Toxoplasma gondii infection inhibits the development of lupus-like syndrome in autoimmune (New Zealand Black × New Zealand White) F1 mice

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of autoantibodies and lupus nephritis. In the present study using New Zealand Black (NZB) × New Zealand White (NZW) F1 (NZBW F1) mice, we planned to investigate the effects of *Toxoplasma gondii* infection on the progress of lupus nephritis. Female NZBW F1 mice at the age of 2 months were perorally infected with *T. gondii*. The *T. gondii* infection reduced the number of mice developing proteinuria and immune complex deposits in their kidneys and prolonged their life span. A marked decrease in the levels of IgM and IgG anti-DNA antibodies, especially IgG2a and IgG3 subclasses, was observed in *T. gondii*-infected NZBW F1 mice at 9 months of age. The level of anti-HSP70 IgG autoantibody in the sera of NZBW F1 mice was significantly higher than that in control mice at 9 weeks after *T. gondii* infection. Moreover, NZBW F1 mice treated with anti-self heat shock protein 70 (HSP70) monoclonal antibody were substantially protected against the onset of glomerulonephritis. Further, down-regulation of intracellular expression of IFN-γ and IL-10 was shown in spleen cells of *T. gondii*-infected NZBW F1 mice. This was consistent with the previous data indicating the involvement of Th1-type and Th2-type cytokines in the development of lupus-like nephritis. These results suggest that *T. gondii* infection is capable of preventing the development of autoimmune renal disorder in NZBW F1 mice.

Introduction

Toxoplasma gondii is among the most prevalent chronic parasitic infections in humans, and toxoplasmosis has emerged as an important opportunistic pathogen in immunocompromised patients. Cellular immunity is essential for protection against *T. gondii* infection (1,2). On the other hand, we have demonstrated the production of anti-*T. gondii* heat shock protein 70 (TgHSP70) antibody cross-reactive to self HSP70, and anti-HSP70 autoantibody was seen to deteriorate the host defense against *T. gondii* infection (3,4). Mooveover, it was earlier demonstrated that VH1-JH1 B-1 cells are responsible for anti-HSP70 autoantibody formation in *T. gondii*-infected BALB/c (a resistant strain) and C57BL/6 mice (B6 mice, a susceptible strain) (4).

Systemic lupus erythematosus (SLE) is a disease of generalized autoimmunity characterized by B cell hyperactivity, autoantibody production against self-antigens, in particular antibodies to nuclear antigens and DNA, and immune complex deposition in vital organs leading to glomerular nephropathies and proteinuria (5). NZB × NZW F1 (NZBW F1) mice develop a systemic autoimmune disease characterized by lupus nephritis, vasculitis, lymphadenopathy and splenomegaly, closely resembling human SLE (5,6,7). Beginning at the age of 3–4 months, autoantibodies, including anti-DNA antibodies, are developed in these mice and, by 7–8 months, immune complex deposits form in the kidneys. They die of the disease within 1 year (7,8).

The precise etiology of SLE is not yet understood, but genetic, hormonal, environmental and immunoregulatory factors, as well as infectious agents, are thought to contribute to the manifestation of the disease (9–11). Several groups have reported that viral infections might lead to exacerbation of autoimmune diseases (12,13), although the precise mechanisms remain unclear. Lloyd *et al.* (14) demonstrated that malaria is accompanied by the production of a number of autoantibodies, including some that react with DNA, and DNA-reactive antibodies in malarial infection have the potential to participate in the formation of immune deposits in nephritic malarial kidneys. Conversely, other groups have reported that malaria infection of NZBW F1 mice, one of the best models for SLE animals, retarded the development of their autoimmune disease (15–17).

The aim of this study was to examine the effect of *T. gondii* infection on the development of glomerulonephritis in NZBW F1 mice. We report here that the pathogenic isotypes (IgG2a and IgG3) of the anti-DNA antibodies in the serum of *T. gondii*-infected NZBW F1 mice were significantly reduced and the intracellular expression of Th1-type cytokine (IFN-γ) and Th2-type cytokine (IL-10) in the spleen cells of *T. gondii*-infected NZBW F1 mice was markedly diminished. Additionally, NZBW F1 mice injected with anti-HSP70 autoantibody showed significant retardation of the development of glomerulonephritis. An ameliorating effect of *T. gondii* infection on lupus-like syndrome in NZBW F1 mice was observed.

Methods

Mice and T. gondii strain

Eight-week-old female C57BL/6 (B6, susceptible strain), BALB/c (resistant strain), NZB, NZW and NZBW F1 mice were purchased from SLC (Hamamatsu, Japan). B6, BALB/c, NZB, NZW and NZBW F1 mice were perorally infected with 10 *T. gondii* cysts of Fukaya strain as previously described (3,18).

Monoclonal antibody (mAb) production, purification and immunization

Non-cross-reactive anti-TgHSP70 mAb (TgNCR C2, IgG2a) and cross-reactive anti-HSP70 autoantibody (TgCR 20, IgG3) were established as previously described (3). TgNCR C2 and TgCR 20 hybridoma clones were injected in pristine primed BALB/c mice, respectively. TgNCR C2 and TgCR 20 mAbs were purified from mouse ascites on protein G–Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. NZBW F1 mice were intraperitoneally immunized with 100 µg TgNCR C2 mAb or 100 µg TgCR 20 mAb twice a month from the age of 2 months. Similarly, NZBW F1 mice were intraperitoneally treated with an isotype-matched control mAb, designated GL 113 (anti-β-galactosidase) (IgG2a, Promega, Madison, WI). At the age of 8 months, protein concentrations in urine, anti-dsDNA antibody formation in the sera of immunized mice and kidney sections were analyzed.

Proteinuria, histopathology and immunofluorescence

Protein concentration in urine was evaluated semiquantitatively using albumin reagent strips (Hema-Combistix; Bayer/

Sankyo, Tokyo, Japan) on a monthly basis. Mice with proteinuria > 100 mg/dl were scored as positive.

Renal lesions were graded in a blinded manner (19). For light microscopy, kidneys of NZBW F1 mice uninfected and *T. gondii*-infected at 2 months of age were fixed in 4% paraformaldehyde, and 4-µm paraffin sections were stained with periodic acid Schiff (PAS) and H & E at the age of 9 months. Glomerular lesions were graded as follows: 1+ = mild focal mesangial hypercellularity alone; 2+ = moderate mesangial hypercellularity; 3+ = complex endocapillary hypercellularity sometimes with mild sclerosis or necrosis; 4+ = severe endocapillary hypercellularity glomerulonephritis with necrosis or crescent formation. Scores ⩾ 1+ were positive.

To detect deposition of immune complexes at glomeruli, frozen sections were incubated for 30 min at room temperature with FITC-labelled goat antibodies to mouse IgM, IgG, or C3 (ICN Pharmaceuticals, Costa Mesa, CA).

