Vaccination with autoreactive CD4^{+}Th1 clones in lupus-prone MRL/Mp-*Fas*$^{lpr/lpr}$ mice

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Abstract

We studied the efficacy of T cell vaccination with $CD4^+\alpha\beta$Th1 clones in lupus-prone MRL/Mp-*Fas*$^{lpr/lpr}$ (MRL/*lpr*) mice. $CD4^+\alpha\beta$ Th1 clones, dna51 (Vβ8.3) and rnp2 (Vβ14), which stimulated anti-dsDNA or U1 ribonucleoprotein (U1RNP) antibody (Ab) production respectively, were isolated from splenocytes of MRL/*lpr* mice. Antinuclear Ab kinetics, renal function, renal histology, survival rate, and lymphocyte subpopulations in the spleen were monitored after intravenous adoptive transfer of IL-2-stimulated (s-) or irradiated (i-) clones to 3 week old female MRL/*lpr* mice. Anti-idiotypic humoral and T cell responses against the transferred autoreactive Th1 clones were determined in parallel. Compared with PBS-treated MRL/*lpr* mice, anti-dsDNA Ab titers, and the activity index for lupus nephritis were all decreased in MRL/*lpr* mice vaccinated with i-dna51 cells, whereas survival rate was not improved. The numbers of $CD4^+$Vβ8.3 T cells in the spleen were also significantly decreased in these mice. Anti-idiotypic Abs recognizing a 12 amino acid sequence of clone dna51 T cell receptor Vβ8.3-complementarity-determining region (CDR) 3 were detected in the MRL/*lpr* mice that received i-dna51 or s-dna51 cells. These Abs suppressed dna51 cell proliferation, as well as cytotoxicity of $CD8^+$T cells against dna51. The present study suggests that vaccination with $CD4^+\alpha\beta$Th1 clone, dna51, elicits anti-idiotypic T cell and humoral responses against dna51 in MRL/*lpr* mice, although the immunoregulatory effects on lupus may be limited.

Key words: T cell vaccination, anti-dsDNA antibodies, lupus nephritis

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, remitting and relapsing, multisystem autoimmune disease that predominantly affects women. Currently, there is substantial evidence that the adaptive and innate immune systems both contribute to the development of SLE [1, 2]. It is widely accepted that the uncontrolled hyperactivity of autoreactive T and B cells and sustained production of anti-nuclear antibodies (Abs) and immune complexes (ICs) are involved in the pathogenesis of lupus. Both anti-dsDNA Abs and ICs contribute to lupus nephritis, and recently DNA-containing ICs have been shown to trigger Toll-like receptor 9, the receptor for bacterial CpG DNA in B cells and dendritic cells [2, 3].

T cells play a central role in the complex series of events culminating in systemic tissue damage in SLE. In human lupus nephritis, restricted T cell receptor usage was shown in infiltrating T cells, which exhibit evidence of recent activation [4]. Studies in SLE patients and lupus-prone animals have characterized lupus T cells as activated autoreactive T cells, presenting with abnormalities of T cell signaling and costimulation, and regulatory T cell dysfunction [5, 6]. Anti-dsDNA and chromatin Abs are serological hallmarks of SLE and their presence is used to help define lupus nephritis. Such disease-associated antinuclear Ab production is confirmed to be oligoclonal, affinity matured, high titer, class switched, and MHC class II-restricted, strongly suggesting cognate T cell help and T cell-derived cytokine-driven affinity maturation. Whereas lupus B cells are also over-activated, pathogenesis in lupus-prone mice requires autoreactive $CD4^+$ $\alpha\beta$ T cells that help autoreactive B cells [7-9]. Such autoreactive T cells that help B cells produce anti-dsDNA Ab production have been characterized, and limited numbers of T cell epitopes defined on the autoantigens [10, 11].

It is noteworthy that autoreactive T cells can escape thymic selection and are

detectable in the peripheral blood of both SLE patients and healthy individuals [12]. Therefore, breakdown of peripheral tolerance results in the expansion of autoreactive T cells in the periphery and can contribute to the development of autoimmunity in lupus. Defects in activation-induced cell death, ineffective control of elimination by regulatory cells, and the abnormal activation of antigen presenting cells (APCs) such as dendritic cells, are all involved in ensuring the persistence of expanded autoreactive T cells.

Taken together, targeting treatment against peripheral autoantigen-specific T cells associated with autoantibody stimulation could represent an effective immunotherapeutic approach in lupus. T cell vaccination (TCV) to eliminate specific pathogenic autoreactive T cells, for example, by immunization with irradiated autologous T cells in multiple sclerosis (MS) or experimental autoimmune encephalomyelitis (EAE), is an attractive modality [13, 14]. In MS and EAE, autoreactive T cells specific for myelin antigens play an important role in pathogenesis. Immunization with myelin-basic protein-reactive T cells induces an anti-idiotypic $CD8^+$ cytolytic T cell response [14], as well as $CD4^+$ regulatory T cell responses [15], and, to a lesser extent, humoral responses [16]. Based on the results of successful TCV in animal models, clinical trials in a small number of MS patients have been initiated [17].

In the present study, using autoreactive $CD4^+V\beta8.3$ Th1 clones, which we isolated from splenocytes of lupus-prone MRL/*lpr* mice, the impact of variables regulating TCV success in this animal model was investigated.

2. Materials and Methods

2.1. Mice

MRL/MpJ Jms Slc-*Fas*$^{lpr/lpr}$ mice (MRL/*lpr* mice) were purchased from the Sankyo Lab service Corp. (Shizuoka, Japan). These mice were bred and housed in specific pathogen-free facilities at the Graduate School of Medicine, Kyoto University. The Kyoto University Animal Welfare Committee approved all procedures (approval number Med Kyo 07073).

2.2. Generation of autoreactive Th clones from lymphocytes of antinuclear Ab-positive MRL/lpr mice

Autoreactive Th clones were isolated and cloned according to Naiki's method with some modifications [18]. Briefly, a single cell suspension of lymph node cells was prepared from anti-dsDNA Ab- or anti-U1-ribonucleoprotein (U1RNP) Ab-positive MRL/*lpr* mice (age = 3-4 months). The cells were treated with red blood cell lysis buffer (Sigma Chemical Co., St. Louis, MO). Initially, 4 x 10^6/ml cells were cultured in 24-well tissue culture plates with Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum, antibiotics, L-glutamine, and 2-mercaptoehtanol. 2 x 10^6/ml irradiated (30 Gy) syngeneic splenocytes as antigen presenting cells (APCs) were added weekly along with 10 units/ml mouse rIL-2 (R&D systems, Minneapolis, MN) in the absence of foreign antigens. After 1 month, cells were transferred at limiting dilution to 96-well plates at a concentration of 0.5 cells/well. Growing cells (< 5/plate) were then harvested and further expanded in 24-well plates.

2.3. Cytokine ELISA

The isolated Th clones (2 x 10^5/well) were stimulated for 24 or 48 h by plate-bound anti-CD3ε mAb (5 μg/ml, 500A2, Becton Dickinson, San Jose, CA) in 48-well plate. Concentrations of IFNγ and IL-4 in culture supernatants were measured by ELISA according to manufacturer's protocol (Becton Dickinson). All samples were performed in duplicate.

2.4. Flow cytofluorometric analysis

Th clones were analyzed by flow cytometry, using anti-TCR-Cβ (H57-597-PE, Becton Dickinson), anti-CD4 (H129.19-FITC), anti-CD3 (500A2-PE), and anti-B220 (RA3-6B2-FITC) monoclonal antibodies (mAbs). For the determination of the TCR Vβ region, mAb against Vβ3 (KJ25-FITC), Vβ4 (KT4-FITC), Vβ6 (RR4-7-FITC), Vβ7 (TR310-FITC), Vβ8 (F23.1-PE), Vβ8.1/8.2 (MR5-2-FITC), Vβ8.3 (1B3.3-FITC), Vβ9 (MR10-2-FITC), Vβ10 (B21.5-FITC), Vβ13 (MR12-3-FITC), Vβ14 (14-2-FITC) and Vβ17 (KJ23-FITC) were used. Stained cells were analyzed by acquisition on a FACScan® flow cytometer (Becton Dickinson) and analyzed by CellQuest™ software (Becton Dickinson).

For detection of Th clone-binding IgM and/or IgG Abs in sera from MRL/*lpr* mice, 2 μl of serum from each of 10 MRL/*lpr* mice, which received PBS, irradiated clone dna51 (i-dna51) or irradiated clone rnp2 (i-rnp2) cells, was diluted in 180 μl of PBS. This mixture was incubated with the T cell clones for 30 min, and then FITC-conjugated goat anti-mouse (IgM+IgG) Abs used as second Abs. For determination of IgG Abs against T cell clones, a 1:100 dilution of serum from some of the MRL/*lpr* mice, which received stimulated (s-) dna51 or i-dna51 cells, was incubated with the clones for 30 min, and then visualized with FITC-conjugated goat anti-mouse IgG Abs.

