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What is This?
Production of anti-double-stranded DNA antibodies in activated lymphocyte derived DNA induced lupus model was dependent on CD4$^+$ T cells

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Our previous study demonstrated that activated lymphocyte derived DNA (ALD-DNA) could function as an autoantigen to induce production of anti-double-stranded DNA (anti-dsDNA) antibodies in syngeneic BALB/c mice. Here we carefully evaluated the potential role of T cells in the induction of anti-dsDNA antibody. We demonstrated that ALD-DNA could effectively induce production of anti-dsDNA antibodies in vivo and in vitro. In contrast, ALD-DNA could not induce the generation of anti-dsDNA antibodies in nude mice. We further showed that in vivo depletion of CD3$^+$ T cells blocked the induction of anti-dsDNA antibodies in BALB/c mice. Notably, we demonstrated that CD4$^+$ but not CD8$^+$ T cells conferred ALD-DNA to induce anti-dsDNA antibodies. Finally, we demonstrated that adoptive transfer of CD4$^+$ T cells could rescue ALD-DNA induced anti-dsDNA antibodies in nude mice. Our results suggested that T helper cells were required for ALD-DNA to induce anti-dsDNA antibodies. These findings could further our understanding about the immunogenic properties of DNA and throw new light on SLE pathogenesis. Lupus (2012) 21, 508–516.

Key words: SLE; activated lymphocyte derived DNA; anti-dsDNA antibody; CD4$^+$ T cell

Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous disease manifested by the presence of anti-double-stranded DNA (anti-dsDNA) antibodies, which is closely correlated with the clinical syndrome and hence of diagnostic and even prognostic value.1–3 Although much research has placed great emphasis on the induction of anti-dsDNA antibodies, the mechanism underlying the induction of anti-dsDNA antibodies is incompletely elucidated.4,5

Our previous study established a lupus murine model in non-susceptible mice by injection with syngeneic activated lymphocyte derived DNA (ALD-DNA). We demonstrated that compared with untreated lymphocyte derived DNA (termed as UnALD-DNA), concanavalin A activation induced ALD-DNA was capable of inducing an autoimmune disease that closely resembled human SLE including high levels of anti-dsDNA antibodies, glomerulonephritis and proteinuria in non-susceptible BALB/c mice.6 We further found that DNA derived apoptotic lymphocytes were responsible for ALD-DNA to induce the SLE autoimmune disease in BALB/c mice.7 This SLE model excluded the genetic mutation interruptions and was induced by a well-defined auto-antigen, thus was ideal for successive characterization of SLE pathogenesis.

We had revealed that Notch1 signaling-dependent macrophage M2b polarization played a pivotal role in the ALD-DNA induced SLE autoimmune disease.8 Also, we found that serum amyloid P component (SAP), an acute-phase serum protein with binding reactivity to DNA in mice, could effectively alleviate lupus nephritis via modulating anti-dsDNA antibody production and the inflammation followed IC deposition induced by ALD-DNA.9 We further showed that binding...
of SAP to ALD-DNA could switch macrophage phenotypic polarization from proinflammatory M2b to anti-inflammatory M2a via PI3K/Akt-ERK signaling activation, thus exerting the protective and therapeutic interventions on lupus nephritis. However, although much was known about the ALD-DNA induced SLE autoimmune disease, the cellular mechanism underlies the induction of anti-dsDNA antibodies induced by ALD-DNA, which was critical for further understanding of the immunogenic DNA and the SLE pathogenesis, still remains to be elucidated.

To this end, here we carefully evaluated the potential role of T cells in the production of anti-dsDNA antibodies induced by ALD-DNA in vitro and in vivo. We demonstrated that T cells were indispensable for ALD-DNA to induce the production of anti-dsDNA antibody. Importantly, we provided direct evidence to elucidate the crucial role of CD4\(^+\) T cells in the production of anti-dsDNA antibody induced by the ALD-DNA.

Materials and methods

Mice

Female BALB/c mice between six and eight weeks of age were purchased from the Center of Experimental Animals of Fudan University and housed in a pathogen-free mouse colony at our institution. All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, PR China, 1998) and with the ethical approval of the Shanghai Medical Laboratory Animal Care and Use Committee as well as the Ethical Committee of Fudan University.