Measurement of antibodies

B6, BALB/c, NZB, NZW and NZBW F1 mice were perorally infected with 10 *T. gondii* cysts at the age of 2 months as described above. The levels of IgM and IgG anti-ssDNA antibody and anti-dsDNA antibody in the sera of uninfected and *T. gondii*-infected B6, BALB/c, NZB, NZW and NZBW F1 mice at the age of 9 months were determined by ELISA as described previously (20,21). Briefly, wells of ELISA plates (Sumitomo Bakelite Co. Ltd, Tokyo, Japan) were coated for 2 h with 10 µg/ml ssDNA prepared from calf thymus DNA (Sigma Chemical Co., St Louis, MO) or dsDNA [poly(dA-dT)/poly(dA-dT); Sigma]. After washing with PBS and 0.05% Tween 20, plates were blocked with 2% BSA in PBS. 1:200 diluted sera from uninfected and *T. gondii*-infected B6, BALB/c, NZB, NZW and NZBW F1 mice at the same age of 9 months were added for 2 h. After washing with PBS–Tween, plates were overlaid with alkaline phosphatase-conjugated anti-mouse IgM antibody (Tago, Camarillo, CA) or anti-mouse IgG antibody (Chemicon International Inc., Temecula, CA) diluted 1:1000 for 2 h at room temperature. Absorbance at OD_{405} nm was measured. The levels of IgG subclasses with dsDNA in the sera were determined by coating 10 µg/ml dsDNA (Sigma) as described above, using alkaline phosphatase-conjugated rat anti-mouse IgG subclass-specific secondary antibodies (X56, anti-IgG1; R19–15, anti-IgG2a; R12-3, anti-IgG2b; R40-82, anti-IgG3; all from PharMingen, Heidelberg, Germany).

To detect the production of anti-TgHSP70 antibody and anti-HSP70 autoantibody in the sera of *T. gondii*-infected B6, BALB/c, NZB, NZW and NZBW F1 mice, mice were bled via the tail at 2 and 9 weeks post-infection. Sera were collected, and the levels of anti-TgHSP70 antibody and anti-HSP70 autoantibody were tested by ELISA using rTgHSP70 and r mouse (m) HSP70 as described previously (3,4,22). Alkaline phosphatase-conjugated anti-mouse IgM antibody (Tago) and anti-mouse IgG antibody (Chemicon International Inc.) were used as second antibodies.

Detection of intracellular IL-10, IL-4, IFN-γ and IL-12

NZBW F1 mice were perorally infected with 10 cysts of *T. gondii* Fukaya strain at the age of 2 months. At 9 months of age, spleen cells and peritoneal exudate cells (PECs) from

uninfected and *T. gondii*-infected NZBW F1 mice were harvested. Then, the cells were fixed and permeabilized with a Cytofix/Cytoperm kit (PharMingen, San Diego, CA) and stained with 0.1 μg of FITC-conjugated rat anti-mouse IL-10 mAb (JES5-16E3; PharMingen), FITC-conjugated rat anti-mouse IL-4 mAb (BVD-24G2; Immunotech, France), FITC-conjugated rat anti-mouse IFN-γ mAb (XMG1.2; PharMingen), and PE-conjugated rat anti-mouse IL-12 (p40/p70) mAb (C15.6; PharMingen), respectively. Finally, the cells were washed with the permeabilization buffer, and the expressions of IL-10, IL-4, IFN-γ and IL-12 were analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Anti-dsDNA antibody production by splenic B cells from NZBW F1 mice

B220+ cells from spleen of NZBW F1 mice at the age of 2 months were obtained by magnetic cell separation (Miltenyi Biotec, Auburn, CA) as previously described (3,4). The purity of these cells was >98%. To investigate the correlation between anti-HSP70 autoantibody and anti-DNA antibody, splenic B cells were stimulated by LPS (15 μg/ml, Sigma Chemical Co., St Louis, MO) and IFN-α (5 U/ml, PharMingen, San Diego, CA) as previously described (23) without or with anti-HSP70 autoantibody (TgCR 18 mAb, IgM) at 1 μg/ml or 10 μg/ml (3). TgCR 18 mAb was purified from mouse ascites as described above. Similarly, splenic B cells were stimulated by LPS and IFN-γ with isotype-matched control mAb, designated J5/D (IgM) (Promega). After 5 days of incubation, the supernatant was collected and the level of anti-dsDNA IgM antibody or anti-dsDNA IgG antibody was assayed using ELISA as described above.

Statistical analysis

Differences between mean values were analyzed by unpaired Student's *t*-test. *P*-values < 0.05 were considered significant.

Results

Effects of T. gondii infection on survival rate and proteinuria in NZBW F1 mice

As shown in Fig. 1(A), uninfected NZBW F1 mice began to die at the age of 7 months, and by the age of 10 months, 100% of the mice had died. Conversely, 100% of *T. gondii*-infected NZBW F1 survived beyond 9 months of age, and by 10 months 5% had died. The incidence of proteinuria in NZBW F1 mice gradually increased from the age of 5 months without *T. gondii* infection (Fig. 1B), and then increased sharply from 7 months. Proteinuria was not detected in *T. gondii*-infected NZBW F1 mice at the age of 7 months, but from the age of 7.5 months a low and slightly increasing incidence became evident.

Retardation of development of glomerulonephritis in T. gondii-infected NZBW F1 mice

At 9 months, there was marked mesangial cell proliferation and an increase in the mesangial matrix in glomeruli, with concomitant glomerular capillary wall thickening, resulting in obliteration of capillary lumina in uninfected NZBW F1
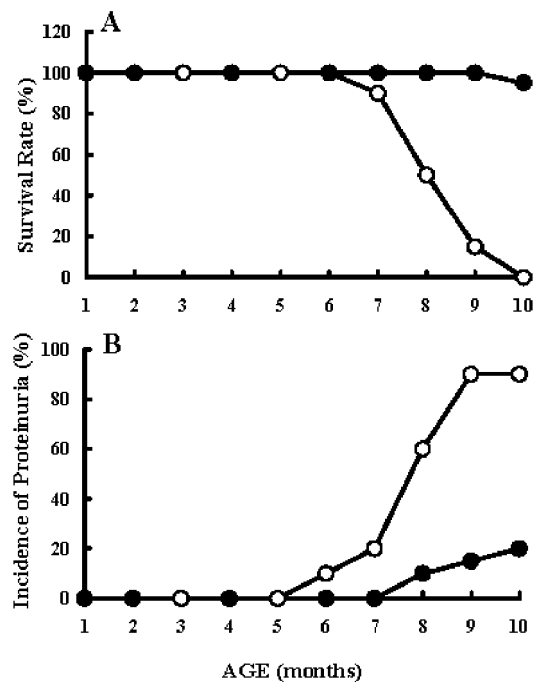

Fig. 1. Effects of *T. gondii* infection on survival and proteinuria in NZBW F1 mice. NZBW F1 mice were perorally infected with 10 *T. gondii* cysts at the age of 2 months as described in Methods. (A) Cumulative mortality in uninfected (open circles) and *T. gondii*-infected (closed circles) NZBW F1 mice. (B) Development of lupus nephritis was analyzed by proteinuria > 100 mg/dl. Twelve uninfected NZBW F1 mice and 20 *T. gondii*-infected NZBW F1 mice were utilized in the experiment.

mice (Fig. 2A and B). In some cases, wire loop lesions, sclerosis, crescent formation and adhesion of the glomerular tuft to Bowman's capsule were noted (grade 4+). However, these changes were less prominent in *T. gondii*-infected NZBW F1 mice (grade 2) (Fig. 2F and G). To verify the development of lupus nephritis in uninfected and *T. gondii*-infected NZBW F1 mice, we examined glomerular deposits of IgM, IgG and C3. IgM (Fig. 2H), IgG (Fig. 2I) and C3 (Fig. 2J) deposits in the glomeruli were suppressed by *T. gondii* infection in NZBW F1 mice at the age of 9 months, as compared with their respective counterparts (Fig. 2C–E) in uninfected NZBW F1 mice. These findings revealed the amelioration of glomerulonephritis in the *T. gondii*-infected NZBW F1 mice at the age of 9 months.

