Immunization with DNA Topoisomerase I Induces Autoimmune Responses But Not Scleroderma-like Pathologies in Mice

PAUL Q. HU, ARTHUR A. HURWITZ, and JOOST J. OPPENHEIM

ABSTRACT. ***Objective.*** Anti-DNA topoisomerase I (anti-topo-I) antibody is a marker of systemic sclerosis (SSc). Anti-topo-I antibody levels are positively correlated with both disease severity and activity. However, its pathogenic role in SSc remains unclear. We investigated whether induction of an autoreactive antibody response is directly pathogenic in mice.

Methods. Autoimmune responses were induced in mice immunized with human recombinant topo-I (rhutopo-I). Both humoral and T cell-mediated autoimmune responses were assessed. Necropsy analyses were performed to determine pathologic changes in immunized mice.

Results. Autoimmune prone SJL and non-obese diabetic mice developed higher humoral autoreactive responses against mouse topo-I than did BALB/c and C56BL/6 mice. Splenic T cells also showed proliferative responses and interferon-γ secretion in response to rhutopo-I. However, serum anti-topo-I antibody levels declined 2 months after the initial immunization. Neither weight loss nor dermal thickening was observed in mice during a followup period of 9 months. Whole-body necropsy analyses, including skin, lung, heart, kidney, gastrointestinal tract, and joints, showed no typical findings of human SSc. Coadministration of anti-CD25 and anti-CTLA-4 antibody with the initial immunization resulted in higher titers of anti-topo-I antibody, but these mice also did not develop SSc-like pathologic features. Development of an anti-topo-I response was not associated with acceleration of the recognized abnormalities in tight-skin mice.

Conclusion. Although tolerance was broken and anti-topo-I antibody was induced by immunization with rhutopo-I in mice, induction of this antibody was not sufficient to induce SSc-like disease. (First Release Oct 15 2007; J Rheumatol 2007;34:2243–52)

Key Indexing Terms:
DNA TOPOISOMERASE I ANTIBODY AUTOIMMUNITY SKIN SYSTEMIC SCLEROSIS

Autoimmune responses with high levels of circulating autoreactive antibodies are commonly detected in patients with rheumatic diseases including rheumatoid arthritis[1-3], Sjögren's syndrome[4], systemic lupus erythematosus (SLE)[5,6], polymyositis, and systemic sclerosis/scleroderma (SSc)[7,8]. Specific autoreactive antibodies are often associated with certain autoimmune diseases or with clinical subsets of a particular disease. For example, anti-double-stranded DNA (dsDNA) antibodies are often detected in patients with SLE[5]. Anti-Jo-1 antibody (histidyl-transfer RNA synthetase) is unique for polymyositis[9]. Anti-DNA topoisomerase I antibody (anti-topo-I) is considered a specific marker for SSc[10]. The association of a specific autoantibody with a distinct disease phenotype has been an intriguing feature of autoimmune connective tissue diseases. The underlying mechanism of induction of anti-topo-I autoantibody production and its potential contribution to the pathogenesis of SSc remains unclear.

Antibody against topo-I is detected more frequently in SSc patients with diffuse (widespread) cutaneous thickening than in those with limited cutaneous thickening[7,11]. In patients with SSc the presence of anti-topo-I antibody is associated with peripheral vascular disease (digital pitting scars), pulmonary interstitial fibrosis, cardiac involvement, coexisting malignancies[12], and HLA-DRB1*11[13]. Recently, we and others have demonstrated a positive correlation between levels of serum anti-topo-I antibody and both disease severity and activity in patients with SSc[14,15]. We and others also showed that autoreactive T cells specific for topo-I can become clonally expanded in patients with SSc, but not in healthy controls[14-16]. In addition, anti-topo-I antibodies have been detected in tight-skin mice (Tsk mice), a mouse scleroderma model[17-19]. These studies clearly indicate a close relationship of autoimmune responses to the pathogenesis of SSc. They have motivated us to investigate whether induction of this humoral autoimmune response can also trigger tissue pathologic changes resembling those of idiopathic SSc.

DNA topoisomerase I is a ubiquitous autoantigen and an

From the Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland, USA.

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P.Q. Hu, PhD, CRTA Fellow; A.A. Hurwitz, PhD, Principal Investigator; J.J. Oppenheim, MD, Laboratory Chief, Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick Cancer Research and Development Center.

Address reprint requests to Dr. J.J. Oppenheim, Laboratory of Molecular Immunoregulation, Center for Cancer Research, National Cancer Institute, Building 560, Room 21-89, Frederick, MD 21702-1201, USA.

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indispensable enzyme involved in DNA replication and protein transcription[20]. Autoimmune responses against topo-I are only detected in the serum of patients with SSc but not in healthy controls[7-11]. The topo-I amino acid sequences are highly conserved in mammals. Human topo-I is a heavily charged nuclear protein with 765 amino acid residues[21]. This enzyme has over 93% sequence identity to the 766 amino acid residues of mouse topo-I (GenBank L20632). It has been demonstrated that immunization using a mutated self-antigen is capable of inducing a more potent autoreactive response than using the *bona fide* autoantigen in mice[22]. Therefore, we immunized mice with recombinant human topo-I (rhutopo-I)[14]. Since different mouse strains are known to respond differently to the induction of autoimmune responses, we compared responses to immunization of autoimmune-prone nonobese diabetic (NOD) and SJL mice to those of BALB/c and C57BL/6 mice.

In our study, mice immunized with human topo-I developed a humoral immune response that was cross-reactive to mouse topo-I, and the magnitude of this response diminished over time. Our results illustrate that tolerance to a nuclear autoantigen can be overcome by immunization with an altered self-antigen. However, induction of this autoreactive antibody specific for a nuclear antigen was not sufficient to induce an autoimmune connective tissue disease.

MATERIALS AND METHODS

Mice. Age and sex matched SJL/Jcr, BALB/cAnNCr, C57BL/6NCr mice (6–8 weeks old) were purchased from the Animal Production Area, Charles River Laboratories Inc. at NCI-Frederick (Frederick, MD, USA). NOD/LtJ and B6.Cg-Fbn1^{Tsk}+/+ Pldn^{pa}/J mice (6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mouse experiments were approved and performed in accord with an animal-use protocol approved by the Animal Care and Use Committee at the National Cancer Institute.