2.5. RNA extraction and sequence analysis of the TCRβ-chain by reverse-transcription PCR

Monoclonality of T cells was confirmed by determination of T cell receptor (TCR) sequence. Total RNA was extracted from the Th clone, and the TCRβ-chain V-D-J sequence was determined by reverse-transcription PCR. Total RNA (5 μg) was reverse-transcribed using AMV transcriptase. The resulting cDNA was PCR-amplified through 40 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 4 min) using probes specific for Vβ8.3 (CAT TAT TCA TAT GGT GCT GGC) and Cβ (CTT GGG TGG AGT CAC ATT TCTC). The amplified cDNA was sequenced directly with Vβ8.3 primer and then matched using the international ImMunoGeneTics (IMGT) database [20].

2.6. Proliferation assays

For Th1 clone-mediated B cell proliferation assays, purified B cells (5 x 10^4/well) were co-cultured with irradiated (20 Gy) Th1 clone, dna51 (2 x 10^4 /well) in 200 μl medium in triplicate using round bottom 96-well plate for 3 days. B cells were purified from splenocytes of MRL/*lpr* mice by Cellect^{TM} Mouse B (Biotex Laboratories Inc., Edmonton, Canada) followed by further T cell depletion using anti-Thy1.2 antibody (HO-13-4) and Low-Tox-M rabbit complement (Accurate Chemical & Scientific Corporation, Westbury, NY; B cell purity >95% as determined by flow cytometry). After 1 μCi of [^{3}H]-labeled thymidine (Amersham, Arlington Heights, IL) was added during the last 16 h of culture, cells were harvested with a semi-automatic cell harvester (Skatron Instruments, Sterling, VA). Incorporated radioactivity was measured using a β-plate scintillation counter (Beckman Instruments, Fullerton, CA). To examine the clone dna51-B cell cognate interaction, anti-CD40L mAbs (MR1, NA/LE, Becton Dickinson) or the isoptype control IgG was incubated in culture for 3 days.

For Th clone proliferation assays in the presence of treated MRL/*lpr* mouse B cells, B cells were purified from splenocytes of Th clone-vaccinated MRL/*lpr* mice. Purified B cells (1×10^5) were irradiated (30 Gy) and then co-cultured with Th clones (1×10^5) for 3 days. After 1 μCi of [^{3}H]-labeled thymidine was added during the last 16 h of culture, cells were harvested and incorporated radioactivity was measured. For functional assay of anti-vaccinated clone Abs, proliferative response of the Th clone (1×10^5/well) in the presence of APCs (5×10^5 of irradiated MRL/*lpr* splenocytes) was examined with T cell vaccination (TCV)-treated MRL/*lpr* mouse sera (1:50 dilution). In addition, to confirm whether anti-vaccinated clone Abs themselves in sera work or not, MRL/*lpr* mouse sera and dna51 Vβ8.3-complementarity-determining region (CDR) 3 synthetic peptides (ASRETGGGDTQY, final concentration = 50 μg/ml) were preincubated before proliferation assay.

2.7. Antinuclear Ab detection by ELISA

Anti-dsDNA Abs were measured according to the method of Rubin [19]. For detection of anti-U1RNP Ab, the MESACUP-2 test® RNP (MBL Laboratories, Co., Nagoya, Japan) was used. Mouse sera were diluted 1:100 with PBS containing 3% bovine serum albumin and incubated at room temperature for 2 h, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) at OD_{405nm} in a microtiter ELISA reader. Using anti-dsDNA Ab- or anti-U1RNP Ab-positive (defined as 300 units) and negative (0 units) standard mouse serum (1:100 dilution), arbitrary units of experimental Ab titer in sample were calculated. In case of MRL/*lpr* mouse sera, arbitrary units of Abs were calculated as ([OD_{405nm} of experimental serum - OD_{405nm} of negative standard] x300 / [OD_{405nm} of positive standard - OD_{405nm} of negative standard]). In *in vitro* helper assay supernatant,

arbitrary units of Abs were calculated as ([OD_{405nm} of experimental wells – OD_{405nm} of no cell wells (background)] x300 / [OD_{405nm} of positive standard - OD_{405nm} of negative standard]).

2.8. In vitro *helper assay*

Th cells (5 x 10^5/well) were co-cultured with 2 x 10^6 of purified MRL/*lpr* B cells in 24-well plates for one week. Culture supernatants were harvested and arbitrary units of anti-dsDNA and anti-U1RNP Abs were determined by ELISA (Refer to *Antinuclear Ab detection by ELISA*). Next, stimulation index was calculated as (Ab arbitrary units of [Th clone+B cells] / Ab arbitrary units of [B cells alone well]). For examining cell contact-dependent Ab production, Th clones (5 x 10^5/well, upper the membrane) and purified MRL/*lpr* B cells (2 x 10^6/well, lower the membrane) were placed separately using a 10μm thin transparent polycarbonate membrane insert (0.4 μm pore size, Becton-Dickinson Labware, Mountain View, CA) in 24-well plate and incubated for one week. Then, membrane insert was removed and supernatant was mixed. Anti-nuclear Ab arbitrary units in supernatant were determined by ELISA and stimulation index was calculated as above.

2.9. Adoptive transfer study with Th1 clones

As controls, stimulated Th1 clones dna51 and rnp2 (1-2 x 10^6 cells) by rIL-2 (10 units/ml for 24 h) (designated as s-dna51 or s-rnp2) were transferred intravenously into each MRL*/lpr* mouse (10 mice in each group receiving Th1 clones). Cultured Th1 clones were stained by trypan blue and more than 95% of non-staining cells in culture were confirmed before transfer. To completely remove APC contamination, cells were used at least 2 weeks after the last addition of APCs. The purity of the transferred cells was

>98% as determined by flow cytometric analysis. For vaccination, the same amount of stimulated Th1 clones was irradiated (20 Gy) before transfer (10 mice in each group receiving Th clones [designated as i-dna51 or i-rnp2]). From the 1st transfer into mice 3 weeks of age, transfers were performed intravenously every three weeks (total of 6 transfers). Serum samples were collected periodically for examining urinary protein amount, serum creatinine level, and anti-nuclear Ab titers. All mice were sacrificed and skin, lungs, and kidneys were collected for histological evaluation at 26 weeks of age. At the same time, splenocyte subpopulations were analyzed by flow cytometry and the groups compared.

2.10. Morphometric analysis and immunohistochemistry

For light microscopy, sagittal kidney sections were fixed by immersion in Carnoy's solution followed by 4% buffered formaldehyde and embedding in paraffin. One-micron-thick sections were stained with hematoxylin-eosin and periodic acid-Schiff. The renal activity index (AI) proposed by Austin et al. [21] was calculated. Briefly, the AI of glomerular abnormalities (cellular proliferation, fibrinoid necrosis/karyorrhexis, cellular crescents, hyaline thrombi/wire loop, and leukocyte infiltration) and tubulointerstitial abnormalities (mononuclear cell infiltration) were calculated separately. Additionally, the number of glomerular cells and the mesangial area were measured quantitatively with a computer-aided manipulator (KS400, Carl Zeiss Vision, Munich, Germany) by counting the nuclei and analyzing the periodic acid Schiff-positive area within the glomerular tuft. More than 10 consecutive glomerular sections, randomly selected in each mouse by scanning from the outer cortex, were examined without knowledge of the origin of the slides, and the mean values were calculated [22]. For immunofluorescence of mouse C3, 1 μm-thick cryostat sections were fixed in acetone.

The sections were washed with phosphate-buffered saline and incubated overnight at 4ºC with FITC-labeled goat anti-mouse C3 (ICN, Aurora, OH).

2.11. Detection of Abs against dna51 Vβ8.3-CDR3 peptides

Synthetic peptides (ASRETGGGDTQY) of dna51 Vβ8.3-CDR3 with coating buffer (pH9.4) (10 μg/ml) were bound to ELISA plates, and wells blocked by 5% bovine serum albumin in PBS. 1:100 diluted sera from MRL/*lpr* mice were incubated for 2 h at room temperature, followed by detection of bound IgG with alkaline phosphatase-conjugated anti-mouse IgG at OD_{405nm} in a microtiter ELISA reader. All assays were performed in triplicate.

2.12. Cytotoxicity assays

Cytotoxicity of $CD4^+$ and $CD8^+$ T cells derived from vaccinated MRL/*lpr* mouse splenocytes against dna51 was determined by Cytotox 96® (Promega Corp., Madison, WI) according to the manufacturer's protocol. $CD4^+$ or $CD8^+$ T cells (effector cells, 2.5-40 $x10^4$ /well) purified using a MACS® isolation kit (Miltenyi Biotec, Inc., Auburn, CA) and dna51 (target cells, $2x10^4$ /well) were incubated in a round-bottom 96-well culture plate for 4 h (experimental wells). Control wells for effector or target cell spontaneous LDH release were set up, and the maximum LDH release control wells of target cells were made. Then, LDH release in supernatant of experimental wells was determined. The percent specific cytolysis was calculated as ([experimental - effector spontaneous - target spontaneous] / [target maximum - target spontaneous]) x100.

