FK506 Inhibits Murine AA Amyloidosis: Possible Involvement of T Cells in Amyloidogenesis

MITSUHARU UEDA, YUKIO ANDO, MASAAKI NAKAMURA, TARO YAMASHITA, SHINGO HIMENO, JAEMI KIM, XUGUO SUN, SHIORI SAITO, TAKIKO TATEISHI, JOAKIM BERGSTRÖM, and MAKOTO UCHINO

ABSTRACT. ***Objective.*** To determine the possibility that T cells represent a potential target for therapy in AA amyloidosis.

Methods. AA amyloidosis was induced in C3H/HeN mice by concomitant administration of $AgNO_3$ and amyloid-enhancing factor (AEF). Mice injected with $AgNO_3$ and AEF received intraperitoneal injections of FK506 (2–200 μg/day). The degree of splenic amyloid deposition was determined by Congo red staining. Serum amyloid A (SAA), interleukin 1ß (IL-1ß), IL-6, and tumor necrosis factor-α concentrations were measured by ELISA. AA amyloidosis was also induced in ICR mice by injection of Freund's complete adjuvant (FCA) and *Mycobacterium butyricum* without AEF. ICR mice injected with FCA and *M. butyricum* also received intraperitoneal injections of FK506 (200 μg/day) to eliminate the possibility that FK506 action might depend upon AEF activity in the amyloid formation. Amyloid deposition was also induced with and without AEF in severe combined immunodeficient (SCID) mice and nude mice to clarify the role of T cells in the mechanism of amyloid formation in AA amyloidosis.

Results. FK506 treatment significantly reduced the amount of amyloid deposition and incidence of amyloidosis without reducing serum SAA and proinflammatory cytokine levels in the murine AA amyloidosis models with and without AEF. SCID mice and nude mice showed resistance to development of AA amyloidosis.

Conclusion. Our findings may provide a new therapeutic strategy for amyloidosis. The results suggested that T cells may play an important role in the mechanism of amyloid formation in AA amyloidosis. (First Release Sept 15 2006; J Rheumatol 2006;33:2260–70)

Key Indexing Terms:
AA AMYLOIDOSIS FK506 SERUM AMYLOID A THERAPY

Amyloidoses constitute a group of disorders of protein metabolism in which normally soluble autologous proteins are deposited in tissue as abnormal insoluble fibrils that cause structural and functional disruptions[1-5]. These amyloidoses are usually characterized by extracellular deposition of amyloid in various tissues and are classified as either localized or systemic disorders[1-5]. The mechanism of amyloid formation in tissues remains to be elucidated, although evidence in support of several hypotheses has been provided[1,5,6].

AA amyloidosis can be induced during the clinical course of chronic inflammatory diseases including rheumatoid arthritis (RA), familial Mediterranean fever, inflammatory bowel syndrome, and chronic infections[2,7-11]. AA amyloidosis is a systemic disorder characterized by extracellular deposition of amyloid-containing serum amyloid A (SAA) and amyloid A protein (AA), proteolytically derived fragments of SAA[12]. SAA is an apolipoprotein that circulates in association with high-density lipoprotein particles[13,14]. The serum concentration of this protein, which is normally < 10 μg/ml, sometimes increases more than 1000-fold during inflammation, and exceeds 1 mg/ml[14,15]. SAA is mainly synthesized by hepatocytes, and its expression is stimulated by proinflammatory cytokines such as interleukin 1ß (IL-1ß), IL-6, and tumor necrosis factor-α (TNF-α)[14]. It is well known that RA is the most frequent cause of AA amyloidosis[16]. In patients with RA, the prevalence rate of AA amyloidosis, determined using the abdominal subcutaneous fat aspiration test to detect amyloid deposits, is from 7% to 26%[17]. Previous studies suggested that the primary objective of therapy for AA amyloidosis should be to lower the SAA levels, and thereby to reduce the supply of amyloid fibril precursor proteins, by using steroids, colchicine, and other antiinflammatory drugs[7,10,11].

From the Department of Neurology and Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto; and Clinical Medicine Section, Department of Clinical Medicine, National Institute for Minamata Disease, Kumamoto, Japan.

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M. Ueda, MD; T. Yamashita, MD, PhD, Assistant Professor; M. Uchino, MD, PhD, Professor, Department of Neurology; Y. Ando, MD, PhD, Associate Professor; S. Himeno, MMedSc; J. Kim, MPharm Sci; X. Sun, MD; S. Saito, MPharm Sci; T. Tateishi, MMedSc; J. Bergström, PhD, Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University; M. Nakamura, MD, PhD, General Manager, Clinical Medicine Section, Department of Clinical Medicine, National Institute for Minamata Disease.

Address reprint requests to Dr. Y. Ando, Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-0811, Japan.
E-mail: yukio@kaiju.medic.kumamoto-u.ac.jp

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However, questions remain about the relationship between SAA concentrations and amyloid formation[6,10,18]. Despite high to elevated serum SAA levels, most patients with chronic inflammatory disease never develop AA amyloidosis. Further, amyloidosis expresses organ specificity: amyloid fibrils are deposited in several specific organs and sites of tissues, but SAA circulates in all tissues through the bloodstream[7-11,16]. In addition to these findings in humans, several strains of mice, such as the A/J strain, are resistant to amyloidosis, while most strains can be useful experimental models for AA amyloidosis[19,20]. These questions remain to be clarified, although several possible genetic and environmental factors may play important roles in the amyloid formation mechanism in AA amyloidosis[2,5,6,10,21].

