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TLR7 modulates anti-nucleosomal autoantibody isotype and renal complement deposition in mice exposed to syngeneic late apoptotic cells

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Abstract

Objectives—The objectives of this study were to determine whether late apoptotic cell material directly induces autoantibodies characteristic of systemic lupus erythematosus (SLE) and to investigate the innate recognition pathways involved.

Methods—B6, B6.MyD88^{−/−}, B6.TLR7^{−/−} and B6.TLR9^{−/−} mice were subcutaneously injected with B6 syngeneic late apoptotic thymocytes (SLATs) without adjuvant on d0, 10, 24 and 37. Sera were tested for IgG antibodies to histones and dsDNA by ELISA and *Crithidia luciliae* indirect immunofluorescence. IgG and C3 deposition in kidney glomeruli was assessed by immunostaining and fluorescence microscopy.

Results—SLAT injections induced anti-dsDNA and anti-histone antibodies of the IgG1 and IgG2b isotypes in B6 but not MyD88^{−/−} mice. TLR7^{−/−} and TLR9^{−/−} mice injected with SLATs produced delayed or slightly more robust responses, respectively. SLAT injections induced IgG deposits in renal glomeruli of B6, TLR7^{−/−} and TLR9^{−/−} mice that were absent in MyD88^{−/−} mice. Unlike B6 and TLR9^{−/−} animals, TLR7^{−/−} mice failed to exhibit IgG co-localized glomerular C3 deposits and demonstrated autoantibodies of primarily the IgG2a isotype.

Conclusions—Late apoptotic cell-induced anti-histone and anti-dsDNA antibodies require MyD88 but not TLR9. Moreover, TLR7 promotes glomerular C3 deposition, possibly through a mechanism of altered antibody isotype switching.

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Keywords

apoptosis; autoantibody; Toll-like receptor; mouse; systemic lupus erythematosus

INTRODUCTION

Cells in early apoptosis are normally not immunogenic, are rapidly cleared from circulation [1,2] and do not induce autoantibody production [3]. Conversely, apoptotic cell clearance can be defective in SLE, resulting in accumulation of circulating nucleosomal material [4–6]. In addition, epitopes of some nuclear antigens targeted in SLE, including histones, become accessible on apoptotic cells [7,8]. Mice genetically defective in apoptotic cell clearance develop lupus-like autoimmunity [9–11] and cells undergoing necrosis can induce production of IgG anti-dsDNA antibodies in both lupus-prone and normal strains of mice [3].

Toll-like receptors (TLR) are innate immune sensors that recognize pathogen-associated molecular patterns and promote immune responses [12]. Nucleic acid binding is a shared characteristic amongst common lupus autoantibody targets. Selection of these targets may be related to triggering of endosomal nucleic acid-binding TLRs including TLRs 3, 7/8 and 9 which bind dsRNA, ssRNA and dsDNA, respectively [12,13]. Of these, only TLRs 7/8 and 9 require the myeloid differentiation adaptor protein MyD88 for signaling [14]. IgG antibodies complexed with chromatin [15,16] or RNA-associated antigens [17–19] trigger B cells and DC through TLR9- or TLR7-dependent pathways.

Autoantibodies to chromatin components were severely diminished or abrogated in MRL^{lpr/lpr} and MRL^{gld/gld} mice deficient in MyD88, suggesting that TLR stimulation is required for autoantibody production in these models [17]. TLR7-deficient MRL^{lpr/lpr} lupus mice lost production of antibodies to the RNA-binding Sm antigen and demonstrated ameliorated disease, while TLR9-deficient MRL^{lpr/lpr} mice lost production of anti-nucleosomal antibodies and experienced exacerbated disease [20]. However, these are models of defective, not excessive, apoptosis [21]. Therefore, innate immune sensors inducing anti-nucleosomal antibodies in response to late apoptotic cell stimuli remain unknown. Herein, we utilize a model specifically focused on early events initiating late apoptotic cell-induced autoantibody production. We show that syngeneic late apoptotic thymocytes (SLATs) induce IgG antibodies to histones and dsDNA through a MyD88-dependent mechanism. Unlike results from TLR-deficient MRL^{gld/gld} mice, TLR7 promoted but TLR9 dampened SLAT-induced autoantibodies to nucleosome components. Interestingly, this system revealed that TLR7 has profound influences on IgG isotype and renal complement deposition that may help explain how TLR7 contributes to initiation of lupus renal disease.