2.13. Statistical analysis

Mortality curves were plotted using the Kaplan-Meier method and significance estimated

using the Mantel-Cox log-rank test. For other statistical tests, the nonparametric Mann-Whitney rank sum test or Wilcoxon signed rank sum test were used.

3. Results

3.1. Characterization of the isolated clones dna51 and rnp2

For the present study, two Th clones, rnp2 and dna51, expressing the αβTCR, CD4, and CD3, but not B220 (data not shown) were selected from the isolated 26 clones. Both secreted IFNγ, but not IL-4, indicating that they are Th1 clones (Fig.1A). *In vitro* helper assays demonstrated that clone dna51 augmented the production of anti-dsDNA and clone rnp2 the production of anti-U1RNP Abs (Fig. 1B). Ab secretion was T-B contact-dependent and syngeneic B cell proliferation was mediated by CD40-CD40L costimulation (Fig.1B, C). Screening with mAbs to each Vβ TCR indicated the presence of Vβ14 and Vβ8.3 on clones rnp2 and dna51, respectively. cDNA of the dna51 TCRβ-chain was a 210 base-pair fragment, and Vβ8.3 and Jβ2.6 usage was confirmed by reference to the international ImMunoGeneTics database [20]. The amino acid sequence of the dna51 βTCR junctional region was ASRETGGGDTQY (AGC AGG GAG ACA GGG GGC GGA GAC ACC CAG).

3.2. The effect of T cell vaccination (TCV)

Anti-dsDNA Ab production was suppressed in 18 and 24 week-old MRL/*lpr* mice receiving irradiated (i-) dna51 cells (Fig. 2A). MRL/*lpr* mice that received stimulated (s-) dna51, s-rnp2 or i-rnp2 had titers of anti-dsDNA Abs equal to the PBS-treated animals. Anti-U1RNP Ab secretion was stimulated in MRL/*lpr* mice that had received s-rnp2 and s-dna51 cells compared to PBS-treated controls (Fig. 2B). This result is consistent with a previous report of CD40L-independent anti-U1RNP Ab stimulation [23].

Compared to PBS-treated animals (Fig. 3A, F), glomerular cell infiltration, activity

index (AI) in glomerular lesions, and immune complex deposition were all suppressed in MRL/*lpr* mice receiving i-dna51 (Fig. 3C, H, and Table 1). Those receiving s-dna51 suffered greater glomerular cell infiltration, increased mesangial areas and higher AI than PBS-treated MRL/*lpr* mice (Table 1). MRL/*lpr* mice receiving s-dna51 had higher total and glomerular AI than s-rnp2 recipients, but the latter tended to manifest higher AI of tubulointerstitial abnormalities than PBS-treated controls (Table 1). Other organs such as skin and lungs suffered damage to a similar extent as the vaccinated and PBS-treated MRL/*lpr* mice (data not shown).

Renal insufficiency was prevented in the i-dna51-vaccinated MRL/*lpr* mice compared to the other groups at 24 weeks (Table 1). Proteinuria at 24 weeks was observed in all the groups and there was no statistically significant difference of protein amounts measured semiquantitatively by a test paper (Terumo Co., Tokyo, Japan) among 5 groups (data not shown). Significant differences in survival rates between clone recipients and PBS-treated MRL/*lpr* mice were not observed until 23 weeks (Fig.4).

3.3. Altered splenocyte subpopulations in the vaccinated MRL/lpr mice

To elucidate mechanisms responsible for TCV-mediated protection in MRL/*lpr* mice, the numbers of total splenocytes, T cell subsets ($CD4^+CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^-$ [double negative], $CD4^+V\beta8.3^+$, $CD4^+V\beta14^+$), and $CD3^-B220^+$B cells were calculated using flow cytometry at 26 weeks. The numbers of $CD4^+V\beta8.3^+$T cells were decreased in MRL/*lpr* mice receiving i-dna51 cells (Fig. 5A). In i-rnp2 recipients, $CD4^+V\beta14^+$ T cells tended to be fewer in number than in PBS-treated MRL/*lpr* mice (data not shown). While total numbers of T cells (including $CD4^+CD8^-$, $CD4^+CD8^+$ and double-negative T cells) were not decreased (data not shown), there were fewer B cells in i-dna51 and i-rnp2-recipients than PBS-treated MRL/*lpr* mice (Fig. 5A). Irradiated B cells from i-dna51 recipients

failed to stimulate dna51 proliferation, whereas irradiated B cells from i-rnp2 recipient MRL/*lpr* mice could enhance dna51 proliferation (Fig. 5B). Thus, B cell function as APC for dna51 appears to be more downregulated in i-dna51 recipients than in i-rnp2 recipients, probably because the percentage of B cells, which can stimulate proliferation of dna51, among total splenic B cells is reduced in i-dna51 recipients.

3.4. Generation of Abs against the T cell clones used to vaccinate the MRL/lpr mice

Sera from i-dna51 recipients contained Ig against the dna51 T cell clone (Fig. 6A). Thus, Ig in sera from i-dna51 recipients strongly reacted with clone dna51 cells, but not with the $CD4^+V\beta14^+$Th1 clone rnp2 (Fig. 6B). Sera from non-autoimmune normal B10.BR/SgSn Slc mice reacted with neither clones (data not shown). Anti-dna51 Abs were present in sera from both i-dna51 and s-dna51 recipients (Fig.6C). To investigate whether anti-dna51 Abs recognize the CDR3 region, we established an ELISA system with synthetic peptides corresponding to the dna51 Vβ8.3-CDR3 region (ASRETGGGDTQY). Anti-[dna51 Vβ8.3-CDR3] Ab titers were significantly higher in sera from mice receiving the s-dna51 or i-dna51 vaccine than in i-rnp2 or s-rnp2 recipients**,** or PBS-treated controls (Fig. 7A). Anti-dna51 Abs in the s- and i-dna51 recipients were found to have inhibitory effects on dna51 cell proliferation (Fig. 7B), and synthetic peptides (ASRETGGGDTQY) reversed the inhibitory effect of dna51 proliferation by anti-dna51 Ab-containing sera (Fig. 7B).

3.5. Cytotoxicity against dna51 mediated by T cells purified from splenocytes of vaccinated MRL/lpr mice

To determine anti-clonotypic T cells in TCV-treated MRL/*lpr* mice, LDH release assay was performed. $CD8^+$ T cells from i-dna51 recipients lysed dna51 used as the vaccine at

lower effector/target cell (E/T) ratios (>=2.5). $CD4^+$ T cells from i-dna51 recipients exerted cytotoxicity against dna51 cells only at the highest E/T ratio tested (=20). Both $CD4^+$ and $CD8^+$ T cells derived from i-rnp2 recipients had little killing effects on dna51.

4. Discussion

In the present study, adoptive transfer of an irradiated $CD4^+V\beta8.3^+$Th1 clone (T cell vaccination, TCV) was shown to decrease activity of IC-mediated glomerulonephritis and regulate anti-DNA Ab production in MRL/*lpr* mice. Our isolated clone, dna51, were thought to be autoreactive, because dna51 survived and proliferated without no exogenous antigens (Fig.7B) and stimulated syngeneic B cells to secrete anti-dsDNA Abs by cognate interaction (Fig.1B, C). This is the first report of vaccination with anti-dsDNA Ab-stimulating autoreactive Th1 clone. Because Th1-predominancy in MRL/*lpr* mice has been repeatedly confirmed [24, 25] and because nucleosome-reactive human T cells produce substantial quantities of IFNγ [11], TCV using autoreactive Th1 cells may be more effective than using Th2 cells. While anti-dna51 TCR-CDR3 Ab production was augmented by adoptive transfer of s-dna51 as well as i-dna51 cells, the number of $CD4^+V\beta8.3^+$ T cells was not decreased and renal disease was not ameliorated in MRL/*lpr* mice receiving s-dna51, s-rnp2, or i-rnp2 cells. Thus, unirradiated clone dna51, or clone rnp2 with or without irradiation, are not effective for TCV in MRL/*lpr* mice. On the other hand, significantly improved survival and proteinuria of TCV-treated animals were not confirmed for any group. The explanations for this may be 1) TCV effects with a single clone was limited, and 2) other MRL/*lpr* diseases such as interstitial pneumonia and severe skin disease [26] were found not to be completely suppressed by TCV with i-dna51.