Splenocytes preparation

Spleens of BALB/c mice were aseptically removed and teased on a nylon mesh immersed in chilled RPMI-1640 medium (Gibco, USA) in a plastic dish. Cells that passed through the nylon mesh were washed twice with RPMI-1640. The erythrocytes were lysed with Tris-NH\(_4\)Cl and the remaining splenocytes were diluted to a final concentration of 2 \(\times\) 10\(^6\) cells/ml and cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Gibco), 2 mmol/L glutamine (Sigma, USA), 100 IU/ml penicillin and 100 \(\mu\)g/ml streptomycin. Apoptotic splenocytes, induced by ConA stimulation (5 \(\mu\)g/ml), were stained by annexin-V-fluorescein isothiocyanate (FITC) (BD Biosciences, USA) and sorted by flow cytometry (BD Biosciences).

DNA extraction and purification

According to our previously described method, genomic DNA from syngeneic apoptotic splenocytes were treated with S1 nuclease (Takara Bio, Shiga, Japan) and proteinase K (Sigma-Aldrich) and then purified using the DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The concentration of DNA was determined by the antibody absorbance (\(A\)) at 260 nm. The final A260/A280 for all of the DNA preparations was more than 1.8. Less than 0.01 U/\(\mu\)g of endotoxin was present in any of the DNA samples, based on a Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA).

Cell isolation

BALB/c mice were primed by subcutaneous injection under the dorsal skin with 0.2 ml of an emulsion containing 50 \(\mu\)g of ALD-DNA [dissolved in phosphate-buffered saline (PBS)] plus complete Freund’s adjuvant (CFA; Sigma). Seven days later, T cells, B cells and macrophages were sorted from the primed splenocytes by flow cytometry using anti-CD3, anti-CD19 and anti-F4/80 antibody respectively (all from eBioscience). Isolation of CD4\(^+\) and CD8\(^+\) T cells was performed by MACS according to the manufacturer’s instructions (Miltenyi Biotec). The purity of all these isolated cells was more than 95%.

In vivo depletion

In vivo depletion of CD3\(^+\), CD4\(^+\) and CD8\(^+\) T lymphocytes was achieved using monoclonal antibodies as previously described. Purified rat anti-mouse CD3 antibody (clone 17A2) was used for CD4 depletion. Rat anti-mouse antibody (clone YTS 191.1) was used for CD4 depletion, while rat anti-mouse antibody (clone YTS 169.4) was used for CD8 depletion. Control mice were treated with the non-immune rat IgG (Santa Cruz).

Adoptive cell transfer

Groups of nude mice were adoptively transferred with CD4\(^+\) or CD8\(^+\) T cells at the dose of 2 \(\times\) 10\(^7\) cells per mouse through intravenous injection respectively, followed by ALD-DNA immunization.
**Mice immunization**

Groups of mice \((n = 6)\) were subcutaneously injected under the dorsal skin with 0.2 ml of an emulsion containing 50 µg of ALD-DNA (dissolved in PBS) plus CFA. Mice receiving an equal volume of PBS plus CFA were used as controls. Mice were given two booster immunizations consisting of 50 µg DNA emulsified with incomplete Freund’s adjuvant at two-week intervals. Serum samples were collected every two weeks.

**Antibody measurement**

Anti-dsDNA antibodies were detected as previously described.\(^7,8\) Briefly, protamine sulfate pre-treated 96-well microtiter plates (Nunc, Denmark) were coated with 50 µg/ml calf thymus dsDNA (Sigma) for 2 h at 37°C and then placed overnight at 4°C. After washing three times with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 5% goat serum in PBST for 1 h, then 100-fold serially diluted (in PBST containing 10% calf serum and 5% goat serum) samples were added and incubated for 2 h at 37°C then with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Southern Biotech, USA). After color development by O-phenylenediamine (OPD; Sigma), the reaction was stopped by 2 M H₂SO₄ and absorbance at 492 nm was measured in a microplate reader (BioLantibody, USA).