Histology. Mouse tissues and organs were harvested and fixed in 10% neutral buffered formalin. Five micrometer paraffin sections were made and stained with hematoxylin and eosin (H&E). Tissue sections were examined by a trained pathologist at the Pathology/Histotechnology Laboratory, SAIC-Frederick, NCI-Frederick.

Purification of topo-I antigens. The rhutopo-I and the domain-fusion proteins (topo-I domains) were produced in Sf9 insect cells infected with recombinant baculovirus and purified using a combination of columns in a fast protein liquid chromatography (FPLC) system as described[14]. Proteins were dialyzed against phosphate buffered saline (PBS) overnight and were stored at –70°C.

Immunization protocols. Mice were immunized subcutaneously on their lower back with 100 µg rhutopo-I emulsified in a total volume of 200 µl complete Freund's adjuvant (CFA) prepared using a high-speed tissue homogenizer. After the initial immunization, mice were boosted twice with rhutopo-I emulsified in incomplete Freund's adjuvant (IFA) at an interval of about 2 weeks. In some experiments, after the second immunization boost, mice received additional immunization boosts with rhutopo-I in IFA. Blood samples were collected by retroorbital bleeding or by bleeding through the tail vein.

In some experiments, mice were treated with antibodies to eliminate regulatory T cells and to block signaling of CTLA4 for further enhancing immune responses. These mice were injected intraperitoneally with 200 µl of diluted ascites of anti-CD25 antibody (clone PC61) at Day 5 and Day 3 before the first immunization and with 200 µl of diluted ascites of anti-CTLA4 antibody (clone 9H10). The antibody-containing ascites were produced according to a described protocol[23].

Anti-topo-I ELISA. ELISA procedures were as described[14] with slight modifications of using 1:5000 horseradish peroxidase conjugated goat anti-mouse Fc antibody for the detection of mouse IgG antibody (Amersham Biosciences UK Ltd., Little Chalfont, England). The rhutopo-I was used to coat the ELISA plates. ELISA plates were developed with TMB substrate (Amersham Biosciences UK). For easy comparison, we measured all serum samples against a pooled sera sample from a group of NOD mice as a standard serum sample. One anti-topo-I antibody unit was defined as the OD_{405} reading of 1/1,000,000 dilution of the serum standard.

Immunoprecipitation. The immunoprecipitation procedures were as described with a slight modification for detecting mouse topo-I[24]. In these experiments, NIH3T3 mouse fibroblast cells were used as the source of mouse topo-I. Ten microliters of serum samples were mixed with 20 µl protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for each sample. The dried gel was exposed to a phosphor screen and the exposure was determined in a Typhoon 8600 Imager (Molecular Dynamics, Piscataway, NJ, USA).

Western blot. The Western blot was essentially as described[24], except NIH3T3 cells were used as the source of antigen for mouse topo-I.

Immunofluorescence staining. NIH3T3 cells were seeded at a concentration of 20,000 per well in a Lab-Teck® II Chamber Slide (Nalge Nunc International, Naperville, IL, USA). Cells were fixed with 2% paraformaldehyde and were permeated with staining buffer (PBS containing 2% FBS plus 0.1% saponin; Sigma, St. Louis, MO, USA). After staining, pictures were taken immediately with identical settings under a fluorescence microscope for all samples.

T cell proliferation. Mouse spleen cells were dispersed by gently pressing spleens in a circular motion between the frosted ends of 2 slides in ice-cold Hanks' balanced salt solution. Total spleen cells were filtrated through a 100 µm nylon screen and washed twice in PBS. Total spleen cells were plated at a concentration of 200,000 cells/well and cultured in RPMI-1640 media supplemented with 10% FBS and penicillin/streptomycin solution for 7 days. In the final 16 h of incubation, 1 µCi of ^{3}H-thymidine was added into each well. Cells were then harvested onto a 1450 glass-fiber membrane using a 1450 MicroBeta Plate Harvester and ^{3}H-thymidine incorporations were counted in a 1450 Trilux MicroBeta Plate Reader (Trilux, Turku, Finland).

ELISPOT. ELISPOT kits for mouse interferon-γ (IFN-γ) and interleukin 5 (IL-5) were purchased from Mabtech Inc. (Mariemont, OH, USA). For positive controls, ionomycin and phorbol myristic acetate were added at a final concentration of 2 µM and 40 ng/ml, respectively. Spots were counted using an Immunospot Analyzer (Cellular Technology, Cleveland, OH, USA) and results typically varied by less than 10% for duplicate wells.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism 4.0 for Windows (GraphPad, San Diego, CA, USA).

RESULTS

Mice immunized with purified rhutopo-I developed marked anti-rhutopo-I IgG responses. Mice were immunized with rhutopo-I emulsified in CFA and boosted with rhutopo-I emulsified in IFA. Antibody responses were measured by anti-topo-I antibody ELISA as described[14] with modification for the detection of mouse anti-topo-I IgG. All serum samples from mice immunized with rhutopo-I were positive for anti-topo-I antibody as compared to naive mice (Figure 1A). We compared all serum samples against a defined anti-topo-I serum sample as a standard, which consisted of pooled serum samples from a group of rhutopo-I-immunized NOD mice (see Materials and Methods). Mice immunized with rhutopo-I developed high anti-topo-I antibody titers, between 5×10^5 to 10^6 in NOD and SJL mice and 10^4 to 10^5 in BALB/c, C57BL/6 mice. The 1-log unit higher anti-topo-I antibody responses developed by the NOD and SJL mice as compared