In the present study, TCV in MRL/*lpr* mice induced 1) clonal depletion of nephritogenic $CD4^+V\beta8.3^+$ T cells, 2) suppression of circulating anti-dsDNA Ab associated with IC-mediated nephritis, and 3) decreased number of B cells as well as

$CD4^+V\beta8.3^+$ T cells. Thus far, only TCV studies using polyclonal autologous lymphocytes against murine and human lupus antigens have been reported. De Alborán et al. used $CD3^+CD4^-CD8^-$ (double negative) *lpr* T cells for vaccination of MRL/*lpr* mice [27]. Because the number of $V\beta8.2^+$ T cells was significantly decreased in these vaccinated MRL/*lpr* mice, $V\beta8.2^+$ T cell involvement in the disease-preventing mechanism was suggested. Ben-Yehuda et al. adoptively transferred spleen cells from MRL/+ mice vaccinated with MRL/*lpr* lymphocytes and reported that production of anti-DNA Abs and glomerulonephritis could be prevented by such "MRL/+-derived protective T cells" that might have been responding to at least one antigen on the MRL/*lpr* cells [28]. Although these previous results showed the importance of a certain lymphocyte subset including T cells both in MRL/*lpr* disease and immunoregulatory mechanisms for TCV, the vaccinate T cells in earlier studies were not well characterized and not clinically applicable for human lupus. Recently, autologous TCV has been tested in Chinese patients with SLE [29]. Purified $CD4^+$ T cells from PBMC were injected after 25Gy irradiation, and clinical improvements together with decreased titers of antinuclear Abs in most patients were reported. Efficacy in that study was shown only for mild lupus manifestations, which can easily be treated with low dose corticosteroid and/or conventional immunosuppressants. TCV with unselected T cells is therefore a questionable therapeutic approach in intractable lupus manifestations, such as lupus nephritis. These therapeutic trials, however, are encouraging and oligoclonal T cells and TCR peptide-based immunotherapy has shown some potential efficacy in MS [30].

Anti-idiotypic Abs against dna51 had suppressive effects on the clone dna51 *in vitro*. These immunoregulatory Abs recognized the Vβ CDR3 of dna51, and T-B cognate help for anti-dsDNA Ab production might thus be inhibited. Monoclonal anti-dna51 Abs could therefore represent therapeutic agents in lupus-prone mice. It was noteworthy that

the number of B cells was also decreased in MRL/*lpr* mice receiving i-dna51. B cells from these i-dna51-vaccinated MRL/*lpr* mice failed to stimulate proliferation of dna51, perhaps because autoantigen-presenting B cell lysis might have occurred *in vivo*. We previously cloned a γδT cell line that down-regulates anti-dsDNA Ab production [31]. One line, GD12, killed activated MRL/*lpr* B cells, an effect dependent on TNFα-TNF-R1 interactions. Killing by GD12 required cognate interaction between the B cell targets and the γδT cells. Although TCV failed to increase γδT cell numbers, a certain subset of γδT cells such as represented by GD12 might be induced by the administration of autoreactive Th1 clones and be responsible for some of the phenomena observed here.

The importance of T cells in lupus has been confirmed by experiments in which thymectomy prevented disease in MRL/*lpr* mice [32, 33]. The clinical utility of autoreactive T cell-targeting treatments in other lupus-prone mice has been repeatedly demonstrated. Diseased (wild-type) MRL/*lpr* mice spontaneously have T cells resembling dna51 (Fig. 6A). In SNF1 mice, histone autoepitopes for nephritogenic autoantibody-producing T cells were identified [34], and their administration shown to delay the onset of severe lupus nephritis in prenephritic mice and to prolong survival and halt the progression of glomerulonephritis even in mice with established lupus nephritis [35]. Also, low-dose nucleosomal peptides have been reported to downregulate murine lupus [36]. Hahn et al. synthesized a peptide based on T cell stimulatory sequences from the V_H regions of murine anti-DNA Abs (pCONS), which could induce immune tolerance in BWF1 mice [37]. Further, Ferrera et al. showed that treatment by pCONS induced TGFβ-producing $CD8^+CD28^-$T cells, which suppressed the antigen-specific stimulation of $CD4^+$ T cells and reduced Ab production [38]. The success of such strategies implies that the repertoire of antigen receptors expressed by autoreactive cells is oligoclonal or restricted. Fujio et al. indicated that TCRαβ and CTLA-4Ig gene-transfected

nucleosome-specific T cells could suppress murine lupus nephritis, strongly suggesting that autoantigen hyperpresentation by antigen-presenting cells contributes to the propagation of lupus [39]. The previous studies clearly demonstrated that lupus nephritis mediated by a particular T cell population could be prevented or reversed by regulatory Abs and/or T cells recognizing the TCR expressed on the pathogenic T cells.

There is evidence that myelin basic protein (MBP)-reactive T cells undergo *in vivo* activation and accumulate in the brain of MS patients. The results of preliminary clinical trials confirm that TCV depletes circulating MBP-reactive T cells [17]. The previous reports demonstrated several regulatory mechanisms of TCV [14-16, 40-42]. An anti-idiotypic T cell response, predominantly by $CD8^+$ cytotoxic T cells, which recognize the immunizing T cell clones, is clearly involved in autoreactive T cell depletion. In EAE, $CD8^+$ T cell-mediated cytotoxicity appeared to be more important than Abs against the MBP-specific T cells [14], while anti-idiotypic humoral responses as well as anti-idiotypic cellular responses were induced [16, 40]. Our data suggests that anti-clonotypic $CD8^+$ T cells are induced by TCV also in MRL/*lpr* mice. While $CD4^+$ T cells appeared to have cytotoxic effects against dna51, a potential physiological effect of $CD4^+$ T cells might be different from that of $CD8^+$ T cells. To answer the important point, which subset of $CD4^+$ T cells proliferates in response to dna51 should be examined. Because $CD4^+$ Th1 cells from i-rnp2-received MRL/*lpr* mice appeared not to be effective, anti-ergotypic T cell response [42], which recognizes non-specific activated T cells, may be different from $CD4^+$ T cell-mediated cytotoxicity in the present study. Rather, $CD4^+$ T cells from i-dna51-received MRL/*lpr* mice may include $CD4^+CD25^+$Treg, which were anti-idiotypic T cells against CDR2 [43]. Alternatively, $CD4^+\gamma\delta$T cells expressing $V\gamma1V\delta1$ may be involved [44]. Why higher effector/target ratio was required for $CD4^+$T cell-mediated dna51 cytolysis is due to the small number or low cytotoxicity of

anti-CDR2 $CD4^+CD25^+$Treg and $CD4^+\gamma\delta$T cells in splenic $CD4^+$T cells. To establish the role of an anti-ergotypic T cell response, cytokine secretion from $CD4^+$T cells in our TCV-treated MRL/*lpr* mice also needs to be determined, whereas IL-10 plays a pathogenic role in SLE [45].

In lupus, it is proposed that oligoclonal T cell subsets are involved in the pathogenesis of nephritis [4, 46]. It remains to be determined whether the deletion of a single clone is sufficient to account for clinical efficacy, because TCR of pathogenic autoreactive T cells from individuals with lupus can recognize more than one nucleosomal peptide epitope [34, 35] in the context of diverse MHC class II molecules. We believe that TCV with a single clone can elicit anti-ergotypic as well as anti-idiotypic response and Cohen et al. reported the beneficial effects of a single clone vaccination on adjuvant arthritis [47]. In MS, however, anti-clonotypic T cell responses induced by oligoclonal TCV are restricted to the vaccinated clone and cannot affect the other MBP-reactive clones not used for vaccination [48]. Of interest, in patients with clinical exacerbation, MBP-reactive T cells, which were originate from clonal origin different from those of T cells persisting before oligoclonal TCV, reappeared [48]. These results indicate that depletion of autoreactive T cell clones causes a shift in the T cell repertoire. Taken together, also in MRL/*lpr* mice or lupus, TCV with as many pathogenic Th clones as possible is supposed to be more effective than TCV with a single clone.

In conclusion, TCV induced both regulatory Abs and cytotoxic $CD8^+$T cells against the vaccinate cells and decreased the activity of MRL/*lpr* lupus nephritis, while the effect with a single clone may be limited. Whereas more extensive knowledge on the detailed regulatory mechanisms remains to be determined, TCV may have potential for suppressing autoreactive T cell targets and altering anti-dsDNA Ab production.

