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Caspase-1 is required for maintenance of marginal zone B cells in pristane-induced lupus

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Abstract

Objective—Caspase-1 is required for nephritis and robust autoantibody development in the pristane model of murine lupus. The objective of this study was to evaluate the immune response and to study the splenic B and T cell populations in wild-type (WT) and caspase-1 $-/-$ mice following pristane injection in order to develop an understanding of why absence of caspase-1 is protective in pristane-induced lupus.

Methods—Immunization responses to NP-Ficoll and NP-Ovalbumin were assessed in WT and caspase-1 $-/-$ mice. *In vitro* IgM and IgG responses to R848 was measured by ELISA. Serum IgM anti-double stranded DNA and IL-1 β was measured by ELISA. B and T cell populations 2 weeks and 6 months following pristane injection were measured by flow cytometry in WT and caspase-1 $-/-$ mice.

Results—Caspase-1 $-/-$ mice generate equivalent IgG responses to NP-Ficoll and NP-Ova antigens when compared to wild-type mice. Additionally, they secrete IgM and IgG in response to TLR7 activation. Pristane injected WT and caspase-1 $-/-$ mice generate robust IgM anti-dsDNA responses. Caspase-1 $-/-$ mice have a significant reduction in marginal zone B cell populations compared to WT 6 months after pristane exposure whereas T cell responses are intact in these mice.

Conclusions—Caspase-1 $-/-$ mice have intact immune responses but do not develop an expanded marginal zone B cell population in response to pristane-induced lupus. This may be one explanation for reduced IgG autoantibody production in these mice.

Keywords

Caspase-1; lupus; B-cell; autoantibody

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune syndrome affecting 72-128/100,000 individuals in the US ¹. A hallmark of SLE is the development of autoantibodies which recognize nuclear components such as double-stranded DNA. These autoantibodies participate in the formation of immune complexes, which deposit in tissues such as the kidney, and cause severe organ damage ².

Recently, a role for the NLRP3 inflammasome has been described in SLE (reviewed in³). Activation of this protein complex results in cleavage and activation of IL-1 β and IL-18⁴. Our group recently showed that in the pristane model of murine lupus, there is an essential role for caspase-1, the key enzyme within the inflammasome complex, for the development of immune complex-mediated nephritis⁵. Importantly, a reduction in IgG autoantibodies recognizing double stranded DNA (dsDNA) was noted in caspase-1 $-/-$ mice exposed to pristane when compared to control. Other groups have also shown that inflammasome inhibition can improve autoantibody profiles in genetic lupus models^{6, 7}, suggesting that activation of the inflammasome contributes to autoantibody development. In this manuscript, we used the pristane model of murine lupus to examine the effects of caspase-1 deficiency on B and T cell populations.

Methods

Mice

All murine studies were reviewed and approved by the University of Michigan's committee on use and care of animals. Caspase 1 $-/-$ mice were generated in the lab of Dr. Richard Flavell, Yale University, and were obtained as a kind gift of Dr. Noriko Tsuji, National Institute of Advanced Industrial Science and Technology, Japan. The mice were backcrossed onto the BALB/c background for at least 8 generations prior to use and bred at the University of Michigan. Wild-type (WT) BALB/c mice were acquired from Harlan and Jackson Laboratory.

Pristane injection

Eight to ten week-old mice were given a single intraperitoneal injection with either 0.5 ml sterile phosphate buffered saline (PBS) as a control or 0.5 ml sterile pristane (Sigma-Aldrich). Mice were euthanized 2 weeks or 6 months post injection.

Immunizations

Eight to ten week old BALB/c and Caspase 1 $-/-$ mice were vaccinated intraperitoneally with an emulsion of 50% V/V Incomplete Freund's adjuvant (Sigma-Aldrich) and either: 100ul PBS, 100ug 4-Hydroxy-3-nitrophenylacetyl (NP)-Ficoll (Biosearch Technologies), or 100ug NP-Ovalbumin (Biosearch Technologies). Serum was collected by saphenous vein bleed at weekly intervals.

ELISA

anti-dsDNA IgM, total IgM and total IgG antibodies were quantified with commercially available kits (Alpha Diagnostic) using manufacturer protocols. NP-specific IgG antibodies were detected by ELISA with 4ug/ml plate bound NP-BSA (Bovine Serum Albumin), ratio > 20 (Biosearch Technologies). Serum IL-1 β was measured via ELISA (E-bioscience).

B cell isolation and culture

B cells from pooled mouse spleens (n=5/group) were purified by negative selection with the EasySep Mouse B cell Isolation Kit (Stem Cell Technologies) and cultured in RPMI 1640 with 10% FCS 50 IU/ml penicillin/50ug/ml streptomycin (Life Technologies) at 1×10^6 cells/well. Cultured B cells were stimulated with the TLR7/8 agonist R848 (Enzo Life Sciences) at 1ug/ml. Four days after stimulation, supernatants were harvested for total IgG and IgM ELISAs.