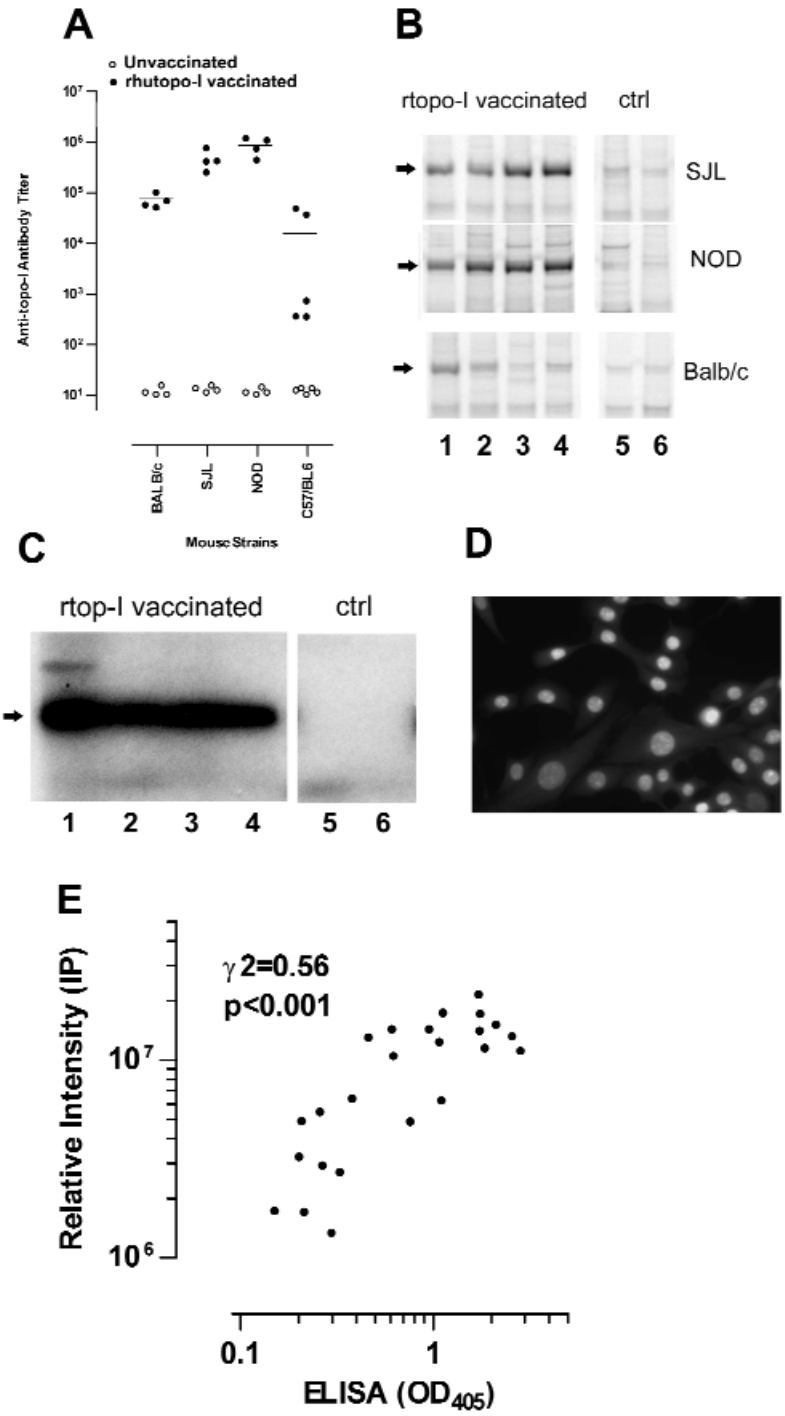

Figure 1. Development of anti-mouse topo-I antibody in mice immunized with rhutopo-I in CFA and boosted twice with rhutopo-I in IFA at an interval of 2 weeks. Two weeks after the second boost, serum samples were taken and analyzed for anti-topo-I antibody titer by ELISA. Each dot represents the titer of an individual mouse (A). Antibodies were cross-reactive to mouse topo-I by the immune-precipitation (IP) using ^{35}S metabolic labeled NIH3T3 (a murine cell line) cell lysate as the source of antigen only in mice immunized with rhutopo-I (lanes 1-4) but not in controls (those immunized with PBS) (lanes 5-6) (B). The antibody cross-reactivity to mouse topo-I in rhutopo-I immunized Balb/c mice was further confirmed by Western blot using the whole cell lysate prepared from NIH3T3 cells (C). A representative immunohistochemical staining using the serum of a rhutopo-I immunized SJL mouse (diluted at 1:50) in NIH3T3 cells was positive (D). The anti-topo-I antibody levels measured by ELISA using rhutopo-I and by immune-precipitation (IP) with ^{35}S labeled murine topo-I were correlated (E).

to the BALB/c and C57BL/6 mice may be related to their increased likelihood of developing autoimmune diseases. Although NOD mice have been reported to produce a number of autoreactive antibodies[25], we did not detect any anti-topo-I antibody in age and sex-matched NOD mice or other mouse strains immunized with adjuvant only or with ovalbumin as a control antigen (Figure 1A).

Immunization with rhutopo-I-induced anti-mouse topo-I responses. We investigated whether the antibody responses induced against human rhutopo-I were cross-reactive with mouse topo-I. Serum samples from rhutopo-I-immunized SJL and NOD precipitated a ^{35}S labeled protein corresponding to a 100 kDa protein, which is the expected molecular weight of mouse topo-I in an immunoprecipitation assay using a cell lysate of NIH3T3 cells, a mouse fibroblast cell line (lanes 1–4 in top 2 panels of Figure 1B). In contrast, samples from unimmunized mice were not reactive with the same protein band using the same lysate, with only some very faint background bands, which were likely due to precipitated nonspecific ^{35}S labeled proteins (lanes 5 and 6 in the top 2 panels of Figure 1B). Under identical conditions, serum samples from rhutopo-I-immunized BALB/c mice were weakly reactive with this protein (lanes 1–4 in bottom panel of Figure 1B). This low reactivity of anti-topo-I antibody to mouse topo-I in the BALB/c mice correlated with the lower anti-topo-I antibody titers in these mice as determined by ELISA (Figure 1A). We next performed Western blots using whole NIH3T3 cell lysate as the source of mouse antigens to confirm if these BALB/c serum samples were indeed reactive to the same protein. All serum samples from immunized animals were reactive with mouse topo-I on a Western blot, as shown by serum sample from rhutopo-I-immunized BALB/c mice in lanes 1–4 of Figure 2B, but not controls (lanes 5 and 6 in Figure 1C). Thus both immunoprecipitation and Western blotting demonstrated that antibody developed after immunization with rhutopo-I in all challenged mouse strains.