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List of abbreviations

MRL/*lpr* = MRL/Mp-*Fas*$^{lpr/lpr}$; SLE = systemic lupus erythematosus; ICs = immune complexes; U1RNP = U1 ribonucleoprotein; APCs = antigen presenting cells; CDR = complementarity-determining region; TCV = T cell vaccination; s-dna51 = stimulated dna51; i-dna51 = irradiated dna51; s-rnp2 = stimulated rnp2; i-rnp2 = irradiated rnp2; MS = multiple sclerosis; EAE = experimental autoimmune encephalomyelitis; TCR = T cell receptor; AI = activity index; MBP = myelin basic protein

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Figure Legends

Fig. 1. Characterization of the isolated Th1 clones by *in vitro* helper assay

A. The isolated Th clones (dna51 and rnp2) secrete IFNγ, but not IL-4 by plate-bound anti-CD3ε mAb-stimulation.

B. Anti-dsDNA Ab and U1RNP Ab in the culture supernatant of each clone (dna51, or rnp2) and purified MRL/*lpr* B cells were shown. In the absence of a 10μm thin transparent polycarbonate membrane insert (M-), the clones dna51 and rnp2 induced anti-dsDNA and anti-U1RNP Abs, respectively. The autoantibody production is inhibited by a membrane insert between T (upper) and B (lower) cells (M+). In the experiment, from OD_{405nm} measured by ELISA, arbitrary units of autoantibodies in supernatants were calculated as ([experimental wells – no cell wells (background)] x300 / [Ab-positive standard mouse serum - Ab-negative standard mouse serum]). Experimental wells include Th clones+B, and B cells alone wells in *in vitro* helper assay. OD_{405nm} of anti-dsDNA and U1RNP Ab-positive standard mouse serum was 2.120 and 1.891, respectively, as 300 units. OD_{405nm} of negative standard normal mouse serum is 0.106 as 0 units. OD_{405nm} of no cell wells (background) was 0.086 and subtracted from OD_{405nm} of experimental wells. Stimulation index was calculated as (anti-dsDNA Ab arbitrary units of [Th clone+B cells] / anti-dsDNA Ab arbitrary units of [B cells alone well]). Data are representative of three independent experiments with similar results.

C. Proliferation of purified MRL/*lpr* B cells in the presence of irradiated dna51 with anti-CD40L mAb (MR1) or isotype control (hamster IgG) was shown. Proliferation of B cells alone was 1045 cpm.

Fig. 2. Anti-dsDNA and U1RNP Ab kinetics in sera from vaccinated MRL/*lpr* mice

A. Stimulated (s-) dna51 (n= 7), s-rnp2 (n= 9), and irradiated (i-) rnp2 (n= 9) recipients have identical titers of anti-dsDNA Abs in comparison with PBS-treated controls at the last observation. At 18 and 24 weeks of age, anti-dsDNA Ab titer in the i-dna51-recipients (n= 10) is significantly lower than the PBS-treated (vehicle) controls (n= 9).

B. Anti-U1RNP Ab production in the vaccinated or PBS-treated MRL/*lpr* mice. s-dna51 and s-rnp2, but not i-dna51 and i-rnp2, recipients have more increased titers of anti-U1RNP Abs in comparison with PBS-treated controls.

*P<0.05, **P<0.01 (compared with PBS-treated MRL/*lpr* mice)

Fig. 3. Renal histology and immunofluorescence study in vaccinated MRL/*lpr* mice

Representative results of hematoxylin-eosin (A-E) and immunofluorescence (mouse complement, C3) (F-J) of glomeruli from 26 week-old MRL/*lpr* mice (magnification = x200). **A, F.** PBS-treated MRL/*lpr* mice (vehicle) developed immune-complex-mediated lupus nephritis. **B, G.** Marked glomerular cell infiltration is observed in stimulated (s-) dna51 recipients. **C, H.** Decreased numbers of glomerular–infiltrating cells and reduced linear deposition of immune complexes can be seen in irradiated (i-) dna51 recipients. **D, I.** In addition to glomerular cell infiltration, tubulointerstitial inflammation is noted in some s-rnp2 recipients. **E, J.** Glomerular cell infiltration is seen in i-rnp2 recipients as well as PBS-treated MRL/*lpr* mice.

Fig. 4. Survival rate of MRL/*lpr* mice treated with TCV.

There is no statistical difference among the 5 groups until 23 weeks. Treated animal numbers were shown in parenthesis.

PBS= PBS-treated MRL/*lpr* mice, s-rnp2= s-rnp2-received MRL/*lpr* mice, s-dna51= s-dna51-received MRL/*lpr* mice, i-rnp2= i-rnp2-received MRL/*lpr* mice, i-dna51= i-dna51-received MRL/*lpr* mice

Fig. 5. Flow cytometric analyses and B cell dysfunction of splenocytes from vaccinated MRL/*lpr* mice

A. $CD4^{+}V\beta8.3^{+}$T cell number in the spleen is decreased in the 26 week-old i-dna51 recipients. Numbers of $CD3^{-}B220^{+}$B cells in splenocytes are significantly decreased in the i-dna51 and i-rnp2 recipient MRL/*lpr* mice. **P*<0.05 (compared with PBS-treated MRL/*lpr* mice)

B. B cell function as APCs derived from TCV-treated MRL/*lpr* mice is shown. Proliferative response of dna51 was significantly decreased when co-cultured with irradiated B cells (irrB) from i-dna51 recipients compared with when with irrB from i-rnp2. **P*<0.01

Fig. 6. Detection of Abs against Th1 clones in sera from the vaccinated MRL/*lpr* mice

A. Two microliters of serum from each of 10 i-dna51-recipients were diluted in 180 μl of PBS. This reacted strongly with the Vβ8.3$^+$ clone, dna51, used as the vaccine. The same serum derived from the i-dna51-received MRL/*lpr* mice reacted with small number of Vβ8.3$^+$ MRL/*lpr* splenocytes (upper right).

B. Diluted serum from i-dna51-recipients (red, n=10), but not from PBS-treated mice (blue**,** n=10), contains IgM and/or IgG reacting with clone dna51, used for vaccination. Serum from i-rnp2 recipient MRL/*lpr* mice (yellow, n=10) failed to react with dna51 cells. The mean fluorescent intensity (MFI) is shown in each histogram.

C. Reactivities with clone dna51 of representative sera from three s-dna51 (s-dna51- 1-3) and three i-dna51 (i-dna51- 1-3) recipients. Both s-dna51 and i-dna51 recipient MRL/*lpr* sera contained IgG Abs against dna51 cells. PBS-treated = Serum from PBS-treated MRL/*lpr* mouse (vehicle).

Fig. 7. ELISA and proliferation study using synthetic peptides identical to the dna51 Vβ8.3-CDR3 region

A. Anti-[dna51 Vβ8.3-CDR3 (ASRETGGGDTQY)] Ab titers are significantly higher in sera from s-dna51 and i-dna51 recipients than s-rnp2, i-rnp2 recipients, and PBS-treated MRL/*lpr* mice after 18 weeks.

*P<0.05, **P<0.01 (compared with PBS-treated MRL/*lpr* mice).

B. Inhibitory effects of TCV-treated MRL/*lpr* mouse sera on dna51 proliferation are shown. Anti-dna51 Ab-positive sera from s- and i-dna51 recipients, but not anti-rnp2 Ab-positive sera from s- and i-rnp2 recipients, decreased proliferation of dna51 cells in the presence of APCs. Preincubation of the clone dna51-derived Vβ8.3-CDR3 synthetic peptides (ASRETGGGDTQY, final concentration = 50 μg/ml) and anti-dna51 Ab-positive sera (50 μl of 1:50 dilution) abrogated the suppression of dna51 cell proliferation.

*P<0.05, **P<0.01 (between the indicated groups); $^{(\mu)}$#P<0.05 (compared with dna51 proliferation in the presence of serum from PBS-treated MRL/*lpr* mouse)

Fig. 8. Cytotoxicity against dna51 mediated by purified T cells

LDH release assay shows that $CD8^+$ T cells purified from i-dna51 recipient at an E/T ratio of 2.5, but not from i-rnp2 recipient MRL/*lpr* mouse splenocytes, kill dna51 cells. At the highest E/T ratio (=20), $CD4^+$ T cells from i-dna51 recipient MRL/*lpr* mice also lyse dna51. Data are representative of three independent experiments with similar results.

Table(s)

Table 1. Renal pathological changes and serum creatinine in the vaccinated MRL/*lpr* mice

	Activity index			Glomerular cell number (/glm)	Mesangial area (mm^2/glm)	Serum creatinine (mg/dl)
	total	glomerular	tubulointerstitial			
PBS-treated (n= 9)	7.6±1.3	5.9±0.8	1.7±1.0	34.4±5.7	953±147	0.168±0.036
s-dna51 (n= 7)	10.3±2.3*	8.2±1.9*	2.1±0.3	46.0±9.3*	1124±49*	0.173±0.037
i-dna51 (n= 10)	3.2±1.7*	1.5±0.4**	1.7±0.8	25.7±3.4*	847±143	0.120±0.032*
s-rnp2 (n= 9)	7.0±1.6	3.9±1.2	3.1±0.5	30.1±6.2	871±155	0.182±0.111
i-rnp2 (n= 9)	7.3±2.0	4.8±1.6	2.5±1.0	31.3±2.4	884±66	0.157±0.041

*P<0.05, **P<0.01 (compared with PBS-treated MRL/*lpr* mice)

