematosus*§

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Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease affecting multiple organs. Glucocorticoids (GCs), the potent anti-inflammatory drugs, remain as a cornerstone in the treatment for SLE; nevertheless, their clinical efficacy is compromised by the side effects of long term treatment and resistance. To improve the therapeutic efficacy of GCs in SLE, it is important to further decipher the molecular mechanisms of how GCs exert their anti-inflammatory effects. In this investigation, FOXO3a was identified as a molecule that was down-regulated in the course of SLE. Of interest, GC treatment was found to rescue FOXO3a expression both in SLE mice and in SLE patients. Gain- and loss-of-function studies demonstrated that FOXO3a played a crucial role in GC treatment of SLE via inhibiting inflammatory responses. Further studies showed that the up-regulation of FOXO3a by GCs relied on the suppression of pI3K/AKT-mediated FOXO3a phosphorylation and the arrest of FOXO3a in the nucleus. Finally, our data revealed that FOXO3a played an important role in GC treatment of SLE by suppressing pro-inflammatory response, and targeting FOXO3a might provide a novel therapeutic strategy against SLE.

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disorder, affecting virtually every organ (1, 2). Lupus nephritis is one of the most severe manifestations of SLE, causing substantial morbidity and mortality (1–4). To date, the precise pathogenesis of SLE still remains obscure; however, increasing evidence indicates that the excessive inflammation caused by immune complex deposition may play a key role in the progress of SLE (5). By far, the mainstay therapeutic strategy for SLE is the usage of glucocorticoids (GCs) (6, 7). GCs are small lipophilic compounds, which are well known to inhibit the pro-inflammatory responses, and are used in the treatment of many immune-mediated inflammatory diseases (8, 9); nevertheless, the underlying molecular mechanisms still remain elusive. Further deciphering the mechanisms whereby GCs suppress inflammation will lead to a better understanding of the inflammatory responses in SLE disease and may facilitate the development of new anti-inflammatory agents in the future. As GCs may cause some side effects (such as osteoporosis, skin atrophy, and glaucoma) in a time- and dose-dependent manner (10, 11), and not every patient responds sufficiently to GC treatment (12), deeper insights into the molecular mechanisms of how GCs exert their anti-inflammatory activities may also help improve the therapeutic efficacy for these patients.

FOXO3a belongs to a family of forkhead box class O (FOXO) transcription factors, which are involved in diverse physiopathological processes, such as metabolism, apoptosis, oxidative stress resistance, and senescence. In mammals, there are four FOXO proteins, FOXO1, FOXO3a, FOXO4, and FOXO6. FOXO1, FOXO3a, and FOXO4 are widely distributed and are expressed in most tissues, whereas FOXO6 expression is largely restricted to neural cells (13–16). In recent years, accumulating evidence has demonstrated the critical roles of several FOXO family members in immunoregulation, although their function may be diverse and in some cases even antagonistic (15–18). For example, FOXO1 has often been showed to activate NF-κB (19, 20); however, evidence indicates that FOXO3a and FOXO4 may play an inhibitory role in autoimmune and inflammatory diseases by repressing NF-κB activity (21, 22). Mice with a defect in FOXO3a were found to predispose to a spontaneous multisystemic inflammatory syndrome, accompanied by an up-regulated production of pro-inflammatory cytokines (21, 23). Accumulating evidence also indicates the expression of FOXO3a may be associated with diverse inflammatory diseases, such as asthma, ulcerative colitis, and Crohn’s disease, and...
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rheumatoid arthritis in clinical patients (24–26). Of interest, it is also suggested that FOXO3a may be involved in some GC-mediated biological processes (27, 28).

Using protein antibody array and Western blotting analysis, we found that FOXO3a was down-regulated in the course of SLE, which could be reversed by treatment with GCs. Of importance, FOXO3a was revealed to play a crucial role in GC treatment of SLE via inhibiting pro-inflammatory responses. Further studies showed that the up-regulation of FOXO3a by GCs relied on the suppression of FOXO3a phosphorylation and its ensuing degradation in cytoplasm. Moreover, it was found that the nucleus-arrested FOXO3a played an important role in GC-mediated inhibition of NF-κB activity, which might involve its interaction with NF-κB p65 protein.