Effect of T. gondii infection on anti-ssDNA antibody and anti-dsDNA antibody formation in NZBW F1 mice

Although 100% of NZB mice succumbed 3 weeks after *T. gondii* infection and 100% of *T. gondii*-infected NZW mice survived more than 10 months (data not shown), 95% of lupus-prone NZBW F1 mice survived more than 10 months after *T. gondii* infection. High levels of IgM (Fig. 3A) and IgG (Fig. 3B) anti-ssDNA antibodies in the sera of uninfected NZB, NZW and NZBW F1 mice were observed
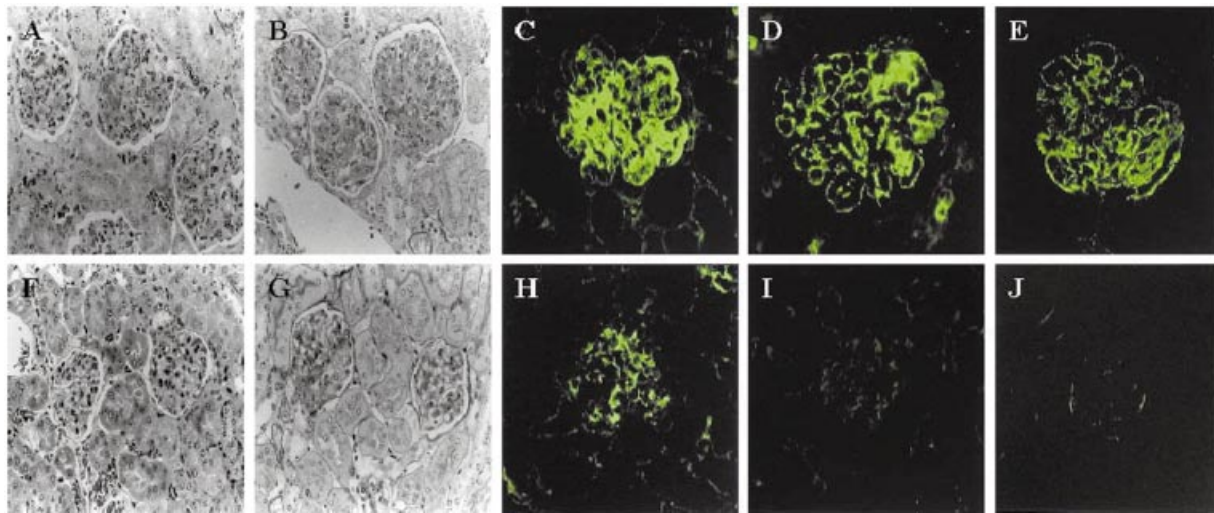
Fig. 2. Renal histopathology and immunofluorescence staining. NZBW F1 mice were perorally infected with 10 *T. gondii* cysts at the age of 2 months. Sections of kidney from uninfected and *T. gondii*-infected NZBW F1 mice were analyzed at the age of 9 months. (A) A kidney section of uninfected NZBW F1 mice subjected to staining with H & E (original magnification, ×350). (B) A kidney section of uninfected NZBW F1 mice subjected to staining with PAS. (original magnification, ×350). (C–E) Sections of kidney from uninfected NZBW F1 mice subjected to immunofluorescence staining with anti-mouse IgM (C), anti-mouse IgG (D), and anti-mouse C3 antibody (E) (original magnification, ×400). (F) A kidney section of *T. gondii*-infected NZBW F1 mice subjected to staining with H & E (original magnification, ×350). (G) A kidney section of *T. gondii*-infected NZBW F1 mice subjected to staining with PAS (original magnification, ×350). (H–J) Sections of kidney from *T. gondii*-infected NZBW F1 mice subjected to immunofluorescence staining with anti-mouse IgM (H), anti-mouse IgG (I), and anti-mouse C3 antibody (J) (original magnification, ×400).
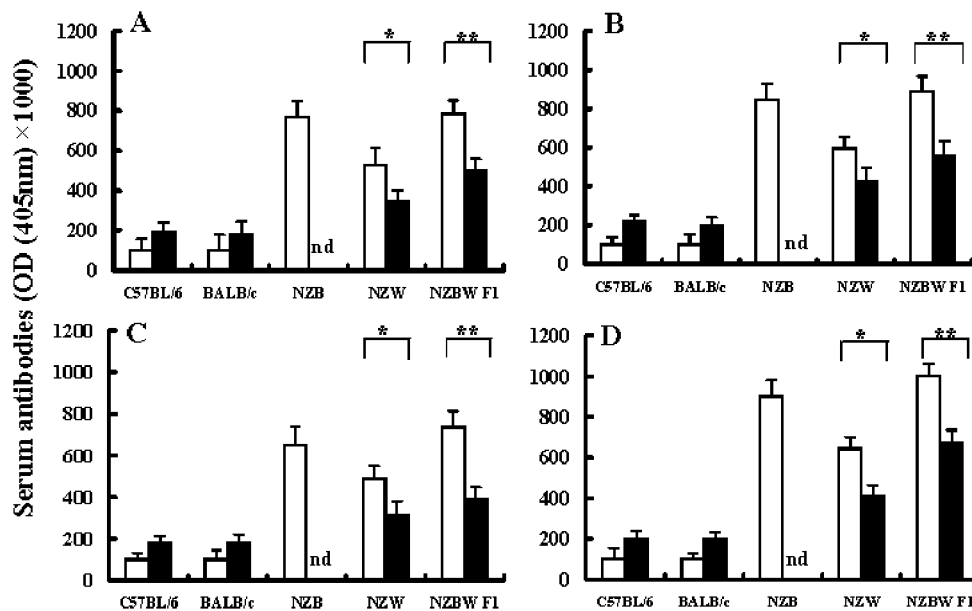

Fig. 3. Anti-ssDNA antibody and anti-dsDNA antibody production in the sera of uninfected and *T. gondii*-infected NZBW F1 mice and other control strains at the same age of 9 months. Mice were perorally infected with *T. gondii* at the age of 2 months as described above. The production of IgM (A) and IgG (B) anti-ssDNA antibody from uninfected (open squares) and *T. gondii*-infected (closed squares) mice was measured by ELISA. The formation of IgM (C) and IgG (D) anti-dsDNA antibody from uninfected and *T. gondii*-infected mice was analyzed as previously described. The mean ± SD of 12 uninfected and 20 *T. gondii*-infected mice of each strain are presented. *$P < 0.05$; **$P < 0.01$ compared with the sera from uninfected mice. nd, no data.

at the age of 9 months. At this age, their levels in the sera of *T. gondii*-infected NZW and NZBW F1 mice were significantly lower compared with those of uninfected NZW and NZBW F1 mice. Similarly, the levels of IgM (Fig. 3C) and IgG (Fig. 3D) anti-dsDNA antibodies in the sera of uninfected NZB, NZW and NZBW F1 mice were high, but their formation in the sera of NZW and NZBW F1 mice after *T. gondii* infection was inhibited.

Reduced production of IgG1, IgG2a and IgG3 anti-dsDNA antibodies in *T. gondii*-infected NZBW F1 mice

To determine whether the IgG subclass pattern of anti-dsDNA antibodies was altered in *T. gondii*-infected NZBW F1 mice, sera from NZBW F1 mice uninfected or infected with *T. gondii* at the age of 2 months were examined at the age of 9 months. Although the level of IgG2b anti-dsDNA antibody was in a similar range in the sera of uninfected and *T. gondii*-infected NZBW F1 mice (Fig. 4C), the levels of IgG1 (Fig. 4A), IgG2a (Fig. 4B) and IgG3 (Fig. 4D) of *T. gondii*-infected NZBW F1 mice were decreased, and especially the formation of IgG2a and IgG3 was significantly reduced.