Figure(s)
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Fig.1

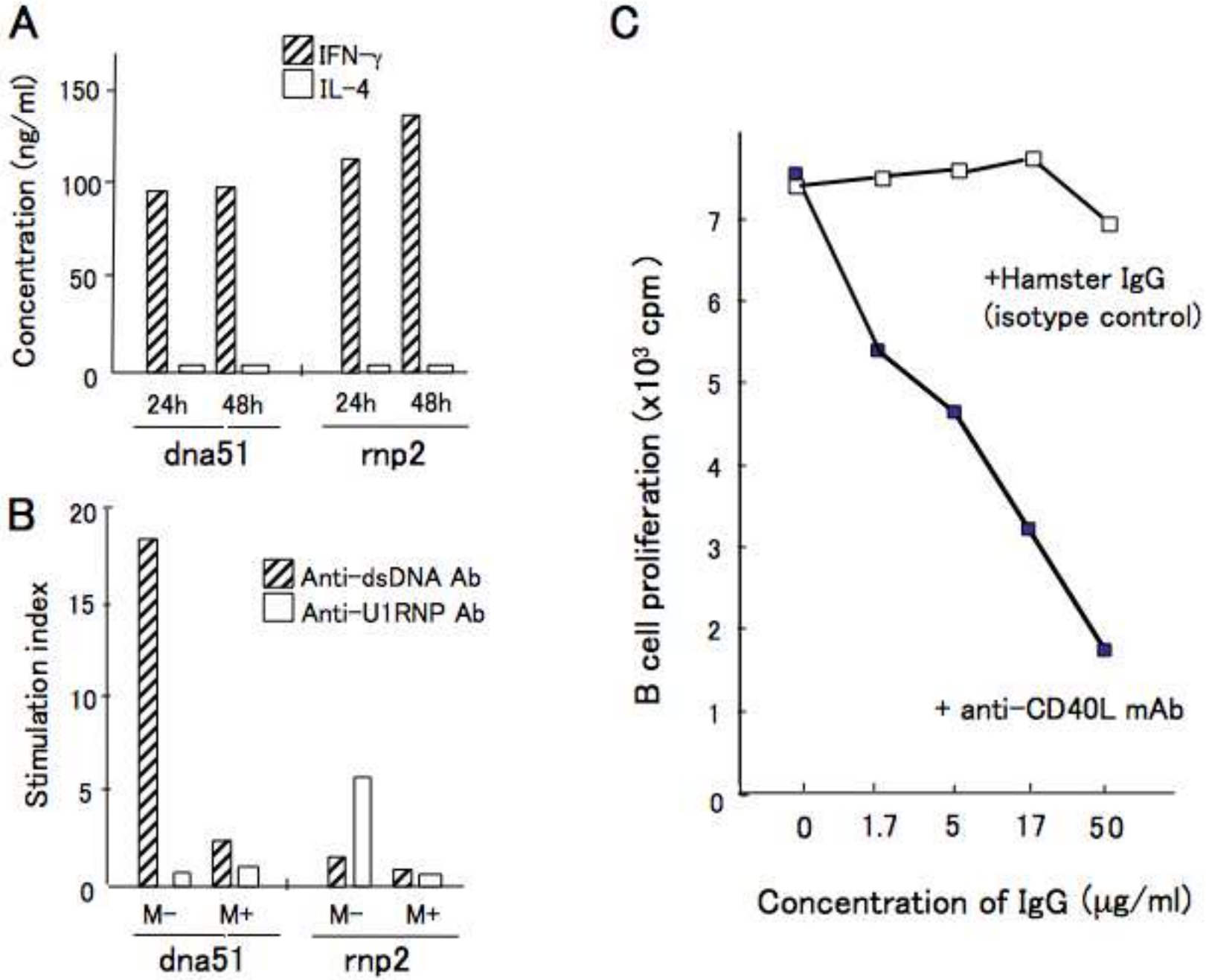

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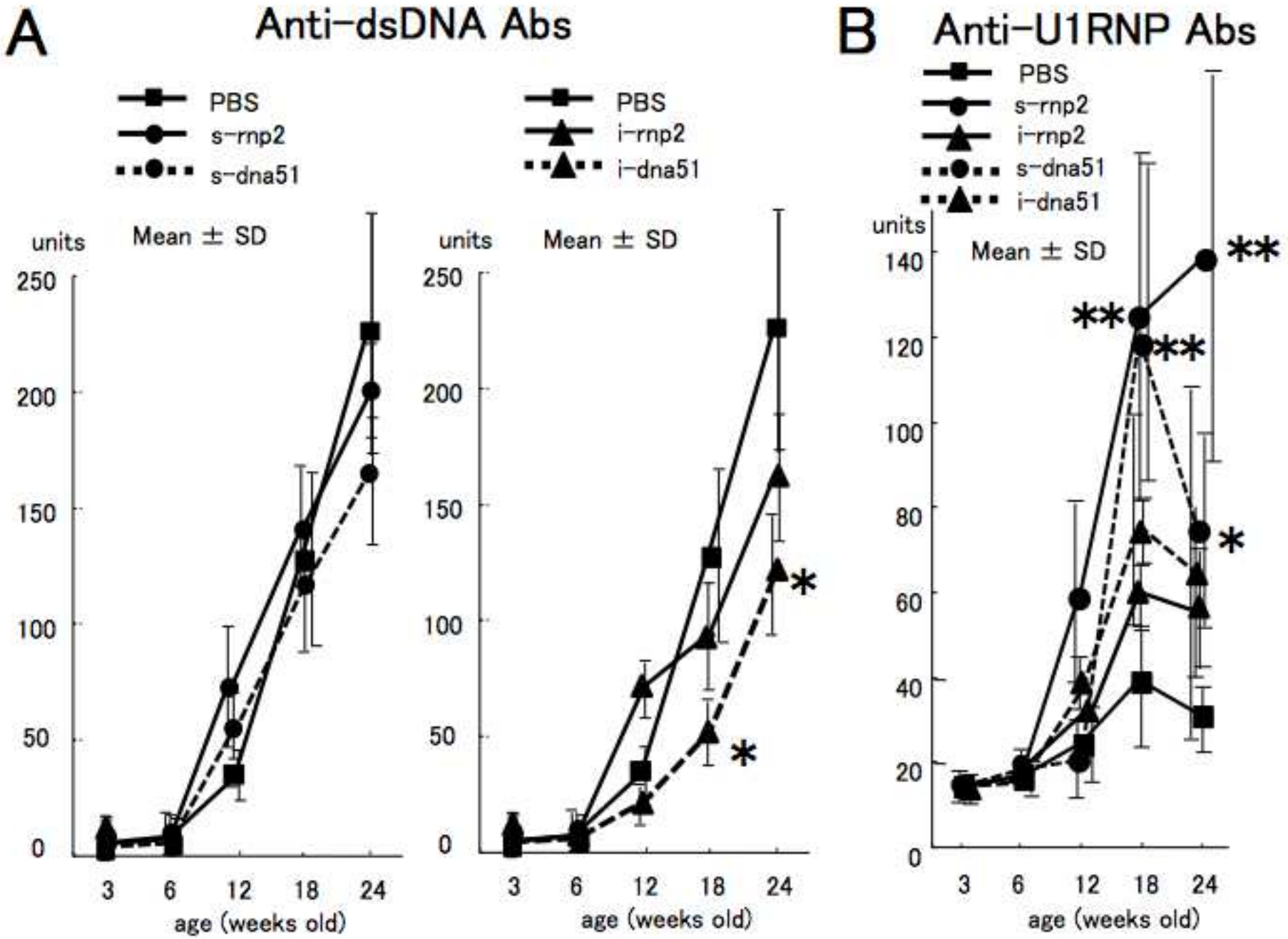
Fig. 2