**ELISA measurement of the proteinuria**

Proteinuria was measured with the Bradford assay using a commercially available kit (GeneRay Biotech, Shanghai, China), according to the manufacturer’s specification.

**Evaluation of the renal pathology**

Eight weeks after the first DNA immunization, histopathology assessment was done on 4 µm sections of paraaffin-embedded, formalin-fixed kidneys stained with hematoxylin and eosin. Glomerulonephritis was evaluated using light microscopy by two pathologists who were blinded to the study.

**Statistical analysis**

Statistical significance was assessed using Student’s \(t\)-test or Mann–Whitney \(U\)-test unless otherwise noted, and data are given as mean ± SD unless otherwise noted. Statistical analyses of data were performed using the GraphPad Prism (version 4.0) statistical program. \(p < 0.05\) was taken as significant.

**Results**

**ALD-DNA could induce anti-dsDNA antibodies in syngeneic BALB/c mice but not in nude mice**

To confirm the potential role of ALD-DNA in induction of anti-dsDNA antibodies, groups of BALB/c mice were immunized with ALD-DNA and the serological anti-dsDNA antibodies were assayed by ELISA. As shown in Figure 1(a), ALD-DNA effectively induced production of anti-dsDNA antibodies in syngeneic BALB/c mice. Furthermore, we showed the dose dependent manner of ALD-DNA induced production of anti-dsDNA antibodies (Figure 1(b)). We further showed that ALD-DNA primarily induced IgG anti-dsDNA antibodies but had no significant effect on IgM, IgA or IgE anti-dsDNA production (Figure 1(c)). Isotypic analysis of IgG anti-dsDNA antibodies revealed that the predominant IgG isotype was IgG1, with lower level of IgG2a and IgG2b detected. In contrast, the level of IgG3 anti-DNA was generally comparable to the background level (Figure 1(d)).

To evaluate the potential role of T cells in induction of anti-dsDNA antibodies, groups of BALB/c nude mice were immunized with ALD-DNA and then anti-dsDNA antibodies were determined. As shown in Figure 1(e), we found that ALD-DNA could not induce production of anti-dsDNA antibodies in nude mice, suggesting that T cells were required for production of anti-dsDNA antibodies induced by ALD-DNA.

**Depletion of T cells blocked the production of anti-dsDNA antibodies induced by ALD-DNA**

We further established an in vitro induction system to characterize the induction of anti-dsDNA antibody. Splenocytes were isolated from ALD-DNA primed mice and then co-cultured with an increasing dose of ALD-DNA for seven days. We demonstrated that the production of anti-dsDNA antibodies was positively correlated with the administered dose of ALD-DNA (Figure 2(a), \(r = 0.86, p < 0.05\)). The optimal dose of ALD-DNA to induce the production of anti-dsDNA antibodies in vitro was 50 µg/ml. Then 50 µg/ml of ALD-DNA was co-cultured with the primed splenocytes for nine days and the anti-dsDNA antibodies were assayed on day 1, day 3, day 5, day 7 and...
day 9. We found that the production of anti-dsDNA antibody had reached its peak on day 7 (Figure 2(b)).

To further address the potential role of T cells in ALD-DNA induced anti-dsDNA antibody, T cells were depleted from the primed splenocytes. We found that depletion of T cells from the splenocytes significantly abrogated the ALD-DNA induced production of anti-dsDNA antibodies in vitro (Figure 2(c)). To confirm this result, T cells, B cells and macrophages isolated from the primed splenocytes were co-cultured with ALD-DNA. As shown in Figure 2(d), we found that ALD-DNA failed to induce the production of anti-dsDNA antibodies without T cells, suggesting that T cells were indispensable for ALD-DNA to induce the production of anti-dsDNA antibody.

To further confirm the critical role of T cells in induction of anti-dsDNA antibodies, groups of BALB/c mice were treated with monoclonal antibodies against CD3 to deplete T cells in vivo and immunized with ALD-DNA. Confirmation of depletion of T cells was analyzed using FACS (data not shown). We found that in vivo depletion...
of T cells dramatically abrogated the generation of anti-dsDNA antibodies induced by ALD-DNA in syngeneic mice (Figure 2(e)).