Anti-HSP70 autoantibody formation in *T. gondii*-infected NZBW F1 mice

The level of anti-TgHSP70 IgM antibody had increased significantly in the sera of B6, BALB/c, NZW and NZBW F1 mice at 9 weeks after *T. gondii* infection (Fig. 5A). Similarly, the level of anti-TgHSP70 IgG antibody had also markedly increased in B6, BALB/c, NZW and NZBW F1 mice at 9 weeks post-infection. The level of anti-TgHSP70 IgG antibody in the sera of *T. gondii*-infected NZBW F1 mice was significantly higher than that in *T. gondii*-infected B6, BALB/c NZW mice (Fig. 5B). Anti-HSP70 IgM (Fig. 5C) and IgG (Fig. 5D) autoantibodies in the sera of B6, BALB/c, NZB, NZW and NZBW F1 mice were analyzed 9 weeks after *T. gondii* infection. The sera levels of anti-HSP70 IgM autoantibody and anti-HSP70 IgG autoantibody were increased in NZBW F1 mice and the control strains after infection. The level of anti-HSP70 IgG autoantibody in the sera of NZBW F1 mice was significantly higher than that in control mice at 9 weeks after *T. gondii* infection.

Intracellular cytokine expression in spleen cells and PECs from uninfected and *T. gondii*-infected NZBW F1 mice

In order to determine the influence of *T. gondii* infection in Th1 and Th2 subsets of NZBW F1 mice, NZBW F1 mice were perorally infected with *T. gondii* at the age of 2 months. The expressions of IL-10, IL-4, IFN-γ and IL-12 in spleen cells and PECs from uninfected and *T. gondii*-infected NZBW F1 mice were observed at the age of 9 months (Fig. 6). High levels of IL-10 and IFN-γ expression were detected in the spleen of uninfected NZBW F1 mice, but their expressions in *T. gondii*-infected NZBW F1 mice were significantly decreased. The expression of IL-4 in spleen cells of uninfected and *T. gondii*-infected NZBW F1 mice was low and unchanged, respectively. Low expressions of IL-10 and IL-4 in PECs of uninfected and *T. gondii*-infected NZBW F1 mice were observed. IFN-γ in PECs of uninfected NZBW F1 mice was expressed at a high level, whereas that in *T. gondii*-infected NZBW F1 mice was markedly decreased. In this study, the expression of IL-12 was not detected in PECs from either uninfected or *T. gondii*-infected NZBW F1 mice at the age of 9 months.

Ameliorating effect on lupus-like syndrome in Tg CR 20 mAb immunized NZBW F1 mice

In order to further understand the relationship between high levels of anti-HSP70 autoantibody in *T. gondii*-infected NZBW F1 mice and the suppression of lupus-like syndrome in NZBW
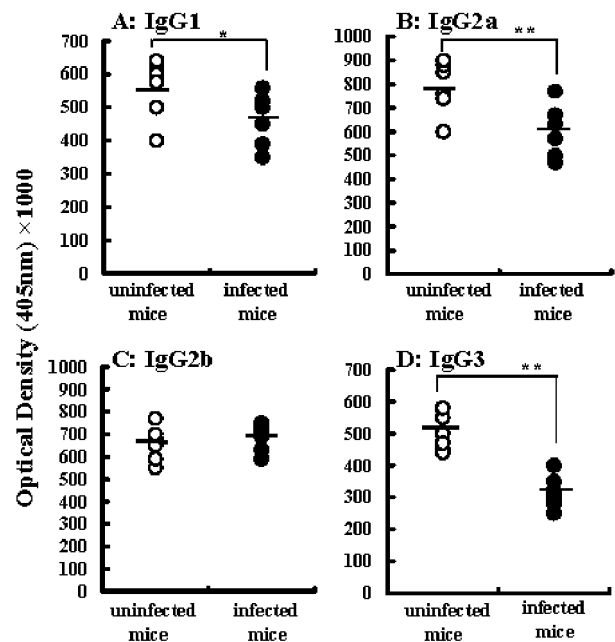

Fig. 4. Analysis of anti-dsDNA antibody IgG subclass formation in the sera of uninfected and *T. gondii*-infected NZBW F1 mice at the age of 9 months. NZBW F1 mice were perorally infected with *T. gondii* at the age of 2 months. The production of IgG1 (A), IgG2a (B), IgG2b (C) and IgG3 (D) antibodies in the sera of uninfected (open circles) and *T. gondii*-infected (closed circles) NZBW F1 mice was detected by ELISA. Six mice of each group were observed in the experiment. Horizontal lines are the mean for each group. *$P <$ 0.05; **$P <$ 0.01 compared with the sera from uninfected NZBW F1 mice.

F1 mice, NZBW F1 mice were administered with Tg NCR C2 mAb or Tg CR 20 mAb from the age of 2 months. 100% NZBW F1 mice treated with Tg NCR C2 mAb were observed with severe proteinuria (>100 mg/dl) at the age of 8 months. In contrast, the percentage of severe proteinuria in NZBW F1 mice immunized with Tg CR 20 mAb from the age of 2 months was low (Fig. 7A). The production of anti-dsDNA antibody in the sera of Tg CR 20 mAb-injected NZBW F1 mice was significantly decreased when compared with those of Tg NCR C2 mAb-injected NZBW F1 mice (Fig. 7B). Moreover, glomerular deposits of IgG (Fig. 7C) were suppressed in NZBW F1 mice immunized with Tg CR 20 mAb. Glomerular deposits of IgG from NZBW F1 mice immunized with Tg NCR C2 mAb did not show any reduction at the age of 8 months (Fig. 7C). The ameliorating influence on glomerulonephritis was not detected in NZBW F1 mice treated with isotype-matched control mAb (data not shown). In order to examine the effect of anti-HSP70 autoantibody on anti-DNA antibody formation by B cells from NZBW F1 mice, an *in vitro* culture method of anti-DNA antibody production of NZBW F1 splenic B cells stimulated with LPS and IFN-γ was utilized. The level of anti-dsDNA IgM antibody was not changed in the culture supernatant from B cells stimulated with LPS and IFN-γ plus TgCR 18 mAb, compared with that from B cells stimulated with LPS and IFN-γ plus isotype-matched control mAb (J5/D) (Fig. 8). The levels of anti-dsDNA IgG antibody were low. Thus,
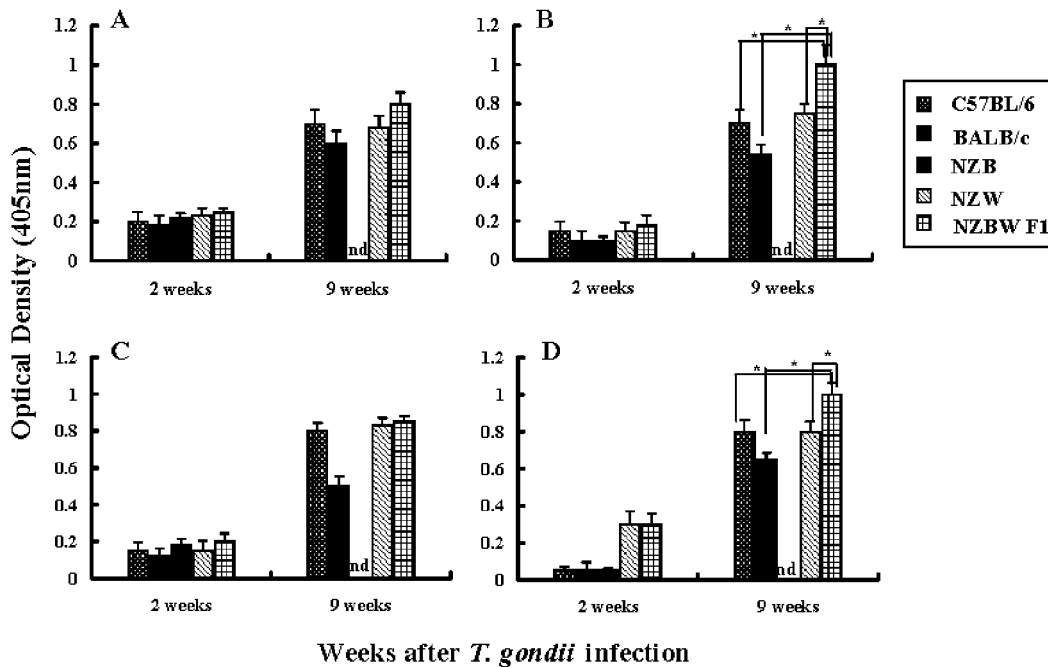
Fig. 5. Anti-HSP70 autoantibody formation in *T. gondii*-infected NZBW F1 mice. (A and B) Isotype specificity of anti-TgHSP70 antibody generated in *T. gondii*-infected mice. NZBW F1 mice and other control strains were perorally infected with 10 *T. gondii* cysts of the Fukaya strain. Production of anti-TgHSP70 IgM antibody (A) and anti-TgHSP70 IgG antibody (B) in the sera of NZBW F1 mice and other control mice was detected by ELISA as described above at 2 and 9 weeks after *T. gondii* infection. (C and D) Isotype specificity of anti-HSP70 autoantibody produced in *T. gondii*-infected mice. NZBW F1 mice and other control strains were perorally infected as described above. Production of anti-HSP70 IgM autoantibody (C) and anti-HSP70 IgG autoantibody (D) in the sera of NZBW F1 mice and other control mice was detected by ELISA. Data represent mean ± SD of three to five mice in three separate experiments. $*P < 0.05$ compared with sera from other control strains. nd, no data.