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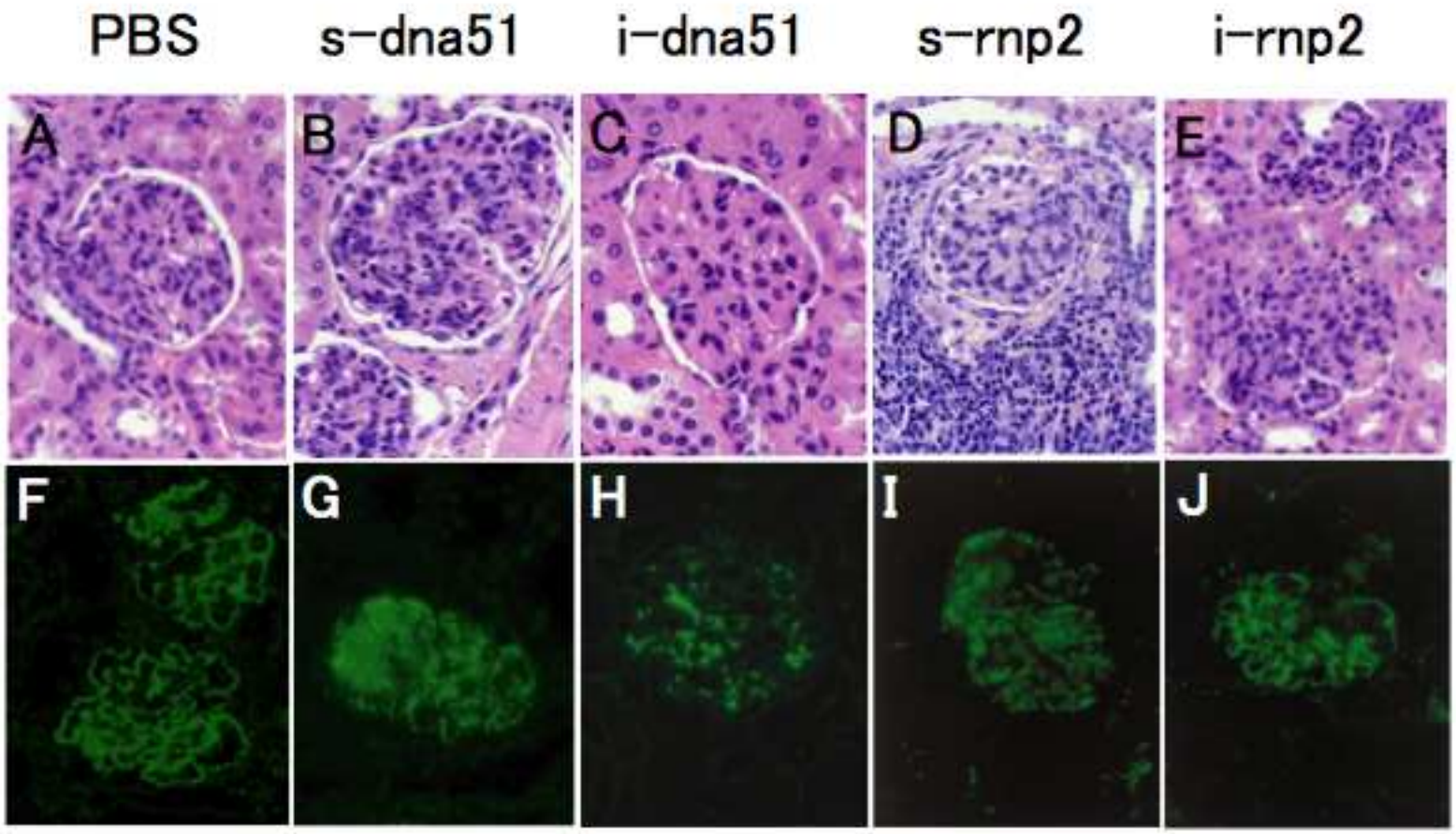