To confirm the production of autoantibody against the mouse topo-I in rhutopo-I-immunized mice, we tested serum reactivity on fixed NIH3T3 cells. All serum samples from rhutopo-I-immunized mice produced the typical anti-topo-I immunofluorescence staining in both HEK293 cells and in NIH3T3 cells. A representative immunohistochemical staining of NIH3T3 cells with a rhutopo-I-immunized SJL mouse serum sample is shown in Figure 1D. A representative serum sample from a control SJL mouse (immunized with ova as a control antigen) was negative under the same conditions (results not shown). These results further confirmed the development of anti-topo-I antibody in mice immunized with rhutopo-I.

Based on the results from ELISA, immunoprecipitation, Western blotting, and immunohistochemical analysis, we concluded that mice immunized with rhutopo-I developed autoantibody against mouse topo-I. Although we evaluated a variety of immunoassays for the detection of anti-topo-I antibody, ELISA was both sensitive and effective for this purpose. Results from the ELISA correlated very well with the immunoprecipitation results ($\gamma^2 = 0.56$ and $p < 0.001$, Figure 1E). We therefore used ELISA for the measurement of anti-topo-I antibody in subsequent experiments.

Multiple epitopes on topo-I were targeted. After topo-I was originally identified as an autoantigen in SSc and its cDNA sequence was cloned[21], a number of "major" antibody epitopes on different portions of topo-I were reported and their contributions to the development of anti-topo-I autoimmunity were examined[17,26-30]. While it is still not clear which particular epitope on topo-I is related to the development of SSc, we have demonstrated that human anti-topo-I antibodies target multiple epitopes, mainly on the DNA binding domains, and that this pattern remains unchanged during the course of the disease[24]. We proceeded to determine if the anti-topo-I antibodies developed in mice also target the DNA binding domains of topo-I.

We took advantage of previously established ELISA to determine antibody molecular reactivity patterns in mice. Anti-topo-I antibodies from different mouse strains showed distinctive reactivity patterns. Antibodies produced by BALB/c mice were targeting the core domain I (aa 207–441) of topo-I (Figure 2A). However, antibodies from C57BL/6 mice showed a lower reactivity toward the same core domain of topo-I (Figure 2B). In NOD and SJL mice, the mouse antibodies were quite distinct and markedly reactive to the core domains II and III (aa 433–636) and the linker-C-terminal domain (aa 625–765; Figure 2C, 2D). Notably, although different antibody reactivity patterns are seen in different strains of mice, all the mouse anti-topo-I antibodies were reactive with the N-terminal domain (aa 1–213; Figure 2A-2D). In addition, mouse anti-topo-I antibodies are also directed against multiple epitopes on the DNA binding domains of topo-I, which is similar to the antibody reactivity pattern of human anti-topo-I antibody.

T cells responded to rhutopo-I in immunized mice. Anti-topo-I IgG responses in mice are indicative of the activation of antigen-specific T helper cells. We performed lymphocyte proliferation and ELISPOT assays for IFN-γ to detect these cellular immune responses. As shown in Figure 3A, spleen cells from a representative group of immunized mice exhibited a dose-dependent T cell proliferative response to rhutopo-I. Lymph node cells from rhutopo-I-immunized mice also showed proliferative responses to rhutopo-I. We did not detect any T cell response to rhutopo-I in control mice immunized with either PBS or ova (data not shown). Spleen cells demonstrated a proliferative response to topo-I and also secreted IFN-γ when stimulated with rhutopo-I, as shown by an ELISPOT assay in Figure 3B. Together, these results demonstrated that T cells of immunized mice responded to rhutopo-I.

Changes of anti-topo-I antibody levels in mice immunized with rhutopo-I. We collected serial serum samples from immunized mice and determined anti-topo-I antibody levels over time. Anti-topo-I antibodies were detectable at Week 3