anti-HSP70 autoantibody did not affect anti-dsDNA antibody production directly.

Discussion

In the present study, we clearly demonstrated that *T. gondii* infection was sufficient to significantly protect against severe lupus nephritis in NZBW F1 mice. Autoantibodies of the IgG class and particularly IgG anti-DNA antibodies are believed to play a major role in the pathogenesis of lupus nephritis (23,25). It has been reported that the levels of circulating and glomerular deposited IgG3 were associated with glomerulonephritis in the MRL-Fas[lpr] mouse model of SLE (26), and complement-fixed IgG2a anti-DNA antibodies were demonstrated to be predominant nephritogenic autoantibodies in NZBW F1 mice (27). Moreover, several groups recently revealed that the reduction in anti-DNA antibody, especially in pathogenic IgG2a and IgG3, prevented autoimmune renal injury and increased survival in NZBW F1 mice (28,29). The present data were consistent with the previous data indicating correlation between the down-regulation of IgG2a and IgG3 anti-DNA antibodies and the amelioration of glomerulonephritis in NZBW F1 mice (12,14).

Cytokines have been suggested to play an important role in the immune dysregulation observed in lupus-prone mice and SLE patients (30,31). Peng *et al.* (32) reported that Th1 and Th2 cells were equally important in SLE, whereas Hasegawa

et al. (33) recently demonstrated that IFN-γ but not IL-4 contributed to the development of lupus in NZBW F1 mice. Although IL-12 is a potent regulator of Th1 and Th2 subset development, defective IL-12 production in NZBW F1 mice and SLE patients has been reported (34,35). An amplification loop consisting of B cells and Th cells as a primary event in lupus pathogenesis was proposed (36). In that loop, autoreactive B cells function as antigen-presenting cells and promote T cell activation. Activated T cells provide further help for B cells, and each cycle might further amplify autoreactive B and T cell clones. Although the treatment of NZBW F1 mice with anti-IFN-γ mAb (37) or anti-IL 10 mAb (38) substantially delayed the onset of glomerulonephritis, the basis of cytokine-mediated protection has not yet been elucidated. One possible mechanism of anti-IL-10-mediated protection of NZBW F1 mice against the onset of autoimmunity suggested a protective role for TNF-α in this disease (39,40). Moreover, TNF-α significantly decreased IFN-γ-induced upregulation of MHC class II expression on different mature cells collected from NZBW F1 mice (41). However, there have been some reports that long-term administration of cytokines caused suppression of cell-mediated immunity in NZBW F1 mice (42). Therefore, the long-term toxicity of cytokine treatment remained to be revealed. In the present study, we found that *T. gondii* infection significantly reduced the intracellular expression of Th1 cytokine IFN-γ in spleen cells from NZBW F1 mice at the age of 9 months. At the age of 7 or 8 months, the
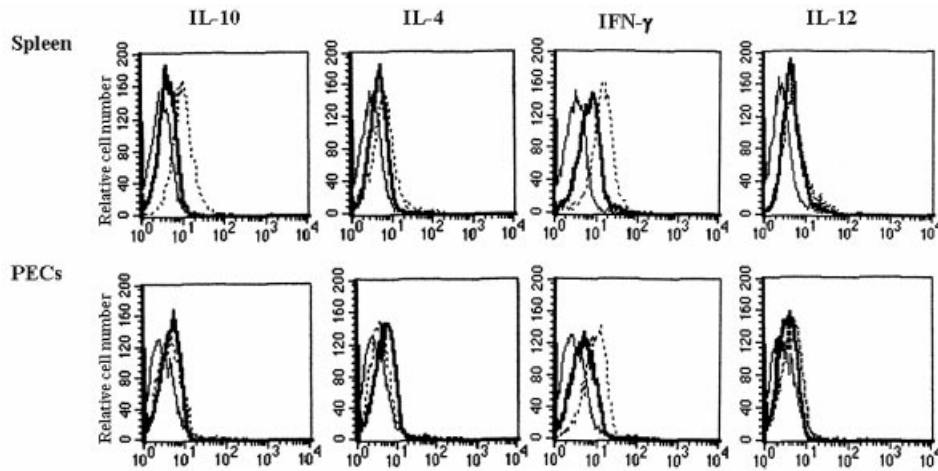
Fig. 6. Intracellular expression of IL-10, IL-4, IFN-γ and IL-12 in spleen cells and PECs from uninfected (dotted line) and *T. gondii*-infected (bold line) NZBW F1 mice was detected by FACS analysis at the age of 9 months. *T. gondii*-infected mice were perorally infected at the age of 2 months as described. The thin lines depict the isotype control-strain cells. Representative results from three independent experiments are shown.
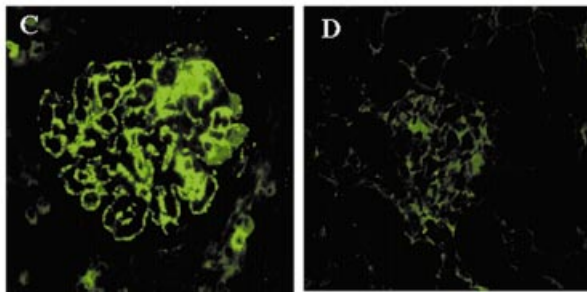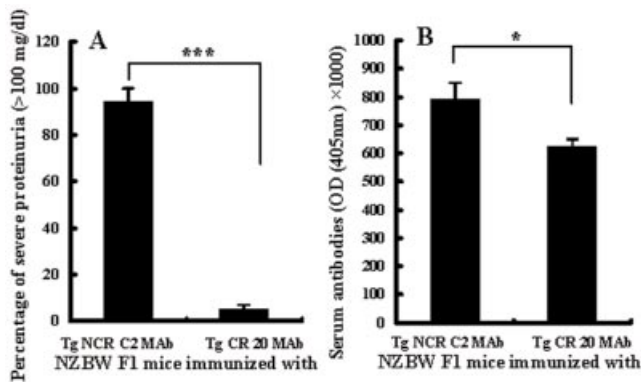

Fig. 7. Effects of TgNCR C2 mAb and TgCR 20 mAb on proteinuria, anti-dsDNA antibody formation and renal immunofluorescence staining in NZBW F1 mice at the age of 8 months. (A) Cumulative incidence of severe (>100 mg/dl) proteinuria in Tg NCR C2 mAb (*n* = 20) and Tg CR 20 mAb (*n* = 20) injected NZBW F1 mice at the age of 8 months. ***P < 0.005. (B) The production of anti-dsDNA antibody in the sera of Tg NCR C2 mAb (*n* = 20)-injected and Tg CR 20 mAb (*n* = 20)-injected NZBW F1 mice was detected by ELISA as described in Fig. 3. *P < 0.05. (C and D) Sections of kidney from TgNCR C2 mAb (C) and TgCR 20 mAb (D) immunized NZBW F1 mice subjected to immunofluorescence staining with anti-mouse IgG (original magnification, ×400).