Experimental Procedures

**Human Samples**—A total of 21 patients (male/female, 2/19; median age, 42 years) with SLE and 12 age- and gender-matched healthy individuals (male/female 1/11; median age, 41 years) were recruited. All patients took prednisone (0.5–1 mg/kg/day orally) as part of their routine therapy for 4 weeks in combination with oral hydroxychloroquine or other immuno-suppressants to achieve a smooth reduction in GC dosage after 4 weeks. After the 4-week GCs treatment, the peripheral blood samples and urine samples were collected from SLE patients after informed consent. The diagnosis of SLE was established according to the 1982 revised American College of Rheumatology criteria (29). This study was approved by the Ethics Committee of Fudan University and performed in compliance with the Helsinki Declaration.

**Mice**—Six-week-old female BALB/c mice were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were housed in a specific pathogen-free room under controlled temperature and humidity. All animal experiments were conducted according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People’s Republic of China, 1998) and with the approval of the Ethical Committee of Fudan University (Shanghai, People’s Republic of China).

**Cell Culture**—RAW264.7 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 2 mM glutamine and 10% FBS (Hyclone, Logan, UT) in a 5% CO₂ incubator at 37 °C. Primary peritoneal macrophages were obtained from 6-week-old female BALB/c mice and were maintained in DMEM (Invitrogen) supplemented with 10% FBS. For the preparation of renal macrophages, murine renal tissues were dispersed in RPMI 1640 medium containing 5% FBS and 0.1% collagenase (Sigma) at 37 °C for 30 min, followed by progressive sieving to obtain single-cell suspensions. The CD11b+/F4/80 high macrophages were further sorted from single-cell suspensions using a FACSaria (BD Biosciences) with FITC-labeled anti-F4/80 and phycoerythrin-labeled anti-CD11b (BD Biosciences). The purity of cells was more than 90%, as determined by flow cytometry (FACSCalibur; BD Biosciences).

**DNA Preparation**—Activated lymphocyte-derived apoptotic DNA (ALD-DNA) and unactivated lymphocyte-derived DNA (UnALD-DNA) extraction and purification were performed as described previously (30–38). In brief, for the generation of activated lymphocyte-derived apoptotic DNA (ALD-DNA), splenocytes from 6- to 8-week-old female BALB/c mice were seeded at 2 × 10⁶ cells/ml in 75-cm² cell culture flasks and cultured for 6 days in the presence of concanavalin A (5 mg/ml) to induce apoptosis. The apoptotic cells were stained with FITC-labeled annexin V (BD Biosciences) and propidium iodide (Sigma) and then sorted using a FACSAria (BD Biosciences). Genomic DNAs from syngeneic apoptotic splenocytes were treated with S1 nuclease (TaKaRa, Dalian, China) and then sorted using the DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Unactivated lymphocyte-derived DNA (UnALD-DNA) was extracted from unactivated (resting) splenocytes using the same methods.

**Generation of SLE Murine Model**—The SLE murine model was generated as described previously (30–38). Briefly, 6-week-old female BALB/c mice were immunized s.c. with ALD-DNA (50 μg/mouse) plus complete Freund’s adjuvant (Sigma) on day 1, followed by s.c. injection of ALD-DNA (50 μg/mouse) emulsified with incomplete Freund’s adjuvant (Sigma) on days 14 and 28 for a total of three times. Serum and urine samples were collected every 2nd week for further experiments. Eight weeks after initial immunization, mice were sacrificed, and kidneys were collected for further cellular function and tissue histology analysis.