Flow cytometry. (Surface Stain, Intracellular cytokine)

Samples were analyzed on an LSR II (BD Biosciences). Data was analyzed with FlowJo V10 software (Tree Star). The following antibodies were used: Pe/Cy7-conjugated anti-CD19 (6d5), FITC-conjugated anti-CD21/CD35 (7E9), PE-conjugated and Pacific Blue-conjugated anti-CD23 (B3B4), PE-conjugated anti-CD138 (281-2), APC-conjugated anti-CD45R/B220 (RA3-6B2), APC-conjugated anti-CD3 (17A2), were from Biolegend. FITC-conjugated anti-CD4 (GK1.5), and FITC-conjugated anti-CD8a (53-6.7) were from BD biosciences. For intracellular cytokine assays, splenocytes were incubated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience) or control splenocytes were treated with Brefeldin A Solution (Biolegend) for four or 16 hours at 37°C with 5% CO₂. Following antibody staining, splenocytes were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) prior to labeling with PE-conjugated anti-IL-17A (TC11-18H10.1) (eBioscience).

Statistics.

Mean differences between populations were compared by two-tailed Student's unpaired *t*-test using Prism Software version 6.01 (GraphPad).

Results

Previously, our observations had indicated that following pristane injection, caspase-1 $-/-$ mice had significantly abrogated anti-dsDNA IgG responses when compared to WT mice⁵. To determine whether this lack of response indicated a deficiency in the general caspase-1 $-/-$ B cell antibody response to antigens, we examined vaccine responses to T-independent (NP-Ficoll) and T-dependent (NP-ovalbumin) antigens. Incomplete Freund's adjuvant was used as it does not stimulate the inflammasome as part of activating the immune response⁸. As shown in Figure 1A, WT and caspase-1 $-/-$ mice generate comparable IgG antibody responses to T-independent and T-dependent foreign antigens. These results indicate that caspase 1 is not required for B cell production of IgG in a T independent manner and that B and T cells do not require caspase-1 to mount IgG responses to protein antigens.

Generation of pristane-induced lupus is dependent on TLR7 signals⁹, so we next examined B cell responses to R848, a TLR7 ligand. Figure 1B demonstrates that caspase-1 $-/-$ CD19+ cells generated a robust and two-fold greater IgM response *in vitro* following TLR7 stimulation when compared to WT, but both WT and caspase-1 $-/-$ mice generated equivalent IgG responses. As we had detected decreased IgG dsDNA responses in caspase-1 $-/-$ mice *in vivo*⁵, we next examined whether dsDNA IgM antibody formation was similarly affected in pristane exposed mice. Caspase-1 $-/-$ mice produced slightly more (albeit non-significantly more) IgM dsDNA 6 months after pristane exposure when compared to WT mice (Figure 1C). These data suggest that while caspase-1 $-/-$ B cells respond to TLR7 agonists as well as or better than WT B cells, dsDNA autoantibody generation *in vivo* following pristane injection requires caspase-1 for robust generation of IgG but not IgM dsDNA antibodies.

In order to better understand which properties of the B cell compartment may contribute to anti-ds-DNA IgG responses following pristane exposure, we examined splenic B cell populations at two weeks and 6 months post pristane exposure. The two week time point has been used previously to understand early responses to pristane^{10, 11}, and we and others have shown that WT mice develop lupus-like phenotypes, including induction of type I interferon responses, vascular damage, and increased glomerular hypercellularity and immune complex deposition, by 6 months post pristane exposure^{5, 9, 12}. All of these phenotypes are abrogated in caspase-1 $-/-$ mice⁵. The generation of IL-18⁵ and IL-1 β (Figure 1D) following pristane injection is also reduced in caspase-1 $-/-$ mice. As shown in Figure 2, the number of CD19+ cells was slightly but significantly decreased in caspase-1 $-/-$ mice two weeks after pristane exposure. However the number of follicular or marginal zone B cell did not differ significantly in WT or caspase-1 $-/-$ mice two weeks following pristane injection. Plasma cell populations were infrequent and remained thus in the spleen at two weeks. At 6 months post pristane exposure the number of total B cells, follicular B cells, and plasma cells did not differ between WT and caspase-1 $-/-$ mice. Surprisingly, the number of marginal zone B cells was significantly decreased in caspase-1 $-/-$ at this time point. These data suggest that loss of marginal zone B cells, which are expanded in lupus mice and are known to secrete anti-dsDNA antibodies¹³ may contribute to decreased anti-dsDNA IgG production following pristane exposure in caspase-1-deficient mice.

T cell populations were also examined. As shown in figure 3, pristane induced a short-term decline in splenic CD4 and CD8 T cell populations in WT mice and a significantly greater decline in CD4 populations in caspase-1 $-/-$ mice; however, by 6 months, no significant differences existed between PBS and pristane-exposed mice for WT or caspase-1 $-/-$ populations (Figure 3A-D). Th17 populations were also examined as serum [IL-17] is decreased in pristane-exposed caspase-1 $-/-$ mice⁵. As shown in Figure 3E, caspase-1 $-/-$ mice had slightly more Th17 cells at baseline than WT, but upon exposure to pristane, both WT and caspase-1 $-/-$ had equivalent Th17 populations.