Fig. 8. Effects of anti-HSP70 autoantibody on anti-DNA antibody formation *in vitro*. Splenic B cells from NZBW F1 mice at the age of 2 months was purified and stimulated with LPS and IFN-γ plus TgCR 18 mAb as described in Methods. The levels of anti-dsDNA IgM antibody (closed squares) and anti-dsDNA IgG antibody (open squares) were detected by ELISA. Data represent mean ± SD of three to five mice in three separate experiments.

cytokine production pattern in *T. gondii*-infected NZBW F1 mice was observed to be similar to those at 9 months (data not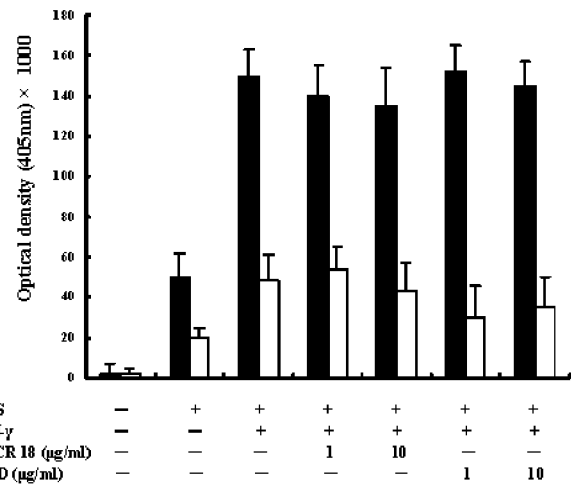 shown). IFN-γ has been reported to be a cytokine of central importance in murine lupus by promoting the switch of IgM and IgG2a subclass, leading to glomerular injury, and by inducing apoptosis of renal parenchymal cells (43). Thus, *T. gondii* infection inhibited IFN-γ expression, leading to reduced IgG2a anti-DNA antibody formation and IgG2a deposition in glomeruli, resulting in the amelioration of lupus nephritis in NZBW F1 mice. On the other hand, the intracellular expression of IL-10, a Th2 cytokine, significantly decreased in *T. gondii*-infected NZBW F1 mice. Ishida *et al.* (38) reported that the administration of anti-IL-10 antibody delayed the onset of autoimmunity in NZBW F1 mice via inhibition of autoantibody production. Therefore, we postulated that pathogenic T cells were involved in the reduction of anti-DNA antibody formation by *T. gondii* infection in NZBW F1 mice.

The complexity of cytokine regulation and the imbalance of Th1 and Th2 subsets in SLE animal models and human SLE are well recognized (38,44–48). As previously described, *T. gondii* infection up-regulated IFN-γ production in wild-type mice (4,49). However, the mechanisms of how *T. gondii* infection in NZBW F1 mice suppresses both Th1 and Th2 responses are not clear. Several groups previously reported that lactic dehydrogenase virus (LDV)-infected NZBW F1 mice exhibited reduction of class II antigen expression on macrophages (50), and that the APC activities of macrophages were suppressed by LDV infection (51). Others and we have previously shown that *T. gondii* down-regulates MHC class II gene expression and antigen presentation to CD4+ T lymphocytes in extracerebral antigen-presenting cells (49,52–55). Lüder *et al.* (56) indicated that *T. gondii* considerably inhibits the IFN-γ-induced MHC class II expression in rat astrocytes and microglial cells at the level of gene transcription, since down-regulation coincided with reduced transcript levels of the most critical transacting factor of class II transcription CIITA. Inappropriate MHC class II expression by endogenous APC of the CNS contributes to the pathogenesis of autoimmune disorders, e.g. multiple sclerosis or experimental allergic encephalomyelitis (57). Hence, impaired APC function may be one of the factors responsible for the suppression of both Th1 and Th2 differentiations in *T. gondii*-infected NZBW F1 mice.

HSP is a family of ubiquitous and phylogenically highly conserved proteins (58). Autoreactivity to HSP is often associated with autoimmune pathology (59). Anti-HSP70 autoantibody produced by VH1-JH1 B-1 cells was detected in *T. gondii*-infected BALB/c and B6 mice (3,4). Additionally, Hayakawa *et al.* (60) previously reported that autoantibody-producing B cells were easily detected in the peritoneal cavity as CD5+ B cells in autoimmune-prone NZBW F1 mice. Our previous study showed that a high level of anti-heat shock cognate protein (HSC) 71 autoantibody was detected in sera from patients with Vogt-Koyanagi–Harada disease (VKH), a systemic disorder that affects various organs and is believed to be an autoimmune disease (61). A high level of anti-HSP70 IgG autoantibody in the sera of NZBW F1 mice was observed after *T. gondii* infection. In order to clarify the relationship between anti-HSP70 autoantibody and lupus-like syndrome, NZBW F1 mice were treated with anti-HSP70 autoantibody from the age of 2 months twice a month. At the age of 8 months, NZBW F1 mice showed significant retardation of the development of glomerulonephritis. Therefore, anti-HSP70 autoantibody formation in *T. gondii*-infected NZBW F1 mice was another important factor contributing to retardation of the development of glomerulonephritis. The direct involvement of self-HSP70 molecules in the pathogenesis of SLE disease remains to be further revealed. One possibility is that anti-HSP70 autoantibody may block the high expression of MHC class II gene induced by HSP70. Down-regulation of antigen presentation to CD4+ T lymphocytes may contribute to the amelioration of glomerulonephritis in NZBW F1 mice. Taken together, these results indicated that *T. gondii* infection inhibited the development of lupus-like syndrome of NZBW F1 mice. Not only reducing the production of IFN-γ and IL-10 but also anti-HSP70 autoantibody formation contributed to retardation of the development of glomerulonephritis in

T. gondii-infected NZBW F1 mice. This is the first report regarding the favorable effect of *T. gondii* infection on glomerulonephritis of NZBW F1 mice.

Abbreviations

B6	C57BL/6
CR	cross-reactive
HSP70	heat shock protein 70
HSC71	heat shock cognate protein 71
LDV	lactic dehydrogenase virus
NCR	non-cross-reactive
NZB	New Zealand Black
NZW	New Zealand White
NZBW F1	(New Zealand Black × New Zealand White) F1
PAS	periodic acid Schiff
PEC	peritoneal exudate cell
TgHSP70	*Toxoplasma gondii* HSP70
VKH	Vogt–Koyanagi–Harada disease

References

1 Suzuki, Y., Sher, A., Yap, G., Park, D., Neyer, L. E., Liesenfeld, O., Fort, M., Kang, H. and Gufwoli, E. 2000. IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with *Toxoplasma gondii*. *J. Immunol.* 164:5375.