**Protein Antibody Array**—The protein expression profiles of ALD-DNA-stimulated RAW264.7 cells in the absence or presence of GCs were analyzed using an antibody array kit (Full Moon Biosystem, Inc., Sunnyvale, CA). Briefly, protein samples obtained from RAW264.7 cells were biotinylated using biotin reagent dissolved in N,N-dimethylformamide. Biotin-labeled protein samples were then conjugated to the antibody microarray slides, followed by incubation with FITC-streptavidin solution. Slides were scanned using the GenePix 4000 Array Scanner, and the images were analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA).

**Gene Silencing in Vitro and in Vivo**—To down-regulate the FOXO3a expression in RAW264.7 cells, cells were transfected with FOXO3a-specific siRNA (catalogue number 6302; Cell Signaling Technology) using mouse macrophage nucleofector kit (Lonza Amaza, Cologne, Germany) according to the manufacturer’s instructions. To inhibit the FOXO3a expression in lupus mice, 6-week-old female BALB/c mice were randomized after initial immunization, mice were sacrificed, and kidneys were collected for further cellular function and tissue histology analysis.

**Preparation of Adenovirus and Adenoviral Delivery in Mice**—Adenoviruses encoding FOXO3a (Ad-FOXO3a) and the control (Ad-Control) were obtained from Hanheng Biological Technology (Shanghai, China). The adenovirus delivery in mice was performed as described previously (39). To examine the therapeutic effects of FOXO3a, each mouse received
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an intravenous injection of 100 µl of Ad-FOXO3a or Ad-Control (3 × 10³ plaque-forming units, pfu) 2 days before ALD-DNA immunization.

Anti-dsDNA Antibody and Proteinuria Examination—For the detection of anti-dsDNA levels, ELISA plates were pretreated with protamine sulfate (Sigma) and then coated with calf thymus dsDNA (Sigma). After incubation with mouse sera, levels of anti-dsDNA antibodies were examined with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL), followed by the measurement of enzymatic color reaction at 450 nm. Proteinuria of the mice was determined by the BCA protein assay kit (Thermo Scientific) according to the manufacturer’s instructions.

ELISA—To assess the levels of TNF-α, IL-6, and MCP-1 in sera of GC-treated mice, ELISAs were performed according to the manufacturer’s instructions (eBioscience, San Diego).

Histology—Murine renal tissues were fixed in 4% paraformaldehyde (Sigma), processed, and coated in paraffin. H&E staining of renal tissue sections was performed according to the manufacturer’s instructions and assessed by a pathologist blinded to treatment group. The kidney score of glomerulonephritis was determined by using the ISN/RPS2003 classification (40). Pictures were acquired with a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) with magnification ×200.

Immunofluorescence—Fluorescent staining of cryosections was used for autoantibody deposition analysis in the glomeruli. Sections were fixed in acetone for 10 min and incubated with FITC-conjugated goat anti-mouse IgG antibody (catalogue number sc-2010; Santa Cruz Biotechnology, Santa Cruz, CA) or FITC-conjugated C3 antibody (catalogue number orb102204; Biorbyt Corp., Cambridgeshire, UK) for 30 min. Pictures were acquired with a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) with magnification ×200.

Immunohistochemistry—Paraffin-embedded kidney tissue sections were cut at 4 µm, placed on 3-aminopropyltriethoxysilane-pretreated slides. Sections were then deparaffinized and rehydrated according to the standard protocol. For immunohistochemistry analysis, sections were incubated with anti-Hexon (catalogue number LS-C63694; LSBio, Seattle, WA) or anti-FOXO3a (catalogue number 2497; Cell Signaling Technology), followed by the incubation of biotin-labeled secondary antibody and avidin-biotin complex. Peroxidase stain was developed with 3,3’-diaminobenzidine solution and counterstained with hematoxylin. Pictures were acquired with a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) with magnification ×200.

NF-kB DNA Binding Assay—The nuclear factor-κB (NF-κB) p65 DNA binding assay was performed according to the manufacturer’s instructions (catalogue number 10007889; Cayman Chemical Co.). Briefly, 10 µg of nuclear extract, diluted to 100 µl, was added to the wells coated with oligonucleotides containing the NF-κB consensus binding site. For the detection of activated NF-κB, antibodies against the p65 were used, followed by HRP-conjugated secondary antibody. The colorimetric readout (450 nm) was done with an ELISA plate reader (Thermo Scientific).