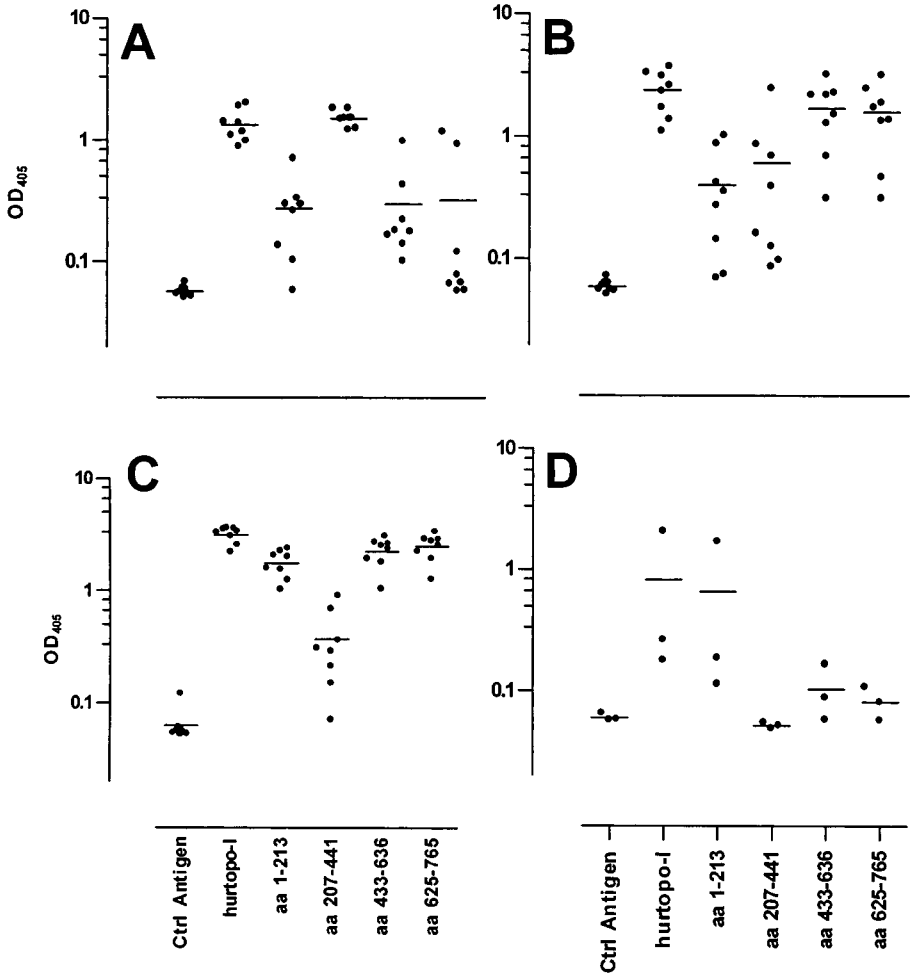

Figure 2. Multiple epitope regions on topo-I were targeted by autoantibodies from mice immunized with rhutopo-I. Epitope regions recognized by antibodies were determined by ELISA either using the rhutopo-I or using individual topo-I domains. The reactivity pattern of serum samples from rhutopo-I-immunized BALB/c (A), SJL (B), NOD (C), and C57BL/6 (D) mice to different epitope regions of topo-I were presented as OD_{405} values.

(Day 22) after the initial immunization. The antibody titer increased up to 2 log units by 7 days after the second boost on Day 36, and remained stable over the next month, declining thereafter in both BALB/c mice (Figure 3C) and SJL mice (Figure 3D). Thus the level of anti-topo-I antibody is not self-perpetuating but is dependent on persistent stimulation by rhutopo-I.

Effect on anti-topo-I antibody titers in SJL mice of additional immunization boosts or treatment with anti-CD25 antibody plus anti-CTLA4 antibody. We tried to prolong and further enhance the levels of anti-topo-I antibody induced in SJL mice by boosting these mice with additional immunization with rhutopo-I in IFA every 2 months after the second boost. These mice maintained their levels of anti-topo-I antibody for a longer period of time of up to 180 days (Figure 4A).

In addition to administering additional immunizations, we tried to enhance this anti-topo-I antibody response by inhibiting some of the suppressive immunoregulatory mechanisms. It has been reported recently that the *in vivo* elimination of CD25-positive regulatory T cells accelerated development of autoimmune diseases in a number of animal models[31], and that blockade of CTLA-4 signaling on antigen-activated T cells enhanced anti-tumor immune responses[32]. We therefore administered antibodies against CD25 (clone PC61 to deplete Tregs) and CTLA4 (clone 9H10 to block inhibitory signal of activated T cells) to enhance anti-topo-I autoimmune response. Treatment with anti-CD25 antibody significantly decreased the percentage of CD25-positive cells from over 5% to 0.5–1% in spleen and lymph node lymphocyte populations (data not shown). These treatments resulted in 5.1-fold increase of antibody titer ($p < 0.05$) in a group of SJL mice and 1.7-fold increase of anti-topo-I antibody titer ($p = 0.12$) [which was not statistically significant for this small group of sample ($n = 4$)] (Figure 4B). Interestingly, this same treatment did not alter the antibody titer ($p = 0.4$) in BALB/c mice (Figure 4B). In both SJL and NOD mice, antibody levels still