METHODS

Mice

Six wk old female C57BL/6J (B6; Jackson Laboratory, Bar Harbor, USA), MyD88^{-/-} [22], TLR9^{-/-} [23] and TLR7^{-/-} mice on the B6 background were maintained under pathogen-free barrier conditions. MyD88^{-/-} mice were backcrossed to B6 >12 generations and TLR7^{-/-} and TLR9^{-/-} mice ≥ 8 generations. All studies were approved by the OMRF IACUC.

Syngeneic late apoptotic thymocytes (SLATs)

Apoptotic thymocytes (≥65% Annexin V⁺ and ≥ 50% AnnexinV⁺PI⁺) were prepared by γ -irradiation and overnight culture as described [24]. Mice were injected with 4×10⁷ AnnexinV⁺ cells in PBS subcutaneously on d0, 10, 24 and 37.

Detection and isotyping of IgG anti-dsDNA and histone serum antibodies

Anti-dsDNA and anti-histone IgG was quantified by ELISA (Alpha Diagnostic, San Antonio, USA). *Crithidia luciliae* slides were from Inova Diagnostics Inc., San Diego, USA. In blocking experiments, 50 μ l aliquots of diluted sera were pre-incubated with purified genomic mouse DNA for 1.5h.

Antibodies in pooled sera were isotyped by ELISA with isotype-specific secondary antibodies conjugated to alkaline phosphatase (Southern Biotechnology Associates Inc., Birmingham, USA).

Immunofluorescent detection of endogenous renal IgG and Complement C3

Bisected kidneys were frozen in 50:50 OCT:TFM (Triangle Biosciences, Durham, USA) and fixed in buffered formalin. Cryosections were stained and evaluated for endogenous IgG and C3 complement as described [25].

Statistical Analysis

Non-parametric and parametric data were evaluated using Mann-Whitney and Student's t-tests, respectively.

RESULTS

SLAT-induced anti-DNA and anti-histone antibodies require MyD88

Because SLE patients produce high-titer, IgG antibodies to dsDNA and histones [26,27], we determined whether these specificities could be induced by injection of mice with SLATs. B6 and MyD88^{-/-} mice (n=5 mice/group) were injected with adjuvant-free SLATs on d0, 10, 24 and 37 and evaluated for production of IgG antibodies to nucleosome components. Anti-dsDNA IgG reactivities were significantly increased in serum samples of B6 mice at d28 and d42 but were unchanged at all time points in MyD88^{-/-} mice, indicating that MyD88 is imperative for anti-DNA antibody production (Fig 1A). *C. luciliae* indirect immunofluorescence revealed antibody binding predominantly at the kinetoplast rim in 3 of 5 (60%) B6 mice (Fig. 1B, left) that was inhibited by pre-incubation of sera with as low as 12.5 ng of genomic mouse DNA (Fig. 1B, left inset), suggesting anti-dsDNA specificity of low affinity. Kinetoplast binding was absent in all MyD88^{-/-} mice (0/5; Fig. 1B).

IgG antibodies to total histones were produced in SLAT-injected B6 mice at d42 and were significantly increased compared to MyD88^{-/-} mice at all time points (Fig. 1C). No antibodies to other extractable nuclear antigens were detected (data not shown). Thus, induction of anti-nucleosomal autoantibodies by SLATs requires MyD88.

SLAT-induced anti-dsDNA antibodies are promoted by TLR7

To determine whether MyD88-dependent SLAT-induced autoantibodies require either TLR9 or TLR7, TLR9^{-/-}, TLR7^{-/-} and B6 mice were injected with SLATs on d0, 10, 24 and 37 and evaluated for IgG antibodies to dsDNA and histones.

SLATs induced anti-dsDNA IgG antibodies in both TLR9^{-/-} and TLR7^{-/-} mice (Fig. 2A). Though anti-dsDNA ELISA reactivity in TLR9^{-/-} mice was equivalent to matched B6 controls at all time points (Fig. 2A, left), anti-dsDNA antibodies in TLR7^{-/-} mice were significantly lower than B6 controls at d42 (Fig. 2A, right).