**CD4^+ T cells but not CD8^+ T cells were indispensable for production of anti-dsDNA antibodies induced by ALD-DNA**

To further address which subset of T cells was responsible for the critical role in induction of anti-dsDNA antibodies, CD4^+ and CD8^+ T cell subsets were deprived from the ALD-DNA primed splenocytes. We found that deprivation of CD4^+ T cells dramatically abrogated the anti-dsDNA antibodies production induced by ALD-DNA in vitro, which could be restored by the re-administration of CD4^+ T cells (Figure 3(a), p < 0.05). In contrast, CD8^+ T cell subset had no significant effect on the ALD-DNA induced...
anti-dsDNA antibody responses (Figure 3(b)). To confirm this result, B cells were co-cultured with ALD-DNA treated macrophages with CD4⁺ or CD8⁺ T cells respectively. We found that CD4⁺ but not CD8⁺ T cells were required for ALD-DNA to induce the anti-dsDNA antibody response (Figure 3(c)).

To confirm the crucial role of CD4⁺ T cells in production of anti-dsDNA antibodies in vivo, groups of BALB/c mice were depleted of CD4⁺ or CD8⁺ T cell subsets in vivo respectively using monoclonal antibodies and then immunized with ALD-DNA. Confirmation of their depletion efficiency was analyzed using FACS (data not shown).

Figure 3  CD4⁺ T cells were responsible for the production of anti-dsDNA antibodies (Anti-dsDNA Ab). Primed splenocytes were deprived of CD4⁺ (a, *p = 0.011, **p = 0.0009) or CD8⁺ (b) T cells and cultured with 50 µg/ml of ALD-DNA for 7 days. (c) B cells were co-cultured with ALD-DNA treated macrophages in the presence of CD4⁺ or CD8⁺ T cells respectively for 7 days. *p = 0.018. (d) Groups of intact (control), CD4-depleted, and CD8-depleted BALB/c mice were immunized with ALD-DNA. Error bars indicate standard deviation of triplicate measurements.
We found that depletion of CD4⁺ but not the CD8⁺ subset dramatically abrogated the ALD-DNA induced production of anti-dsDNA antibodies in syngeneic BALB/c mice (Figure 3(e)).

Adoptive transfer of CD4⁺ T cells rescued the production of anti-dsDNA antibodies induced by ALD-DNA in nude mice

To confirm the crucial role of CD4⁺ T cells in generation of anti-dsDNA antibodies induced by ALD-DNA, groups of nude mice were adoptively transferred with CD4⁺ or CD8⁺ T cells and then immunized with ALD-DNA. We found that adoptive transfer of CD4⁺ but not CD8⁺ T cells rescued the ALD-DNA induced anti-dsDNA antibody response in nude mice (Figure 4(a)). We further analyzed the isotype of IgG anti-dsDNA antibodies and found the predominant IgG isotype was IgG1, IgG2a and IgG2b, which was similar to that in ALD-DNA immunized BALB/c mice (Figure 4(b)).

To further elucidate the role of CD4⁺ T cells in ALD-DNA induced SLE autoimmune disease, the urine protein and histopathology of kidney of ALD-DNA immunized nude mice which were adoptively transferred with CD4⁺ T cells were determined. As shown in Figure 4(c) and (d), we found that ALD-DNA failed to induce glomerulonephritis and proteinuria in nude mice, which could be rescued by adoptive transfer of CD4⁺ T cells. These results were consistent with the crucial role of T helpers in ALD-DNA induced generation of anti-dsDNA antibodies.
Discussion

SLE is an autoimmune disease characterized by typical involvement of many different organ systems and by immunological abnormalities, notably hyperactive B-cells producing various autoantibodies. A high level of serological IgG anti-dsDNA antibodies, which is closely correlated with the clinical syndrome and hence of diagnostic and even prognostic value, has been reported as the central pathogenic autoantibody involved in SLE pathogenesis.1–3,13 Here we formally demonstrated that CD4+ T cells conferred the production of anti-dsDNA antibodies in lupus induced by ALD-DNA.