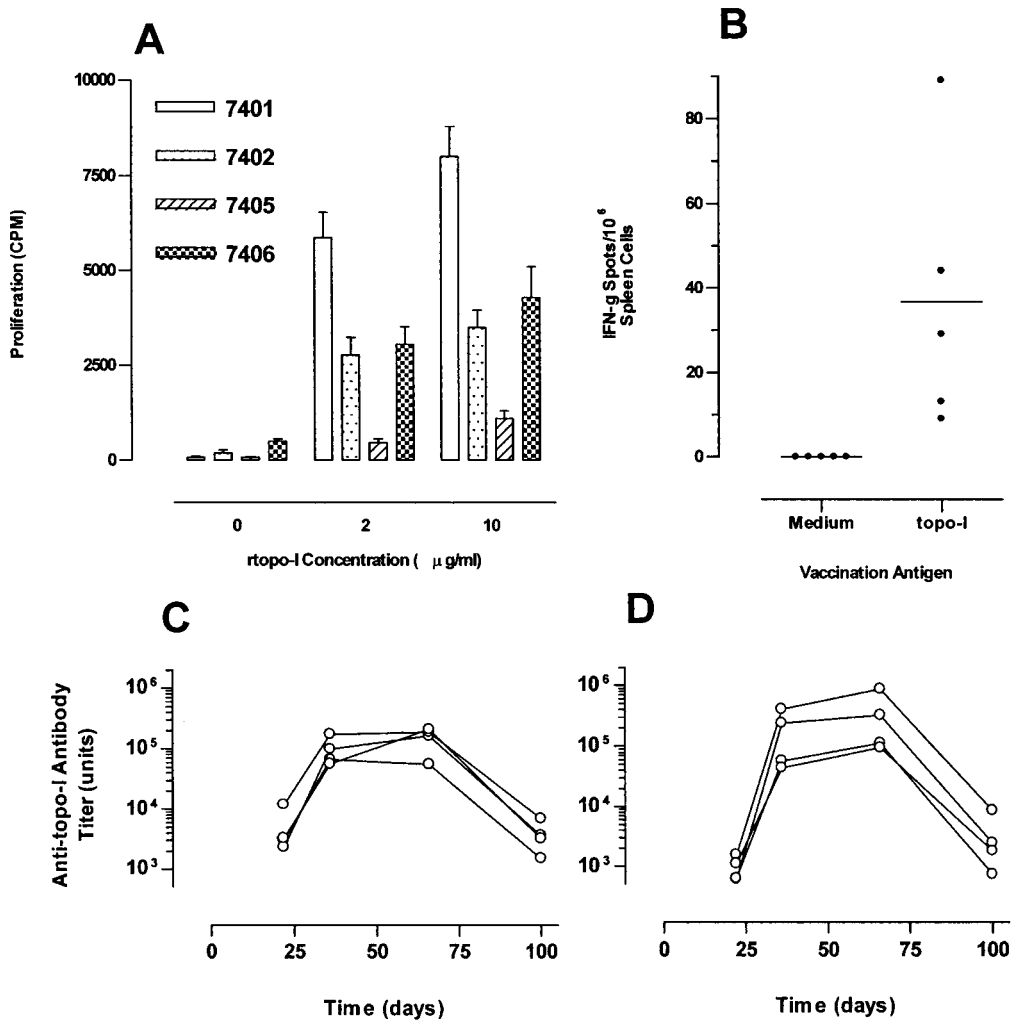

Figure 3. Responses of splenic T cells to topo-I and the levels of anti-topo-I Ab over time in immunized mice. A group of Balb/c mice were immunized with rhutopo-I for 180 days and an additional immunization boost 2 weeks before the experiment. Splenic T cells from these mice were isolated and stimulated with rhutopo-I *in vitro* and cell proliferation was determined by ^{3}H labeled thymidine. Each bar represents the T cell proliferation (an average of 3 triplicate wells) from an individual mouse (A). The secretion of murine IFN-γ of spleen T cells from a group of SJL mice immunized with rhutopo-I was determined by an ELISPOT assay (B). Anti-topo-I antibody levels in rhutopo-I immunized Balb/c (C) and SJL (D) mice were measured at different times by the ELISA.

declined by 2 months, yielding a pattern similar to that of untreated mice, as shown in Figure 3C and 3D.

Effect of immunization with rhutopo-I on tissue histology. Our results showed the development of anti-topo-I autoimmune responses in immunized mice. We investigated whether mice with autoimmune responses to topo-I developed pathological changes resembling human SSc. We examined mice 1–2 times every week for changes in body weight. Mice immunized with rhutopo-I (Figure 5A) or with ova (Figure 5B) exhibited no weight loss during an observation period of up to 200 days after the initial immunization. Skin samples were taken from the upper back, close to the neck, of each immunized mouse and were sectioned and stained with H&E. Dermal thicknesses were measured using a microscope. As shown in Figure 5C, the mean dermal thickness was similar in the 2 groups of SJL mice immunized with either rhutopo-I or ova. We also performed total-body necropsies and examined the heart, kidney, liver, lungs, esophagus, stomach, intestine and colon, foot joints, spleen, and lymph nodes in SJL, BALB/c, and NOD mice 4 months after immunization and in the group of SJL mice that received repeated immunization boosts for an extended period of time. All tissue sections were examined by a veterinary pathologist. No abnormalities characteristic of SSc were detected in the immunized mice. Further, those immunized SJL and NOD mice, treated with antibodies against CD25 and CTLA-4, had increased levels of anti-topo-I antibody but still did not develop scleroderma-like skin or pathologic changes in other organs.

Anti-topo-I autoimmune responses in Tsk mice. Tsk mice are considered a mouse SSc model[33,34]. Anti-topo-I antibodies have been detected more frequently in older Tsk mice (over 10 months old)[17]. We tested the hypothesis that induction of an

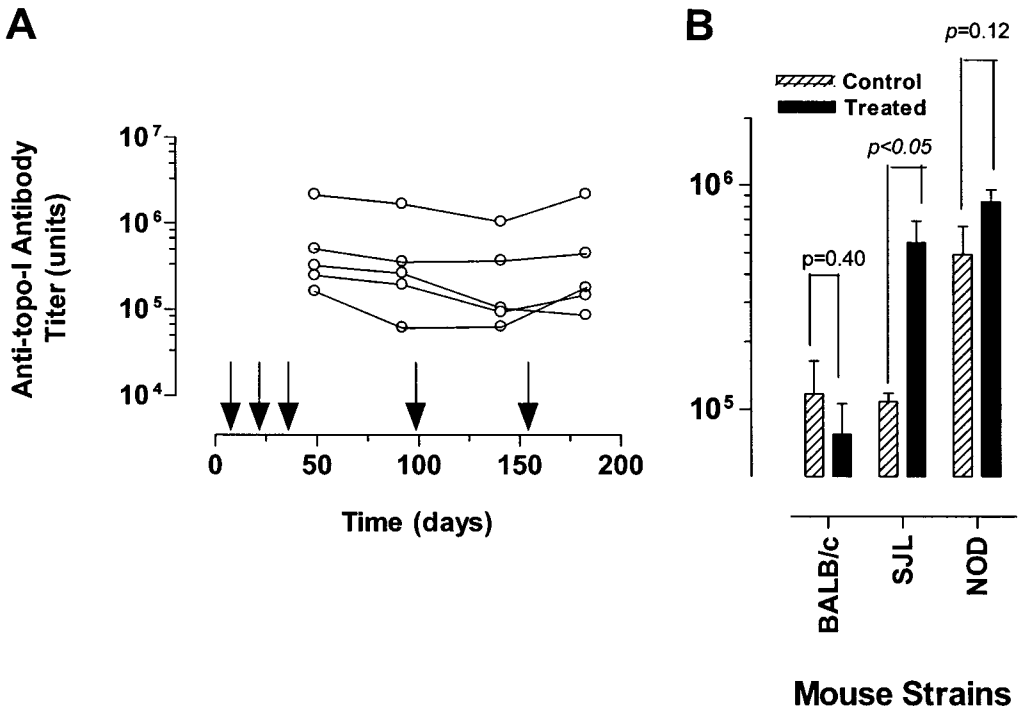

Figure 4. Anti-topo-I antibody levels of mice received additional immunization or treated with anti-CD25 antibody plus anti-CTLA4 antibody. Antibody levels of a group of SJL mice (each line represents the antibody levels of a single mouse over time) received additional immunizations with rhutopo-I in IFA around Day 100 and Day 150 were determined by the ELISA (each arrow indicates a single immunization) (A). The antibody levels were compared in Balb/c, SJL and NOD mice treated with or without anti-CD25 before immunization plus anti-CTLA4 antibody after immunization (n = 4) (B).