C. luciliae kinetoplast rim staining was observed in 3 of 10 (30%) B6 mice (Fig. 2B, left) and 0 of 5 TLR7^{-/-} mice (Fig. 2B, right). Significantly, 3 of 5 (60%) TLR9^{-/-} mice demonstrated classic homogenous kinetoplast binding (Fig. 2B, center). All kinetoplast staining was

effectively blocked by pre-incubation of sera with murine genomic DNA (Fig. 2B, left and center insets). Thus, TLR7 promotes while TLR9 modestly inhibits anti-dsDNA antibodies in response to a defined late apoptotic cell stimulus.

Anti-dsDNA isotype analysis of d0 and d42 serum samples revealed that IgG1 and 2b isotypes comprised the responses of B6 and TLR9^{-/-} mice, whereas IgG2a antibodies predominated in TLR7^{-/-} mice (Fig. 2C). IgG2b anti-dsDNA antibody reactivity was increased in TLR9^{-/-} mice (≥ 3 -fold higher) compared to that of B6 or TLR7^{-/-} mice (Fig. 2C). Therefore, TLRs 7 and 9 profoundly influence SLAT-induced anti-dsDNA IgG isotypes.

Anti-histone IgG induced by SLATs is inhibited by TLR7 deficiency

Compared to baseline, anti-histone antibodies were significantly increased in B6 and TLR9^{-/-} mice at d28 and d42 (Fig. 3A, left), and in TLR7^{-/-} mice at d42 only (Fig. 3A, right). Moreover, anti-histone IgG was significantly reduced in TLR7^{-/-} compared to B6 controls at d28 and d42 (Fig. 3A, right). Thus, TLR7 contributes to the SLAT-induced anti-histone IgG response.

IgG1 and 2b isotypes comprised the anti-histone responses in B6 and TLR9^{-/-} groups, with IgG2b antibodies being increased 12-fold in d42 sera of TLR9^{-/-} mice (Fig. 3B). In contrast, TLR7^{-/-} mice demonstrated a 6-fold increase in anti-histone IgG2a at d42 compared to other groups (Fig. 3B).

TLR7 promotes glomerular C3 deposition in SLAT-injected mice

Because anti-histone and anti-dsDNA autoantibodies are implicated in SLE-associated renal pathogenicity [28–31], we determined whether mice injected with SLATs demonstrated evidence of renal immune complex deposition (Fig. 4A). B6, TLR9^{-/-} and TLR7^{-/-} mice all demonstrated glomerular IgG deposits of similar extent (Fig. 4B). However, severe glomerular C3 staining was abrogated in TLR7^{-/-} mice (Fig. 4C). Conversely, glomerular IgG deposits of all B6 and TLR9^{-/-} mice invariably co-localized with C3 (Fig. 4A). Unimmunized age- and sex- matched B6 control mice displayed no IgG or C3 deposition in renal sections (data not shown). Thus, TLR7 promotes antibody-associated complement deposition in renal glomeruli.

DISCUSSION

High titer IgG ANA is a unifying feature amongst patients with SLE. Although late apoptotic/secondarily necrotic cells promote autoantibodies in lupus, the innate immune signals initiating the autoimmune response to this stimulus are unclear. Previous studies in MRL^{lpr/lpr} mice do not address this question since these mice do not display apoptotic cell excess [21]. We show that subcutaneous delivery of excess SLATs without adjuvant results in IgG autoantibodies to dsDNA and histones in normal inbred mice, thus providing a model system for investigating innate immune pathways that contribute to loss of tolerance to late apoptotic cells.

Anti-dsDNA and anti-histone responses in SLAT-injected B6 mice required MyD88. In contrast to the reported abrogation of these responses in TLR9^{-/-} MRL^{lpr/lpr} mice [32], we demonstrated that TLR9 is, in fact, not required for production of anti-dsDNA or anti-histone IgG induced by SLATs. In this model, TLR9-deficiency significantly increased anti-nucleosomal IgG2b isotype antibodies (Figs. 2C and 3B) and enhanced anti-dsDNA antibody binding to *C. luciliae* kinetoplasts. The capacity of TLR9 to dampen nucleosomal autoantibody responses to SLAT insult highlights poorly understood immunoregulatory properties of this TLR.

Counter to responses in SLAT-injected TLR9^{-/-} mice and TLR7^{-/-} MRL^{lpr/lpr} mice [20], IgG responses to chromatin components were measurably reduced in TLR7^{-/-} animals, as

evidenced by a kinetic delay in the production of anti-histone antibodies, lower anti-histone antibody reactivity, and the absence of antibody binding to *C. luciliae* kinetoplasts.