In the present study, we confirmed our previous findings which demonstrated that ALD-DNA could induce the generation of anti-dsDNA antibodies.6–10 In fact, accumulating data has indicated the potential role of DNA in the induction of anti-dsDNA antibodies.14–16 An increasing body of data indicated that DNA was a complex macromolecule with immunological properties that have been both misconstrued and under-appreciated.17,18 In recent years, the conceptualization of DNA’s immunological properties has undergone a remarkable transformation as studies in human and murine systems have demonstrated conclusively that mammalian and bacterial DNA can both potently activate the immune system, which depends on DNA sequence, epigenetic modification, backbone structure and context, including the presence in immune complexes.17,19–21 As such, production of IFN-β in DNase II-null mice suggested that endogenous DNA that escaped DNA fragmentation could activate immune responses.22 A recent study also demonstrated that DNA hypomethylation of self-DNA from aged donors was the cause of increasing its immunogenicity, which resulted in its reaction to dendritic cells inducing the secretion of IFN-α.23

It is well acknowledged that T helper cells are critical for B cell-mediated humoral immune response which was characterized by the generation of IgG antibody induced by the protein based immunogens. However, little was known about the cellular mechanism underlying the induction of antibodies induced by the non-protein based immunogens. Here we conduct in vivo and in vitro experiments to further elucidate the potential role of T cells in production of anti-dsDNA antibodies induced by ALD-DNA. We demonstrated that ALD-DNA could induce the generation of anti-dsDNA antibodies in syngeneic BALB/c mice but not in nude mice. We then showed that depletion of CD3+ T cells blocked the induction of anti-dsDNA antibodies in BALB/c mice both in vivo and in vitro. These data suggested that T cells were indispensable for DNA to induce the anti-dsDNA antibodies. We then demonstrated that CD4+ T cells were responsible for the production of anti-dsDNA antibodies induced by ALD-DNA using the in vitro induction system. To further elucidate the potential role of T helper cells for DNA to induce the specific antibodies, CD4+ and CD8+ T cells were depleted in vivo using monoclonal antibodies as previously described. We found that depletion of CD4+ but not CD8+ T cells dramatically abrogated the ALD-DNA induced production of anti-dsDNA antibodies. Finally, we demonstrated that adoptive transfer of CD4+ T cells could rescue ALD-DNA induced production of anti-dsDNA antibodies in nude mice. All of these findings suggested that T helper cells were also required for DNA to induce the IgG specific antibodies. In addition, we also showed that T helpers were critical for ALD-DNA to induce the SLE autoimmune disease. We presumed that the ALD-DNA specific T helper cells might provide the cytokine environment for B cell differentiation and antibody class switch, or might be able to affect the inhibitory role of regulatory T cells, which ultimately accounted for the onset of SLE autoimmune syndrome. In fact, when the CD4+ T cells were co-cultured with ALD-DNA in the presence or absence of macrophages, we found that the ALD-DNA could effectively induce the proliferation of CD4+ T cells, which was dependent on the presence of macrophages (data not shown). These findings suggested that the processing and presentation of ALD-DNA by antigen presenting cells was required for the activation of CD4+ T cells induced by ALD-DNA. However, the precise mechanism about how CD4+ T cells recognize ALD-DNA and provide help for ALD-DNA to induce the antibodies still remains to be elucidated.

Our previous study has demonstrated that DNA hypomethylation was crucial for ALD-DNA to induce the anti-dsDNA antibodies,7 which was consistent with others’ studies.24–26 Recent findings showed that serological DNA–anti-DNA antibody complex in SLE patients and HMGB1-nucleosome complexes derived from apoptotic cells could effectively activate the immune system.27–31 Thus, we presumed that ALD-DNA might bind to DNA sensors to form a complex which in turn would induce the production of anti-dsDNA antibodies. However, whether there is a unique sequence or
backbone structure of the ALD-DNA and the molecular mechanisms underlying the activation of the immune system induced by ALD-DNA undoubtedly need successive studies.

To conclude, here we extended previous studies by providing direct evidence which demonstrated that CD4⁺ T cells were required for ALD-DNA to induce the anti-dsDNA antibody in lupus. These findings might further our understanding of the immunogenic properties of DNA and the pathogenesis of SLE.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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