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Fig. 4

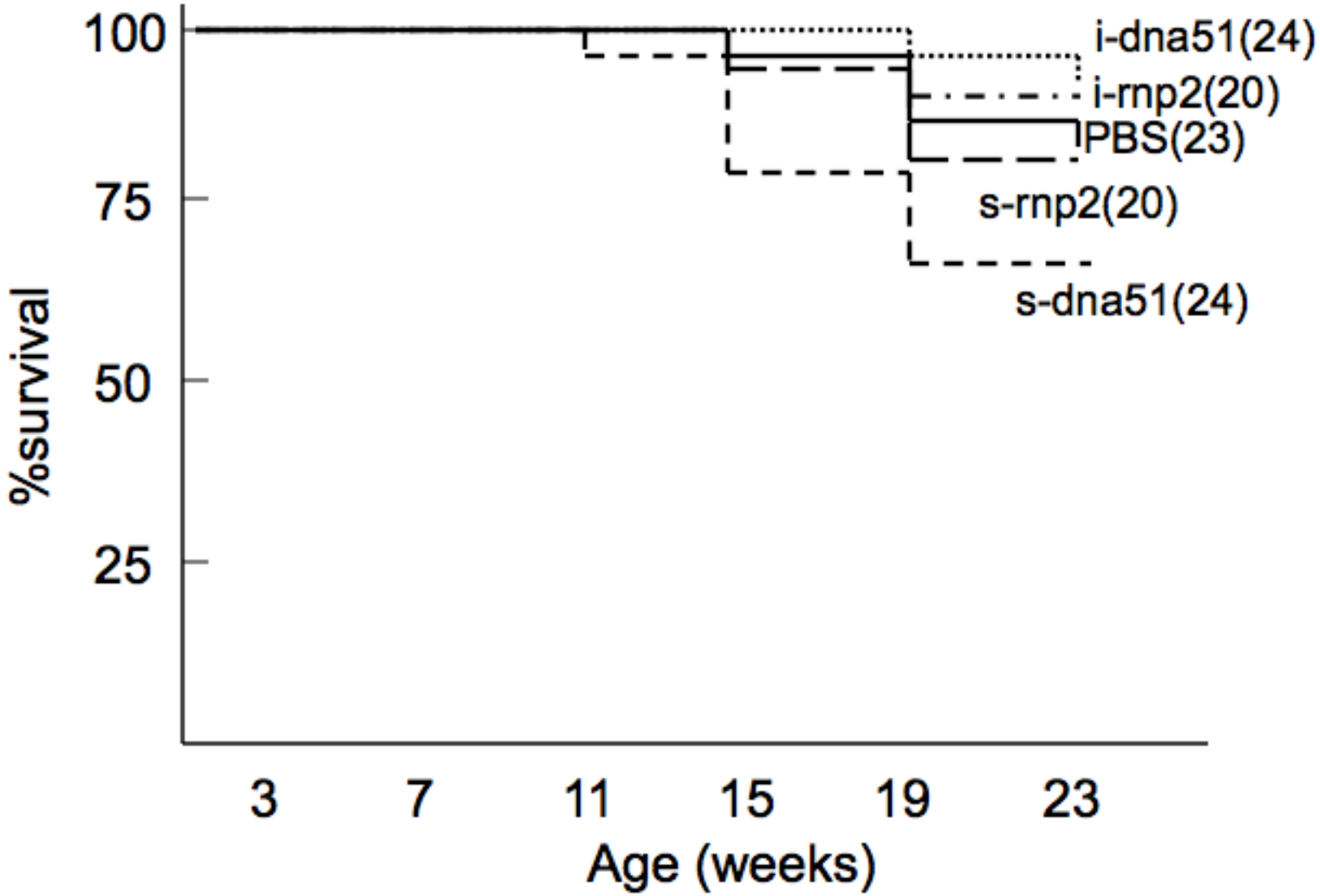

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Fig. 5

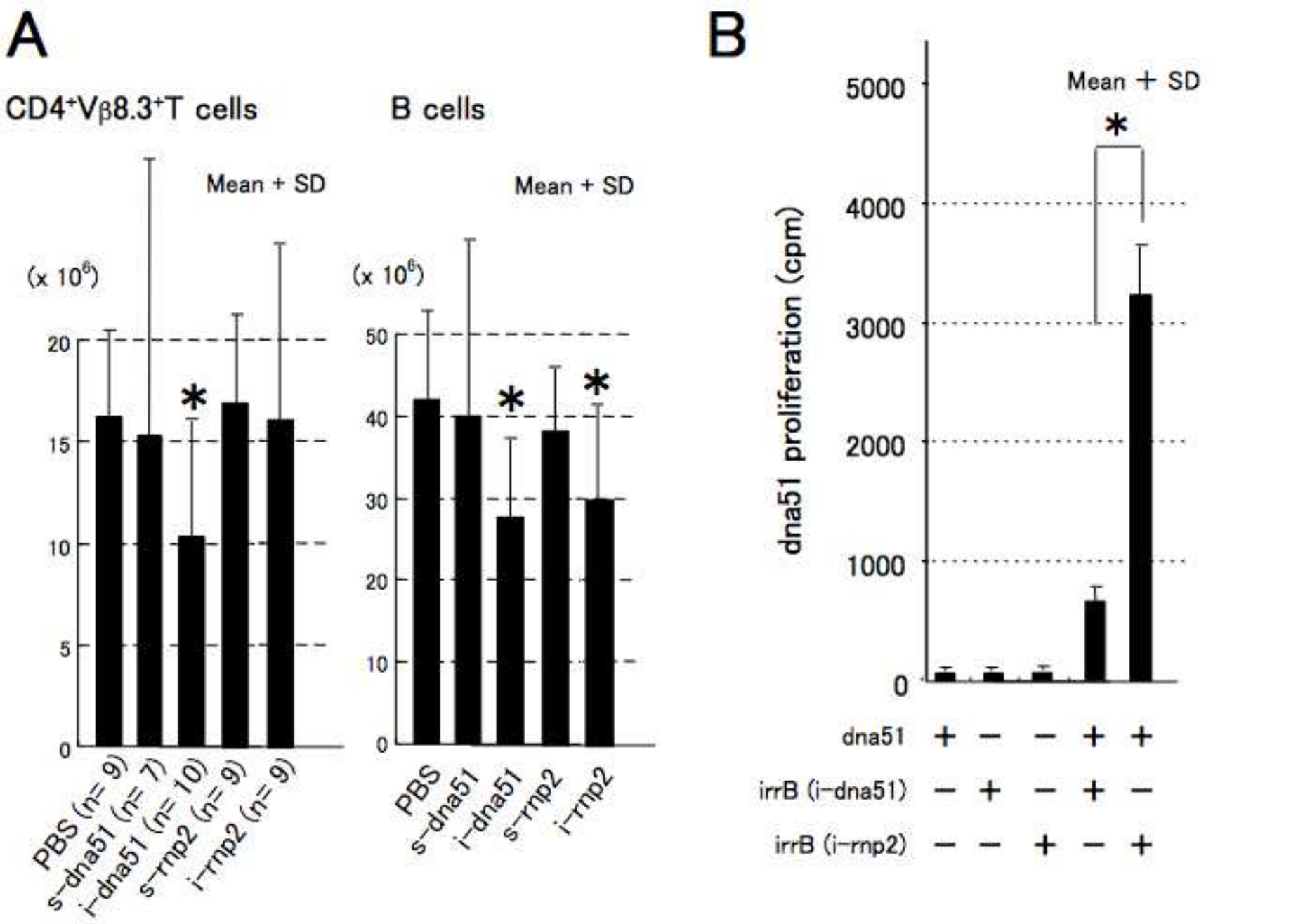

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Fig. 6

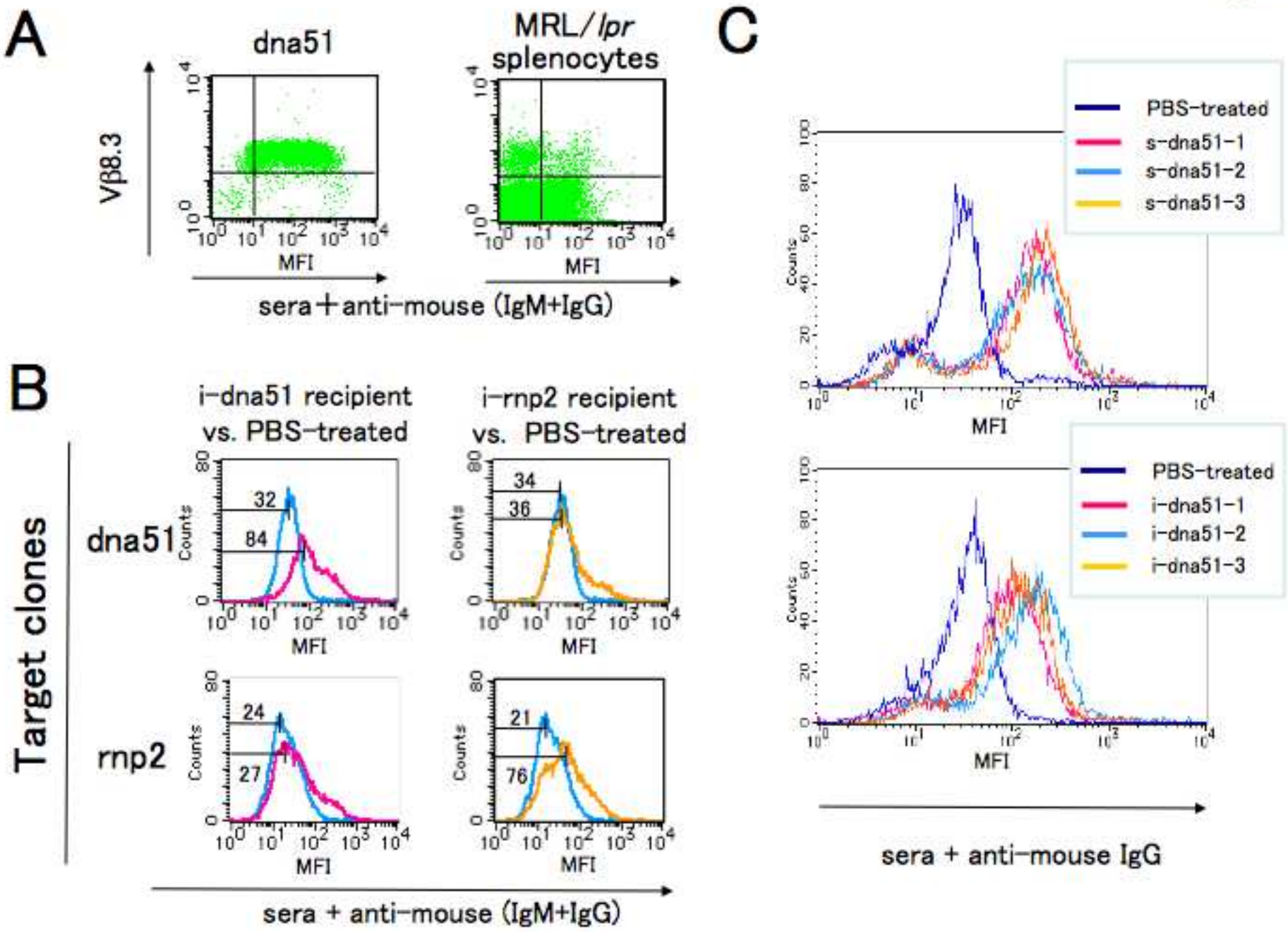

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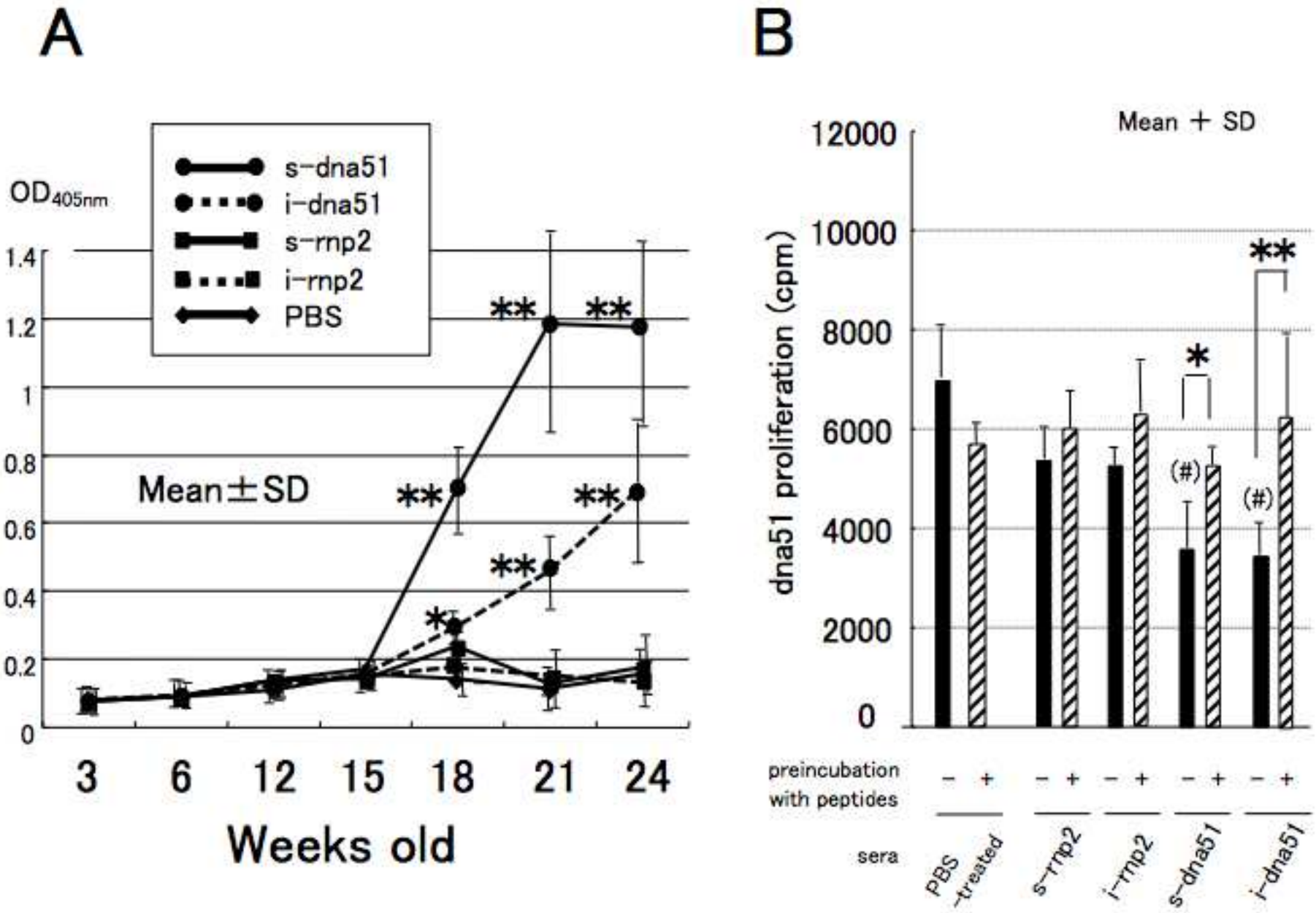
Fig. 7



Figure(s)
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Fig. 8