2 Denkers, E. Y., Gazzinelli, R. T., Hieny, S., Caspar, P. and Sher, A. 1993. Bone marrow macrophages process exogenous *Toxoplasma gondii* polypeptides for recognition by parasite-specific cytolytic T lymphocytes. *J. Immunol.* 150:517.

3 Chen, M., Aosai, F., Mun, H. S., Norose, K., Hata, H. and Yano, A. 2000. Anti-HSP70 autoantibody formation by B-1 cells in *Toxoplasma gondii*-infected mice. *Infect. Immun.* 68:4893.

4 Chen, M., Aosai, F., Norose, K., Mun, H. S. and Yano, A. 2003. The role of anti-HSP70 autoantibody-forming VH1-JH1 B-1 cells in *Toxoplasma gondii*-infected mice. *Int. Immunol.* 15:39.

5 Theofilopoulos, A. N. and Dixon, F. J. 1985. Murine models of systemic lupus erythematosus. *Adv. Immunol.* 37:269.

6 Howie, J. B. and Helyer, B. J. 1968. The immunology and pathology of NZB mice. *Adv. Immunol.* 9:215.

7 Theofilopoulos, A. N. 1992. Murine models of lupus. In Lahita, R. G., ed., *Systemic Lupus Erythematosus*, p. 121. Churchill Livingston, New York.

8 Mozes, E. 1999. Experiment systemic lupus erythematosus: From a pathogenic autoantibody to immunomodulating peptides. In Shoenfeld, Y., ed., *The Decade of Autoimmunity*, p. 109. Elsevier Science, Amsterdam, The Netherlands.

9 Cooper, G. S., Dooley, M. A., Treadwell, E. L., St Clair, E. W., Parks, C. G. and Gilkeson, G. S. 1998. Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosus. *Arthritis Rheum.* 41:1714.

10 Lahita, R. G. 1999. The role of sex hormones in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* 11:352.

11 Wakeland, E. K., Liu, K., Graham, R. R. and Behrens, T. W. 2001. Delineating the genetic basis of systemic lupus erythematosus. *Immunity* 15:397.

12 Van-Ghelue, M., Moens, U., Bendiksen, S. and Rekvig, O. P. 2003. Autoimmunity to nucleosomes related to viral infection: a focus on hapten-carrier complex formation. *J. Autoimmun.* 20:171.

13 Adelman, M. K. and Marchalonis, J. J. 2002. Endogenous

retroviruses in systemic lupus erythematosus: candidate lupus viruses. *Clin. Immunol.* 102:107.

14 Lloyd, C. M., Collins, I., Belcher, A. J., Manuelpillai, N., Wozencraft, A. O. and Staines, N. A. 1994. Characterization and pathological significance of monoclonal DNA-binding antibodies from mice with experimental malaria infection. *Infect. Immun.* 62:1982.

15 Hentati, B., Sato, M. N., Payelle-Brogard, B. S., Avrameas, S. and Ternynck, T. 1994. Beneficial effect of polyclonal immunoglobulins from malaria-infected BALB/c mice on the lupus-like syndrome of (NZB × NZW) F1 mice. *Eur. J. Immunol.* 24:8.

16 Greenwood, B. M., Herrick, E. M. and Voller, A. 1970. Suppression of autoimmune disease in NZB and (NZB × NZW) F1 hybrid mice by infection with malaria. *Nature* 226:266.

17 Wellmann, U., Letz, M., Schneider, A., Amann, K. and Winkler, T. H. 2001. An Ig μ-heavy chain transgene inhibits systemic lupus erythematosus immunopathology in autoimmune (NZB × NZW) F1 mice. *Int. Immunol.* 13:1461.

18 Luo, W., Aosai, F., Ueda, M., Yamashita, K., Shimizu, K., Sekiya, S. and Yano, A. 1997. Kinetics in parasite abundance in susceptible and resistant mice infected with an avirulent strain of *Toxoplasma gondii* by using quantitative competitive PCR. *J. Parasitol.* 83:1070.

19 Richards, H. B., Satoh, M., Jennette, J. C., Okano, T., Kanwar, Y. S. and Reeves, W. H. 1999. Disparate T cell requirements of two subsets of lupus-specific autoantibodies in pristane-treated mice. *Clin. Exp. Immunol.* 115:547.

20 Shao, D. Z., Yamada, S., Hirayama, F., Hirano, H., Ono, S. and Hamaoka, T. 1995. Modulation of B-cell abnormalities in lupus-prone (NZB × NZW) F1 mice by normal bone marrow-derived B-lineage cells. *Immunology* 85:16.

21 Okubo, T., Hagiwara, E., Ohno, S., Tsuji, T., Ihata, A., Ueda, A., Shirai, A., Aoki, I., Okuda, K., Miyazaki, J. and Ishigatsubo, Y. 1999. Administration of an IL-12-encoding DNA plasmid prevents the development of chronic graft-versus-host disease(GVHD). *J. Immunol.* 162:4013.

22 Mun, H. S., Aosai, F. and Yano, A. 1999. Role of *Toxoplasma gondii* HSP70 and *Toxoplasma gondii* HSP30/bag1 in antibody formation and prophylactic immunity in mice experimentally infected with *Toxoplasma gondii*. *Microbiol. Immunol.* 43:471.

23 Ye, Y. L., Suen, J. L., Chen, Y. Y. and Chiang, B. L. 1998. Phenotypic and function analysis of activated B cells of autoimmune NZB × NZW F1 mice. *Scand. J. Immunol.* 47:122.

24 Lefkowith, J. B. and Gilkeson, G. S. 1996. Nephritogenic autoantibodies in lupus: current concepts and continuing controversies. *Arthritis Rheum.* 39:894.

25 Madaio, M. P. and Shlomchik, M. J. 1996. Emerging concepts regarding B cells and autoantibodies in murine lupus nephritis. B cells have multiple roles; all autoantibodies are not equal. *J. Am. Soc. Nephrol.* 7:387.

26 Takahashi, S., Nose, M., Sasaki, J., Yamamoto, T. and Kyogoku, M. 1991. IgG3 production in MRL/lpr mice is responsible for development of lupus nephritis. *J. Immunol.* 147:515.

27 Park, C. L., Balderas, R. S., Fieser, T. M., Slack, J. H., Prud'Homme, G. J., Dixon, F. J. and Theofilopoulos, A. N. 1983. Isotypic profiles and other fine characteristics of immune responses to exogenous thymus-dependent and -independent antigens by mice with lupus syndromes. *J. Immunol.* 130:2161.

28 Kinoshita, K., Yoo, B. S., Nozaki, Y., Sugiyama, M., Ikoma, S., Ohno, M., Funauchi, M. and Kanamaru, A. 2003. Retinoic acid reduces autoimmune renal injury and increases survival in NZB/W F(1) mice. *J. Immunol.* 170:5793.

29 Eilat, E., Zinger, H., Nyska, A. and Mozes, E. 2000. Prevention of systemic lupus erythematosus-like disease in (NZB × NZW) F1 mice by treating with CDR1- and CDR3-based peptides of a pathogenic autoantibody. *J. Clin. Immunol.* 20:268.

30 Theofilopoulos, A. N. and Lawson, B. R. 1999. Tumour necrosis factor and other cytokines in murine lupus. *Ann. Rheum. Dis.* 58:149.

31 Dean, G. S., Tyrrell-Price, J., Crawley, E. and Isenberg, D. A. 2000. Cytokines and systemic lupus erythematosus. *Ann. Rheum. Dis.* 59:243.

32 Peng, S. L., Moslehi, J. and Craft, J. 1997. Roles of interferon-gamma and interleukin-4 in murine lupus. *J. Clin. Invest.* 99:1936.