Western Blotting and Coimmunoprecipitation Assay—Western blotting assay and coimmunoprecipitation assay were performed as described previously (41, 42). Antibodies against β-actin (catalogue number sc-130300), tubulin (catalogue number sc-9104), and lamin B (catalogue number sc-374015) were obtained from Santa Cruz Biotechnology. Antibodies against p-FOXO3a (catalogue number 13129), FOXO3a (catalogue number 2497), p-NF-κB p65 (catalogue number 3033), NF-κB p65 (catalogue number 4765), p-1κBα (catalogue number 2859), 1κBα (catalogue number 4812), p-Akt (catalogue number 9721), Akt (catalogue number 9272), p-JNK (catalogue number 9251), p-ERK1/2 (catalogue number 9101), and p-p38 (catalogue number 9211) were from Cell Signaling Technology (Beverly, MA).

Results

GC Treatment Effectively Ameliorated the Severity of SLE in Both Patients and Mouse Model—To investigate the therapeutic effect of GCs on SLE patients, we detected the clinical indicators of SLE patients before and after GC treatment. It was found that after GC treatment, levels of serum anti-dsDNA antibody (Fig. 1A) and urine protein (Fig. 1B) were decreased; the serum C3 level (Fig. 1C) was elevated; and the SLE disease activity index (SLEDAI) (Fig. 1D) was significantly attenuated. We also tested the therapeutic effect of GCs on SLE using a mouse model, which was established by immunized female BALB/c with an autoantigen, activated lymphocyte-derived apoptotic DNA (ALD-DNA), as described previously (30–38). Results showed that ALD-DNA treatment efficiently induced a typical SLE syndrome in mice, as revealed by the increased concentrations of serum anti-dsDNA IgG (Fig. 1E) and urine proteins (Fig. 1F), the deposition of IgG immune complexes (IgG-ICs) (Fig. 1G) and complement 3 (C3) (Fig. 1H) in mouse kidney, and the more severe kidney pathology (Fig. 1, I and J). Although the UnALD-DNA, similar to PBS, failed to induce these SLE symptoms, of importance the treatment with glucocorticoid dexamethasone (Dex) was found to significantly attenuate the SLE symptoms in this mouse model (Fig. 1, E–J), indicating the ALD-DNA-triggered SLE mouse model could act as a suitable tool for further investigation on GC treatment of SLE.

GC-mediated Amelioration of SLE Is Associated with the Inhibition of Pro-inflammatory Responses—To investigate the mechanism of GC therapy for SLE, we examined levels of pro-inflammatory cytokines before or after GC treatment in SLE patients. The results showed that GC treatment significantly decreased the sera levels of TNF-α, IL-6, and MCP-1 in SLE patients (Fig. 2, A–C). Of importance, GC-mediated down-reg-
ulation of these inflammatory cytokines was negatively correlated with SLEDAI in patients (Fig. 2, D–F). We also examined the effect of GCs on pro-inflammatory responses in the SLE mouse model. Consistent with the data from SLE patients, GC treatment significantly down-regulated the sera concentration of TNF-α, IL-6, and MCP-1 (Fig. 2, G–I), which was negatively associated with kidney score in the mouse model (Fig. 2, J–L).

Results from our group or others demonstrated that the macrophage-mediated inflammatory response played a crucial role in the pathogenesis of SLE (32, 34–38, 43, 44); thus, we investigated the effect of GCs on macrophage inflammatory response. It was found that Dex treatment dose-dependently suppressed the secretion of TNF-α, IL-6, and MCP-1 in both RAW264.7 cells (Fig. 2M) and mouse peritoneal macrophages (Fig. 2N) in response to ALD-DNA stimulation. To exclude the
possibility that GC-mediated attenuation of pro-inflammatory responses triggered by ALD-DNA was due to the effect of GCs on cellular apoptosis, we tested the effect of GCs on the apoptosis of ALD-DNA-stimulated RAW264.7 cells by FACS analysis. It was found that GCs at the doses used in this study did not have significant effect on cellular apoptosis (data not shown).