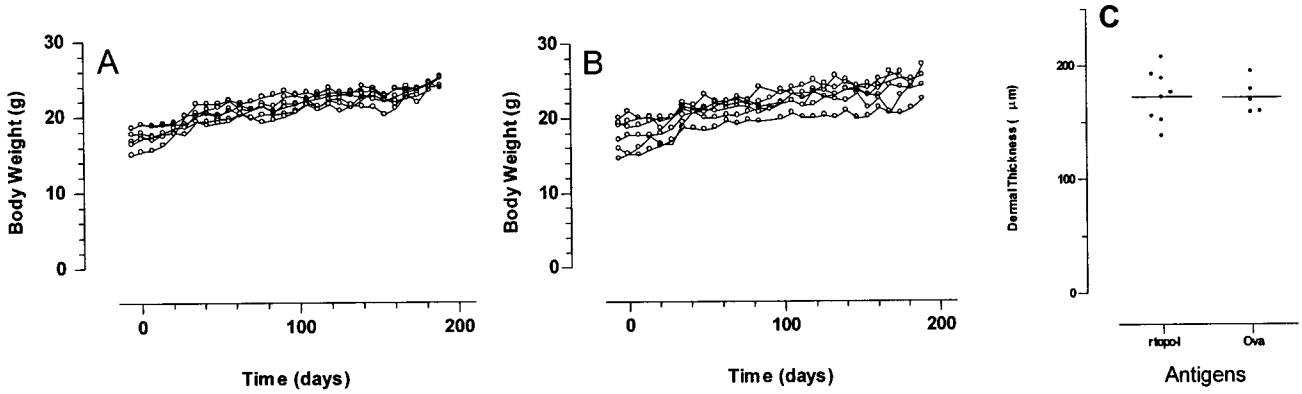

Figure 5. Changes in body weight over time and skin thickness in SJL mice immunized with rhutopo-I or with ova. The body weights of mice immunized with rhutopo-I (A) or with ova (B) were recorded and plotted over time. Each dot represents the weight of a single animal at a certain timepoint. Back skin samples of 2 groups of SJL mice immunized with rhutopo-I or with ova were sampled and sectioned. The skin thicknesses of each group were measured under a microscope. Each closed circle represents the average dermal thickness of 10 skin sections from a single mouse (C).

anti-topo-I autoimmune response will accelerate dermal thickening in Tsk mice.

Tsk mice were immunized with either rhutopo-I or with ova. In contrast to some reports showing naturally occurring anti-topo-I antibody in Tsk mice[17], we did not detect any anti-topo-I antibody by ELISA in Tsk mice younger than 8 months of age immunized with ova (data not shown). After immunization, rhutopo-I-immunized Tsk mice developed anti-topo-I antibody and showed a molecular reactivity pattern (Figure 6A) similar to that of C57BL/6 mice (Figure 2D). The anti-topo-I antibody levels diminished in all Tsk mice beginning at 100 days after immunization (Figure 6B). The antibody levels showed a trend to decrease over time ($p = 0.0027$, one-way ANOVA of linear trend test). The levels of antibody at Day 134 and Day 168 were significantly lower than that at Day 35 ($p < 0.05$). Multiple skin samples were obtained and exam-

ined, but no increased dermal thickness or subdermal hyperplasia was observed in Tsk mice immunized either with rhutopo-I or with ova during a period of over 6 months after the initial immunization (Figure 6C). The dermal thickness of a Tsk mouse (Figure 6D) was similar to that of a SJL mouse as shown in Figure 6E.

DISCUSSION

The role of antinuclear antibodies in the development of autoimmune connective tissue diseases remains under investigation. Clinical evaluation[35,36] and studies of animal models[37] have demonstrated a positive correlation between induction of humoral autoimmune responses and the development of disease. However, recent studies have shown that induction of mouse lupus-like disease can be dissociated from the development of certain autoreactive antibodies in a genetic linkage model[38,39] and in a TLR9 knockout model[40,41]. In these model systems, the development of mouse lupus does not require the production of anti-dsDNA antibody. These 2 recent studies demonstrate that a humoral autoimmune response, in this case antibody against ds-DNA, is not required for the development of lupus in mouse lupus models. These studies do not address whether a particular humoral autoimmune response can trigger autoimmune disease.

We investigated whether initiating an autoimmune response against mouse topo-I can induce pathologic manifestations resembling human SSc. Mice clearly developed high levels of antibody against mouse topo-I in response to immunization with rhutopo-I. However, this autoimmune antibody response diminished after 2 months and was not accompanied by the pathology observed in human SSc of skin thickening or pulmonary fibrosis over an extended observation period of 6–8 months after immunization. In addition, induction of an anti-topo-I autoimmune response did not accelerate the dermal thickening in Tsk mice, a mouse model of SSc. Our results show that anti-topo-I autoimmune responses diminished following cessation of immunization. The induction of humoral and cellular autoimmune responses