Discussion

Several murine models have now demonstrated an important role of the inflammasome in lupus, including the development of nephritis and IgG anti-dsDNA antibodies⁵⁻⁷. Here, we

report that caspase-1 is not required for non-dsDNA antibody responses to T-dependent and T-independent antigens. We also found that caspase-1 is not required for the development of IgM anti-dsDNA antibodies, but is necessary for the maintenance, and possible expansion, of the marginal zone B cell population following pristane exposure.

Marginal zone B cells express high levels of TLRs and polyreactive B-cell receptors which allow them to generate antibody responses to circulating products in a rapid fashion¹⁴. This population is expanded in lupus-prone mice and secretes anti-dsDNA antibodies¹³. Stimulation of marginal zone B cells by TLRs, including TLR7, results in plasmablast differentiation and IgM production as well as generation of class-switched IgG responses¹⁵. Our data detects a trend for expansion of marginal zone B cells following long-term pristane exposure, consistent with a role of these cells in pristane-induced lupus. However, this expansion is absent in caspase-1 $-/-$ mice, which may explain the decrease in IgG dsDNA antibodies following pristane exposure.

The mechanism by which caspase-1 is required for marginal zone B cell expansion may be explained by its activation of IL-18. Recently, a role for IL-18 in proliferation and class switching of the marginal zone B cell population was identified¹⁶. We had previously shown that IL-18 is increased in WT, but not caspase-1 $-/-$ mice following pristane injection, consistent with the role of caspase-1 in IL-18 activation⁵. Thus, absence of IL-18 activation may explain the dramatic decrease in marginal zone B cell populations in caspase-1 $-/-$ mice following lupus induction with pristane and serve as a possible explanation for reduced production of anti-dsDNA antibodies. Alternatively, the reduction of IL-18 and IL-1 β production in marginal zone dendritic cell and macrophage cells in caspase-1 $-/-$ mice may negatively impact B cell proliferation and maturation. Further research should explore the role of the various factors that expand and promote class-switching of marginal zone B cell populations in lupus.

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Figure 1. Caspase-1^{-/-} mice have normal vaccine responses, synthesize IgG and IgM in response to TLR7 agonist and have robust generation of dsDNA IgM antibodies in vivo

A. BALB/c and caspase-1^{-/-} mice were immunized with Incomplete Freund's adjuvant and either: PBS, NP (4-Hydroxy-3-nitrophenylacetyl)-Ficoll (T cell independent), or NP-Ovalbumin (T cell dependent). Sera were harvested at 1, 2, 3, and 4 weeks post inoculation, and antibody responses were determined by ELISA. The graphs represent relative NP specific antibody levels in sera diluted at 1:400 from one to four weeks post immunization. Bars show the mean $\pm$ Standard Deviation. **B.** B cells from spleens of 8-10 week old untreated female mice were stimulated with media or R848 for 72 hours. Culture supernatants were analyzed for total IgM or IgG secretion by ELISA. **C and D.** Six months post inoculation with pristane or PBS, sera were collected from BALB/c and caspase-1^{-/-} mice, and anti-double-stranded DNA IgM antibody titers (C) were and IL-1 β concentrations were measured by ELISA. Each symbol represents an individual mouse. Bars show the mean $\pm$ Standard Deviation. *= $p < 0.05$ using unpaired one-tailed Student's t-test.

**= $p < 0.01$ using unpaired two-tailed Student's t-test.

Figure 2. Caspase-1^{-/-} mice show decreased expansion of Marginal Zone B cells in response to pristane

Lymphocytes isolated from spleens after PBS or pristane injection of female BALB/c and caspase-1^{-/-} mice were stained with anti-CD19, anti-CD21, and anti-CD23 or anti-B220 and anti-CD138. Shown are the frequency of B cells (CD19⁺), follicular (CD19⁺, CD23⁺), marginal zone (CD19⁺, CD21⁺), and plasma cells (B220⁺, CD138⁺) per 10,000 splenocytes. Spleens were analyzed at two weeks and six months post injection. Each symbol on the graph represents findings from one mouse spleen. * = $p < 0.05$ ** = $p < 0.01$ using an unpaired two-tailed Student's *t*-Test.

Figure 3. Pristane induces a transient decrease in the frequency of spleen T cells, and stimulates a chronic Th17 bias independent of caspase-1

Lymphocytes from the spleens of two week (A, C) and six month (B,D) pristane injected female BALB/c and caspase-1^{-/-} mice were stained with anti-CD3, anti-CD4, and anti-CD8. Shown are the frequency of T cells per 10,000 splenocytes. (E) Lymphocytes from six month pristane injected mice were stimulated with PMA and ionomycin or media with Brefeldin A prior to labeling with anti-CD3, anti-CD4, and anti-IL17A. Numbers in the flow charts show percentages of IL-17A positive CD4 T cells from spleens. Data shown for surface staining at the two week time point is comprehensive of five experiments, and data from the six month time point is comprehensive of seven experiments. Intracellular staining for IL-17A is representative of four experiments. * = $p < 0.05$ **** = $p < 0.0001$ using an unpaired two-tailed Student's *t*-Test.