To further confirm the inhibitory effect of GCs on ALD-DNA-triggered macrophage inflammatory response, we examined the production of inflammatory cytokines in CD11b+/F4/80high renal macrophages that were isolated from Dex-treated SLE mice, and we found that renal macrophages from Dex-treated SLE mice secreted much lower levels of TNF-α, IL-6, and MCP-1 than those from vehicle-treated mice (Fig. 2O).

**FIGURE 2.** GC-mediated amelioration of SLE was associated with the inhibition of pro-inflammatory responses. Levels of TNF-α (A), IL-6 (B), and MCP-1 (C) were evaluated by ELISA in SLE patients pre- or post-treated with GCs (n = 21). The correlation between TNF-α (D), IL-6 (E), MCP-1 (F), and SLEDAI was carried out by Pearson correlation analysis in SLE patients. Mouse sera were analyzed for TNF-α (G), IL-6 (H), and MCP-1 (I) by ELISA at 8 and 10 weeks after the initial immunization. The correlation between TNF-α (J), IL-6 (K), MCP-1 (L), and SLEDAI was carried out by Pearson correlation analysis in SLE mice. RAW264.7 cells (M) or peritoneal macrophages (N) were pretreated with vehicle or different concentrations of Dex (0.001, 0.01, 0.1, 1, and 10 µM) for 1 h and then stimulated with ALD-DNA (50 µg/ml) for 24 h. The secretion of TNF-α, IL-6, and MCP-1 in the supernatant was measured by ELISA. Data are presented as mean ± S.E. of at least three independent experiments. *p < 0.05.
FOXO3a Is Identified to Be a Molecule That Is Down-regulated in the Course of SLE, although GC Treatment Rescues Its Expression—To decipher the molecular mechanisms by which GCs inhibited the inflammatory response in SLE disease, a protein antibody array was performed to examine the protein expression profiles in ALD-DNA-stimulated macrophage RAW264.7 cells in the presence or absence of GCs. It was found that FOXO3a, a potential inflammation repressor (21, 23), was one of the molecules that was down-regulated in response to ALD-DNA stimulation, although GC treatment significantly rescued FOXO3a expression (supplemental Table 1). Further Western blotting analysis confirmed that ALD-DNA stimulation down-regulated FOXO3a protein level in RAW264.7 cells and mouse peritoneal macrophages, although GC treatment could rescue FOXO3a expression in a dose-dependent manner (Fig. 3, A and B), consistent with GC-mediated attenuation of inflammatory response in these cells (Fig. 2, M and N). We also examined the effect of ALD-DNA stimulation on FOXO3a expression in renal macrophages isolated from vehicle- or GC-treated SLE mice, and similar results were obtained (Fig. 3C). To further ascertain the significance of FOXO3a in SLE disease, we examined FOXO3a expression in human samples. It was found that, compared with normal controls, FOXO3a protein level was down-regulated in peripheral
blood mononuclear cells (PBMCs) from SLE patients (Fig. 3D); however, after GC treatment, the FOXO3a expression in most SLE patients was significantly up-regulated (Fig. 3E). These data further indicated the possible involvement of FOXO3a in GC treatment of SLE.

**FOXO3a Is Involved in GC Treatment for SLE**—The above data suggested that FOXO3a protein might be involved in GC treatment of SLE; therefore, we investigated the role of FOXO3a in GC treatment of SLE by down-regulating FOXO3a expression in SLE mice. It was found that the FOXO3a protein level in kidney tissue was efficiently down-regulated in FOXO3a siRNA (siFOXO3a)-treated mice (Fig. 4A), and FOXO3a down-regulation significantly attenuated the therapeutic efficacy of GCs in SLE, as demonstrated by the increased concentrations of serum anti-dsDNA IgG (Fig. 4B) and urine proteins (Fig. 4C), the increased kidney deposition of IgG-ICs (Fig. 4D) and C3 (Fig. 4E), and the more severe kidney pathology (Fig. 4, F and G).