33 Hasegawa, K., Hayashi, T. and Maeda, K. 2002. Promotion of lupus in NZB × NZWF1 mice by plasmids encoding interferon (IFN)-gamma but not by those encoding interleukin (IL)-4. *J. Comp. Pathol.* 127:1.

34 Alleva, D. G., Kaser, S. B. and Beller, D. I. 1998. Intrinsic defects in macrophage IL-12 production associated with immune dysfunction in the MRL/++ and New Zealand Black/White F1 lupus-prone mice and the *Leishmania major*-susceptible BALB/c strain. *J. Immunol.* 161:6878.

35 Horwitz, D. A., Gray, J. D., Behrendsen, S. C., Kubin, M., Rengaraju, M., Ohtsuka, K. and Trinchieri, G. 1998. Decreased production of interleukin-12 and other Th1-type cytokines in patients with recent-onset systemic lupus erythematosus. *Arthritis Rheum.* 41:838.

36 Mamula, M. J., Lin, R. H., Janeway, C. A. Jr and Hardin, J. A. 1992. Breaking T cell tolerance with foreign and self co-immunogens. A study of autoimmune B and T cell epitopes of cytochrome c. *J. Immunol.* 149:789.

37 Ozmen, L., Roman, D., Fountoulakis, M., Schmid, G., Ryffel, B. and Garotta, G. 1995. Experimental therapy of systemic lupus erythematosus: the treatment of NZB/W mice with mouse soluble interferon-gamma receptor inhibits the onset of glomerulonephritis. *Eur. J. Immunol.* 25:6.

38 Ishida, H., Muchamuel, T., Sakaguchi, S., Andrade, S., Menon, S. and Howard, M. 1994. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. *J. Exp. Med.* 179:305.

39 Jacob, C. O. and McDevitt, H. O. 1988. Tumour necrosis factor-alpha in murine autoimmune 'lupus' nephritis. *Nature* 331:356.

40 Gordon, C., Ranges, G. E., Greenspan, J. S. and Wofsy, D. 1989. Chronic therapy with recombinant tumor necrosis factor-alpha in autoimmune NZB/NZW F1 mice. *Clin. Immunol. Immunopathol.* 52:421.

41 Watanabe, Y. and Jacob, C. O. 1991. Regulation of MHC class II antigen expression. Opposing effects of tumor necrosis factor-alpha on IFN-gamma-induced HLA-DR and Ia expression depends on the maturation and differentiation stage of the cell. *J. Immunol.* 146:899.

42 Gordon, C. and Wofsy, D. 1990. Effects of recombinant murine tumor necrosis factor-alpha on immune function. *J. Immunol.* 144:1753.

43 Schwarting, A., Wada, T., Kinoshita, K., Tesch, G. and Kelley, V. R. 1998. IFN-gamma receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL-*Fas*^lpr mice. *J. Immunol.* 161:494.

44 Haas, C., Ryffel, B. and Le Hir, M. 1998. IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB × NZW)F1 mice. *J. Immunol.* 160:3713.

45 Kono, D. H., Balomenos, D., Park, M. S. and Theofilopoulos, A. N. 2000. Development of lupus in BXSB mice is independent of IL-4. *J. Immunol.* 164:38.

46 Hagiwara, E., Okubo, T., Aoki, I., Ohno, S., Tsuji, T., Ihata, A., Ueda, A., Shirai, A., Okuda, K., Miyazaki, J. and Ishigatsubo, Y. 2000. IL-12-encoding plasmid has a beneficial effect on spontaneous autoimmune disease in MRL/MP-lpr/lpr mice. *Cytokine* 12:1035.

47 Takahashi, S., Fossati, L., Iwamoto, M., Merino, R., Motta, R., Kobayakawa, T. and Izui, S. 1996. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J. Clin. Invest.* 97:1597.

48 Nakajima, A., Hirose, S., Yagita, H. and Okumura, K. 1997. Roles of IL-4 and IL-12 in the development of lupus in NZB/W F1 mice. *J. Immunol.* 158:1466.

49 Yang, T. H., Aosai, F., Norose, K., Ueda, M. and Yano, A. 1995. Enhanced cytotoxicity of IFN-gamma-producing CD4^+ cytotoxic T lymphocytes specific for *T. gondii*-infected human melanoma cells. *J. Immunol.* 154:290.

50 Hayashi, T., Mori, I. and Yamamoto, H. 1992. Lactic dehydrogenase virus infection prevents development of anti-nuclear antibody in (NZB × NZW) F1 mice; role of prostaglandin

E2 and macrophage Ia antigen expression. *Int. J. Exp. Pathol.* 73:593.

51 Michaelides, M. C. and Simms, E. S. 1980. Immune responses in mice infected with lactic dehydrogenase virus. III. Antibody response to a T-dependent and a T-independent antigen during acute and chronic LDV infection. *Cell. Immunol.* 50:253.

52 Yano, A., Mun, H. S., Yang, T. H., Hata, H., Kobayashi, M., Norose, K., Hayakawa, S., Tagawa, Y., Iwakura, Y., Nakazaki, S. *et al.* 1998. Roles of IFN-γ in effector mechanisms and pathogenicity of HSPs in mice and human infected with *Toxoplasma gondii*. In Tada, I., Kojima, S., Tsuji, M., eds., *9th International Congress of Parasitology*. p. 457. Monduzzi Editore, Bologna, Italy.

53 Yano, A., Aosai, F., Ohta, M., Hasekura, H., Sugane, K. and Hayashi, S. 1989. Antigen presentation by *Toxoplasma gondii*-infected cells to CD4+ proliferative T cells and CD8+ cytotoxic cells. *J. Parasitol.* 75:411.

54 Yang, T. H., Aosai, F., Norose, K., Ueda, M. and Yano, A. 1996. Differential regulation of HLA-DR expression and antigen presentation in *Toxoplasma gondii*-infected melanoma cells by interleukin 6 and interferon gamma. *Microbiol. Immunol.* 40:443.

55 Lüder, C. G., Lang, T., Beuerle, B. and Gross, U. 1998. Down-regulation of MHC class II molecules and inability to up-regulate

class I molecules in murine macrophages after infection with *Toxoplasma gondii. Clin. Exp. Immunol.* 112:308.

56 Lüder, C. G., Lang, C., Giraldo-Velasquez, M., Algner, M., Gerdes, J. and Gross, U. 2003. *Toxoplasma gondii* inhibits MHC class II expression in neural antigen-presenting cells by down-regulating the class II transactivator CIITA. *J. Neuroimmunol.* 134:12.

57 McGeer, P. L., Kawamata, T., Walker, D. G., Akiyama, H., Tooyama, I. and McGeer, E. G. 1993. Microglia in degenerative neurological disease. *Glia* 7:84.

58 Lindquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55:1151.

59 Kenderov, A., Minkova, V., Mihailova, D., Giltiay, N., Kyurkchiev, S., Kehayov, I., Kazatchkine, M., Kaveri, S. and Pashov, A. 2002. Lupus-specific kidney deposits of HSP90 are associated with altered IgG idiotypic interactions of anti-HSP90 autoantibodies. *Clin. Exp. Immunol.* 129:169.

60 Hayakawa, K., Hardy, R. R., Parks, D. R. and Herzenberg, L. A. 1983. The 'Ly-1 B' cell subpopulation in normal immunodefective, and autoimmune mice. *J. Exp. Med.* 157:202.

61 Norose, K., Mun, H. S., Chen, M., Aosai, F. and Yano, A. 2000. Serum antibodies to HSC71 in Vogt–Koyanagi–Harada disease. *Br. J. Ophthalmol.* 84:1434.