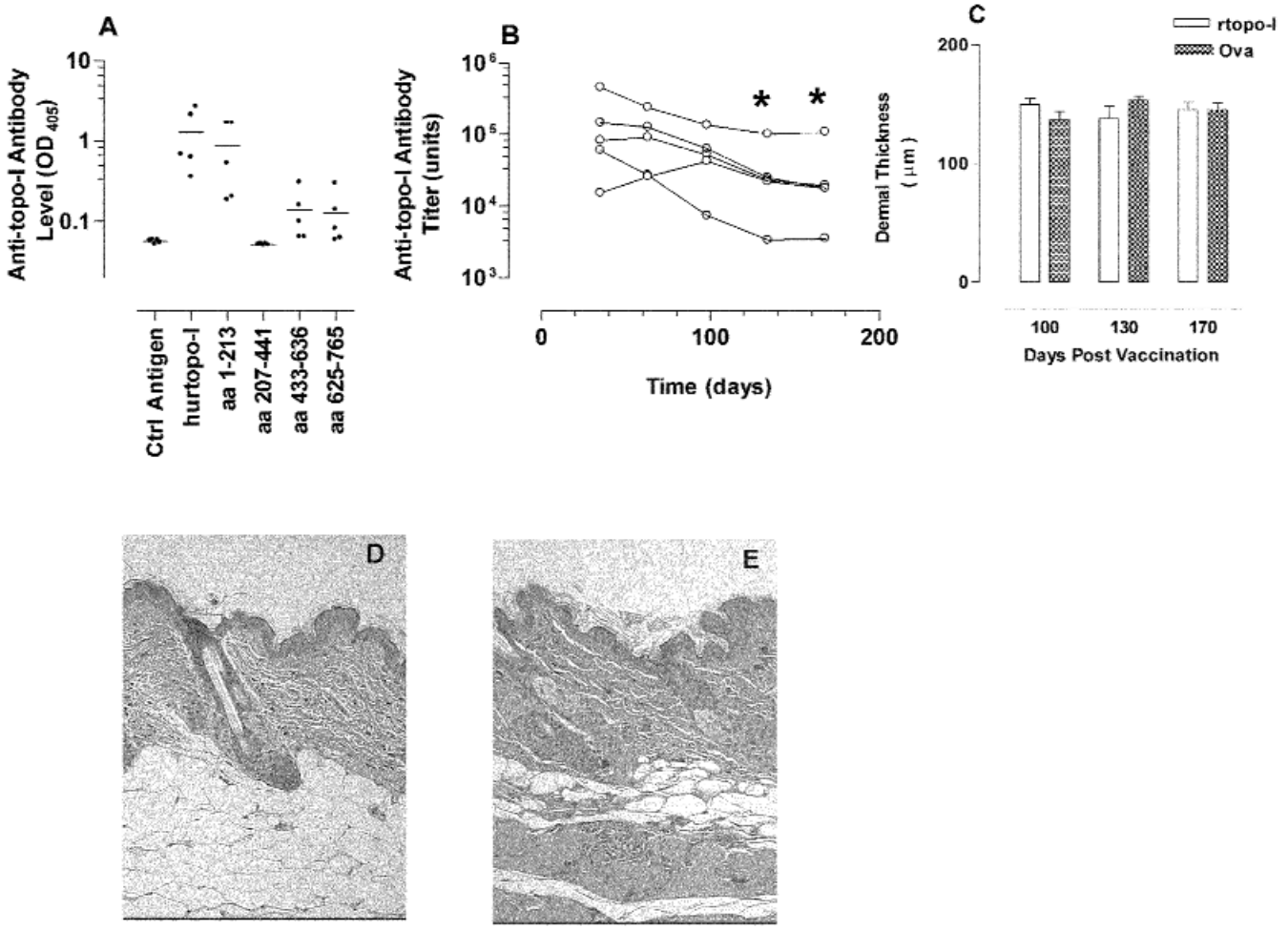

Figure 6. Anti-topo-I autoimmune responses in Tsk mice. Antibody levels and reactivity patterns of rhutopo-I in immunized Tsk mice were measured by ELISA (A). Antibody levels were determined over time in immunized Tsk mice and showed decrease at Day 134 and 168 as compared to that of Day 35 (B). The dermal thickness of Tsk mice immunized with rhutopo-I were measured and compared to those mice immunized with ova (C). The dermal thickness of a Tsk mouse (D) was comparable to that of a SJL mouse (E). * $p < 0.05$, Dunnett's Multiple Comparison Test

against topo-I was not sufficient to trigger SSc-like diseases in mice with different genetic backgrounds including BALB/C, C57/BL6, SJL, NOD, and Tsk mice. These different strains of mice are known to have different susceptibility to the induction of autoimmune diseases and respond differently to immunizations. This result is consistent with a recent article[42] showing an overexpression of CD19 increased anti-topoisomerase I antibody levels, but failed to accelerate skin fibrosis. Additional signals may be required to induce SSc disease.

In addition to vaccinating mice with rhutopo-I in CFA, we further enhanced autoantibody response by suppressing T regulatory cell effects with anti-CD25 and anti-CTLA4. The treatment enhanced anti-topo-I antibody response in NOD and SJL mice, but not in the BALB/c mice. Presumably the autoimmune response is not as stringently regulated in autoimmune mice as in BALB/c mice. Nevertheless inhibition of the regulatory T cells that suppress immune responses and the blockade of CTLA-4 signaling reduced the negative regulation of T cell response. Both treatments are reported to enhance T cell-mediated autoimmune responses[23,31,32]. Our results demonstrated that such an enhancement of autoreactive T cell response can increase but not prolong antinuclear autoreactive antibody levels. However, we still could not detect any pathologic changes related to the SSc over a period of 6 months after the initial immunization and antibody treatments. It is noteworthy that the effect of antibody treatment in our studies may not have been persistent. The anti-CD25 antibody was a rat monoclonal antibody, whereas 9H10 antibody was a hamster monoclonal antibody. We only treated mice with antibodies at the beginning of the immunization based on the concern that repeated exposure to these antibodies may induce neutralizing antibodies against the CTLA-4 and that, after the initial immunization, anti-CD25 antibodies may eliminate activated antigen-specific T cells. The effects of these treatments therefore may have been somewhat transient. It is possible that treatment with mouse monoclonal antibodies will result in even greater anti-topo-I autoimmune responses, but these antibodies are not currently available. The longterm effect of anti-topo-I antibodies together with extended removal of regulatory T cells and the blockade of CTLA4 remains to be determined.

We have demonstrated that induction of humoral and cellular autoimmune responses against topo-I did not trigger SSc-like pathology. However, we cannot rule out the possibility that, under certain pathophysiological conditions, induction of these autoimmune responses can lead to development of SSc. For example, it is possible that immunization with immune complexes consisting of autoreactive antibody and autoantigen together with potent adjuvant can stimulate immune response at the site of inflammation and result in some types of tissue damage, whereas our immunization experiment might not recapitulate such *in vivo* pathophysiological conditions. Autoantigens released from apoptotic cells can be recognized by antinuclear antibodies[43]. The complex of autoantigen and nuclear antibody can induce dendritic cell phagocytosis and presentation of nuclear antigen[44]. Although it is not clear whether complexes of autoantigen and autoantibody deposit in dermal tissue of SSc patients, autoantibodies from patients with SSc were shown to be able to stimulate fibroblasts and to induce proadhesive and proinflammatory phenotype[45]. Interestingly, a recent article suggested that complexes of anti-topo-I antibody and topo-I bind to lung fibroblasts and induce monocyte adhesion to lung fibroblasts[46]. We did test whether anti-topo-I antibodies from our immunized mice bind to the surface of a human embryonic fibroblast cell line, WI-38, but with negative results (data not shown). Whether prolonged and/or repeated exposure to complexes of autoantigen and autoantibody can be pathogenic *in vivo* has therefore not been ruled out by our study. Although we succeeded in breaking tolerance to topo-I autoantigens, additional signals needed to induce SSc remain to be identified.

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