To determine the potential role of FOXO3a in GC-mediated inhibition of macrophage inflammatory response, we down-regulated FOXO3a expression in RAW264.7 cells with FOXO3a-specific siRNA, and we found that FOXO3a down-regulation significantly attenuated GC-mediated inhibitory effect on TNF-α, IL-6, and MCP-1 secretion (Fig. 4, H and I), consistent with GC-mediated inhibition of pro-inflammatory response in SLE disease. Furthermore, we obtained similar results in renal macrophages that were isolated from siFOXO3a-treated SLE mice after the GC treatment (Fig. 4J).
FOXO3a, per se, Can Alleviate the Severity of SLE—Because the GC-up-regulated FOXO3a appeared to be important for the treatment of SLE, we further investigated whether FOXO3a, per se, could alleviate the severity of SLE by using an adenovirus-mediated gene transfer. Results showed that intravenous injection of recombinant FOXO3a-carrying adenovirus led to a strong expression of FOXO3a in the kidney of SLE mice, as shown by Western blot and immunohistochemistry staining (Fig. 5A–D). Eight weeks after initial immunization, IgG deposition (G), C3 deposition (H), and pathological changes (I and J) in mice kidney were evaluated. K, renal macrophages isolated from the indicated immunized mice were incubated with ALD-DNA (50 μg/ml) for 24 h. The supernatants were collected and assayed for TNF-α, IL-6, and MCP-1. Data are presented as mean ± S.E. of at least three independent experiments. *, p < 0.05.

FIGURE 5. FOXO3a overexpression alleviated the severity of SLE disease via attenuating macrophage inflammatory responses. Mice were intravenously injected with 3 × 10^9 pfu of either Ad-FOXO3a or Ad-Control. 72 h later, the exogenous expression of FOXO3a was determined by Western blotting using anti-Hexon (A) and anti-FOXO3a (B). Mice were treated as in A and B. The exogenous expression of FOXO3a was determined by immunohistochemical staining using anti-Hexon (C) and anti-FOXO3a (D). E–J, mice were treated with PBS, ALD-DNA, ALD-DNA + Ad-Control, or ALD-DNA + Ad-FOXO3a, respectively. Levels of the sera anti-dsDNA IgG (E) and the urine protein (F) were measured every 2 weeks. *, p < 0.05 (ALD-DNA + Ad-FOXO3a versus ALD-DNA + Ad-control). Eight weeks after initial immunization, IgG deposition (G), C3 deposition (H), and pathological changes (I and J) in mice kidney were evaluated. K, renal macrophages isolated from the indicated immunized mice were incubated with ALD-DNA (50 μg/ml) for 24 h. The supernatants were collected and assayed for TNF-α, IL-6, and MCP-1. Data are presented as mean ± S.E. of at least three independent experiments. *, p < 0.05.

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Up-regulation of FOXO3a by GCs Relies on the Suppression of FOXO3a Phosphorylation and the Arrest of FOXO3a in the Nucleus—It is reported that the functions of FOXO proteins were subjected to post-translational modifications, such as phosphorylation and acetylation (45–49). Our data showed that ALD-DNA treatment stimulated the phosphorylation of FOXO3a in a dose-dependent manner (Fig. 6A), although it failed to affect its acetylation (data not shown). Our data further showed that ALD-DNA treatment activated the PI3K/AKT and MAPKs (p38, ERK, and JNK) signaling pathways (Fig. 6B), consistent with our previous investigations (32, 34, 50). However, it
was PI3K/AKT, but not MAPKs pathways, that was crucial for FOXO3a phosphorylation triggered by ALD-DNA (Fig. 6C). Reports indicate that the FOXO proteins normally locate in the nucleus, although the phosphorylated modification may lead to their nuclear exportation and degradation (45–48, 51, 52). Therefore, we further investigated whether ALD-DNA treat-
ment could prompt the translocation of FOXO3a into the cytoplasm. As shown in Fig. 6D, ALD-DNA treatment indeed triggered the translocation of FOXO3a from the nucleus into the cytoplasm. Furthermore, it was found that ALD-DNA stimulation enhanced the ubiquitination of FOXO3a (Fig. 6E) and led to its degradation in a proteasome-dependent manner (Fig. 6F).