HMGB1, uric acid and heat shock proteins have been proposed to trigger inflammation to secondarily necrotic cells through TLRs 2 and 4 [33]. However, we failed to detect NF κ B activation in HEK293 cells transfected with murine TLR2, 4 or 5 upon incubation with SLAT preparations *in vitro* (data not shown). The IL-1 and IL-18 receptors (R) also require MyD88 for signaling [34]. We observed that IL-1R-deficient mice injected with SLATs produced levels of anti-nucleosomal IgG that were indistinguishable from contemporary B6 controls (data not shown), ruling out involvement of the IL-1R in this model. IL-18R involvement in the generation of ANA or anti-dsDNA antibodies was absent in two different models of murine lupus [35,36]. Our studies suggest that more than one TLR or an as yet undiscovered MyD88-dependent pathway may contribute to induction of anti-nucleosomal antibodies in response to SLATs.

B6 and TLR9^{-/-} mice demonstrated IgG and complement deposition in renal glomeruli in response to SLAT injection. The invariable association of glomerular IgG and C3 in these mice is consistent with IgG2b complement-fixing antibody isotypes detected in the immune sera of these two strains. Conversely, SLAT-injected TLR7^{-/-} mice had high levels of IgG2a anti-dsDNA and anti-histone antibodies and displayed no glomerular C3 deposition in renal sections, indicating a critical role for TLR7 in promoting C3 deposition in renal glomeruli following SLAT injection. Because TLR7 is not expressed in renal tissue [37] and these mice lacked renal infiltrates (not shown), these findings are consistent with influences of TLR7 on isotype switching and constitute new mechanistic insight into the initiation of renal inflammation by TLR7.

In summary, SLATs induce autoantibodies to chromatin constituents in normal mice in a MyD88-dependent fashion. TLR9 played no essential role in anti-dsDNA antibody production in this model, whereas TLR7 promoted anti-nucleosomal antibodies and renal complement deposition, a feature that reflected TLR7 influence on antibody isotype. These data shed light on the role of TLR7 in lupus autoantibody production and associated renal complement deposition.

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Figure 1. SLAT-induced anti-dsDNA and anti-histone responses in B6 and MyD88-deficient mice IgG anti-dsDNA (A) and anti-histone (C) ELISA reactivity using 1:100 preimmune (d0) and immune (d28 and d42) serum dilutions from individual mice. Bars represent median values. B) Representative IgG *Crithidia luciliae* indirect immunofluorescence using 1:20 serum dilutions. Fractions represent the number of mice with positive kinetoplast binding of the total number tested. DNA inhibition confirmed anti-dsDNA specificity in animals with kinetoplast rim staining (inset). * $p < 0.05$ and ** $p \leq 0.01$.

Figure 2. SLAT-induced anti-dsDNA responses in B6, TLR9- and TLR7-deficient mice
 A) IgG anti-dsDNA ELISA reactivity of sera from individual mice. Bars represent median values. B) Representative *Crithidia luciliae* IgG anti-dsDNA binding specificity using sera diluted 1:20. Other designations, including insets are as in Fig. 1C) IgG isotypes of anti-DNA antibodies as determined by ELISA using pools of pre-immune (d0) and post-immune (d42) sera. * $p < 0.05$ and ** $p \leq 0.01$.

Figure 3. SLAT-induced anti-histone responses in B6, TLR9- and TLR7-deficient mice
 A) IgG anti-histone ELISA reactivity of sera from individual mice. Bars represent median values. B) IgG isotypes of anti-histone antibodies as determined by ELISA using pools of pre-immune (d0) and post-immune (d42) sera. * $p < 0.05$ and ** $p \leq 0.01$.

Figure 4. Immunofluorescent detection of IgG and complement C3 in kidney sections of SLAT-injected B6, TLR9- and TLR7-deficient mice

A) Representative individual and composite images for IgG (Alexa546, red), C3 (FITC, green), and nuclei (DAPI, blue). B) Individual scores and median percentages of glomeruli exhibiting IgG deposition scored as zero-mild (■), moderate (●) and severe (◆). C) Individual scores and median percentages of glomeruli exhibiting C3 deposition scored as zero-mild (■), moderate (●) and severe (◆).