As the above data revealed that GC treatment could efficiently suppress the ALD-DNA-mediated degradation of FOXO3a, we thus wanted to know whether GC treatment could interfere with the above-mentioned degradative processes of FOXO3a. It was found that GC treatment significantly inhibited the ALD-DNA-activated PI3K/AKT signaling pathway and the ensuing phosphorylation of FOXO3a (Fig. 6G). Furthermore, our data revealed that the GC treatment inhibited the ALD-DNA-triggered translocation of FOXO3a into the cytoplasm, leading to the arrest of FOXO3a in the nucleus (Fig. 6H).

**Nucleus-arrested FOXO3a Is Important for GC-mediated Inhibition of NF-κB Activity, Which Might Involve Its Interaction with p65**—Given the importance of NF-κB in pro-inflammatory responses (53), we investigated whether NF-κB activation was involved in ALD-DNA-triggered macrophage inflammatory responses. Results showed that the phosphorylation of NF-κB p65 subunit (p65) was significantly enhanced following ALD-DNA stimulation in RAW264.7 cells (Fig. 7A). The NF-κB p65 transcription factor assay also showed that ALD-DNA treatment significantly enhanced p65 DNA binding activity (Fig. 7B). We investigated the effect of FOXO3a on ALD-DNA-activated NF-κB activation by infecting RAW264.7 cells with adenovirus harboring the FOXO3a gene (Ad-FOXO3a). It was found that overexpression of FOXO3a was found to significantly inhibit the ALD-DNA-activated p65 binding activity in RAW264.7 cells (Fig. 7C). However, FOXO3a overexpression did not enhance IκB phosphorylation and its following degradation (Fig. 7D) nor affect NF-κB p65 levels in the nucleus (Fig. 7E). We therefore speculated whether FOXO3a could physically interact with p65 and thus inhibit p65 DNA binding activity. The immunoprecipitation assay revealed that there existed significant interaction between FOXO3a and p65 no matter the whether the precipitating antibody was anti-FOXO3a or anti-p65, especially after GC treatment (Fig. 7F). Further study showed that GC treatment significantly inhibited ALD-DNA-activated p65 binding activity as expected; however, once the expression of FOXO3a was down-regulated, the Dex-mediated inhibition of p65 DNA binding was significantly attenuated, although the nuclear p65 level in these processes was not significantly altered (Fig. 7G). Taken together, these data suggested that FOXO3a contributed to GC-mediated inhibition of NF-κB activity, which might involve its interaction with p65.

**Discussion**

SLE is a potentially fatal disease characterized by immune complex deposition and the subsequent inflammatory responses. The incidence of SLE worldwide is still on the rise in recent years (1, 2). The discovery of hydrocortisone by Kendall and Reichstein in 1949 and its following successful use in arthritis and SLE patients have made GC treatment the cornerstone for diverse autoimmune diseases (54). However, SLE patients are at a high risk of developing GC-associated adverse side effects, such as osteoporosis and immunosuppression, as they often need to take GCs for many years. In addition, some patients with highly active SLE do not respond to GC therapy (6, 7). Therefore, it is of importance to further decipher the underlying mechanisms by which GCs ameliorate the severity of SLE disease.

In our previous investigations, we have successfully established an SLE model in syngeneic mice by immunization with ALD-DNA (30), and we also demonstrated that excessive macrophage inflammatory responses play a crucial role in the pathogenesis of SLE (32, 34–36, 50). In this investigation, we identified FOXO3a as a molecule that was down-regulated in the course of SLE, although GCs could efficiently rescue its expression both in SLE mice and in SLE patients. We further demonstrated that FOXO3a acted as a negative regulator of ALD-DNA-triggered inflammatory response, and played an important role in GC-mediated inhibition of inflammatory response, as well as in GC-mediated amelioration of SLE disease. Interestingly, FOXO3a was also revealed to be involved in some other GC-mediated biological processes, such as apoptosis (27).

Post-translational modification, especially phosphorylation, played a crucial role in regulating the functions of FOXO proteins. The unphosphorylated FOXO3a has been suggested to be its active form, which mainly localizes in the nucleus and functions as a transcriptional regulator for a variety of genes (15, 51, 52, 55). Our data revealed that ALD-DNA stimulation enhanced the phosphorylation of FOXO3a, and the phosphorylated FOXO3a was then translocated to the cytosol and subjected to ubiquitin-proteasomal degradation. As the down-regulation of FOXO3a protein level may also be due to the inhibition of FOXO3a transcription, we had determined the effect of ALD-DNA stimulation on FOXO3a mRNA level by real time PCR, and we found that ALD-DNA stimulation did not affect the expression of FOXO3a at the mRNA level (data not shown). Our data further revealed that GC treatment significantly attenuated the effect of ALD-DNA on the phosphorylation of FOXO3a, leading to its nuclear localization, and thus rescuing its expression.

It is well established that the NF-κB signaling pathway played a crucial role in controlling the pro-inflammatory response, although GC-mediated suppression on NF-κB activity is considered to be the predominant mechanism by which they exert their anti-inflammatory effects (8). Therefore, we wanted to know whether FOXO3a contributed to GC-mediated inhibition of NF-κB activity. It was found that FOXO3a was indeed involved in GC-mediated inhibition of NF-κB activity triggered by ALD-DNA stimulation, and FOXO3a, per se, was able to attenuate ALD-DNA-triggered NF-κB activity. Two mechanisms may contribute to the FOXO3a-mediated inhibition of NF-κB activity as follows: one is that FOXO3a may inhibit the IκB phosphorylation and its degradation, thus suppressing the translocation of NF-κB into the nucleus; the other is that the nuclear-arrested FOXO3a may interact with NF-κB and thus interrupt...
the binding of NF-κB with its responding cis-element. Our data revealed that FOXO3a did not affect the expression of NF-κB, which controls the NF-κB nuclear translocation, but it could interact with NF-κB subunit p65. Similar to our results, another FOXO family member, FOXO4, was also reported to interact with NF-κB and repress its DNA binding activity (22). It is reported that GC receptor (GR) can bind to NF-κB and inhibit its binding to NF-κB response element, which may need the help of a corepressor (56). Our results did prove the crucial role of GR in GC-mediated attenuation of NF-κB activity (22). D, RAW264.7 cells were treated as in C, and then subjected to Western blotting using antibodies against p-IκB, IκB, FOXO3a, or β-actin. E, RAW264.7 cells were treated as in C, and nuclear proteins were subjected to Western blotting using antibodies against p65, FOXO3a, or lamin B. Data are presented as mean ± S.E. of at least three independent experiments. *, p < 0.05.

In conclusion, we identified FOXO3a as a negative regulator of autoantigen (ALD-DNA)-triggered inflammatory response. Of importance, FOXO3a was revealed to play an important role in GC-mediated inhibition of inflammatory response, as well as in GC-mediated amelioration of SLE disease. Mechanistically, it was found that the up-regulation of FOXO3a by GCs relied on the suppression of PI3K/AKT-mediated FOXO3a phosphorylation and its ensuing degradation in cytoplasm. Furthermore, the nuclear-arrested FOXO3a was revealed to be critical for GC-mediated inhibition of NF-κB activity (Fig. 8). These findings may have important implications in GC ther-
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