It has been generally confirmed that in experimental murine AA amyloidosis models, mice develop amyloid deposits mainly around follicles of the spleen, in which various types of immune cells are present. In addition, it was reported that an amyloid-resistant mouse strain, the A/J strain, had poor mitogenic responses of splenic T cells[22]. Another study provided evidence that a high SAA level induced adhesion and migration of T cells[23,24]. On the basis of these reports, it can be hypothesized that some immune cells, including T cells, may play important roles in amyloid formation mechanisms, and immunogenic stimuli may be among the factors for inducing AA amyloidosis.

We examined the therapeutic effect of an inhibitor of T cell activation, FK506[25,26], on the development of AA amyloidosis in murine models. In addition, we compared the degrees of amyloid formation in severe combined immunodeficient (SCID) mice and nude mice with those for the same strains of mice having a normal immune system. The pathogenesis of AA amyloidosis is also discussed.

MATERIALS AND METHODS

Chemical agents. FK506 was generously supplied by Astellas Pharmaceutical (Tokyo, Japan). Other chemicals used were of analytical grade.

Animals. Male C3H/HeN mice, each 7–8 weeks old and weighing 20–25 g, and male ICR mice, each 7–8 weeks old and weighing 35–40 g, were obtained from Charles River Japan (Kanagawa, Japan). Male CB-17, CB-17-SCID, heterozygous BALB/cA (*nu*/+), and BALB/cA-nude (*nu*/*nu*) mice, 7–8 weeks old and weighing 20–25 g, were obtained from CLEA Japan (Tokyo, Japan). Mice were maintained in a specific pathogen-free environment at the Center for Animal Resources and Development, Kumamoto University. CB-17, CB-17-SCID, BALB/cA (*nu*/+), and BALB/cA-nude (*nu*/*nu*) mice were kept in sterilized microbarrier units under germ-free conditions.

Murine models of AA amyloidosis. Amyloidosis was induced in mice by 2 different methods with and without amyloid-enhancing factor (AEF). (1) Amyloidosis was induced by concomitant administration of $AgNO_3$ and AEF. Briefly, 0.4 ml of 2% $AgNO_3$ was injected into subcutaneous tissue of the back, and 0.3 ml AEF was injected intraperitoneally. AEF was prepared from the spleens of C3H/HeN mice with AA amyloidosis as described[27]. Briefly, the amyloid-laden spleens of C3H/HeN mice were homogenized in 0.15 M NaCl and centrifuged at 15,000 g for 30 min at 4°C, and the supernatant was discarded. This process was repeated 10 times followed by distilled water. Pooled supernatants from the second and the third water extracts were used as AEF. AEF was preserved at –80°C, and the same AEF lot was used throughout all experiments. Mice were killed at 6 days after administration of $AgNO_3$ and AEF. (2) Second, amyloidosis was induced by the method of Ram, *et al*[28] with some modifications[29]. Briefly, 0.25 ml of emulsion made from a mixture of Freund's complete adjuvant (FCA; 1 ml; BD Difco, Franklin Lakes, NJ, USA) and phosphate-buffered saline (PBS; 1 ml) containing 60 mg heat-killed *Mycobacterium butyricum* (BD Difco) was injected into subcutaneous tissue of the back. A second injection of 0.25 ml of emulsion was administered at 14 days after the first injection. These mice were killed at 28 days after the first injection.

Administration of FK506. Mice were injected intraperitoneally with FK506 (2–200 μg) or PBS daily. The dosage regimen of FK506 was comparable to those used in previous studies[30,31].

Detection of amyloid deposition. Tissue samples were fixed with 10% formalin, embedded in paraffin, serially sectioned at a thickness of 3 μm, and placed onto microscope slides. Sections were stained with alkaline Congo red and hematoxylin. Amyloid deposits were confirmed under polarized light for the presence of green birefringence. The degree of amyloid deposition was determined by measuring Congo red-positive areas under polarized light by means of a Macintosh computer using the public domain NIH Image program developed at the US National Institutes of Health (http://rsb.info.nih.gov/nih-image/). Six different visual fields of each spleen specimen at 100× magnification were checked and saved with a digital camera (Olympus Model DP70, Olympus, Tokyo, Japan). Using the NIH Image program, the degree of amyloid deposition was determined as follows. No amyloid deposition: 0%. The average values of Congo red-positive areas in the control mouse group in each experiment were presented as 100%, as described in figure legends below.

Measurement of SAA and proinflammatory cytokines. Serum SAA, IL-1ß, IL-6, and TNF-α concentrations were measured with an ELISA kit (Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions. Blood samples were collected from the femoral veins of mice.

Statistical analysis. Student's t test (2-tailed, independent samples) was used to determine significant differences ($p < 0.05$).

RESULTS

Effect of FK506 on amyloid deposition. In the murine model of AA amyloidosis, daily injections of FK506 significantly reduced the degree of amyloid deposition in comparison with that in PBS-treated control mice. At 6 days after administration of $AgNO_3$ and AEF, severe amyloid deposition around follicles in the spleens of C3H/HeN mice injected with AEF and $AgNO_3$ was detected by Congo red staining under polarized light (Figure 1A). In FK506-treated mice (200 μg/day), amyloid deposition was reduced to very small focal deposits (Figure 1B). Amyloid deposition was observed in the Glisson's capsule and Disse spaces in livers of C3H/HeN mice injected with AEF and $AgNO_3$ (Figure 1C). Amyloid deposition was also reduced to small deposits in the liver in FK506-treated mice (Figure 1D). The degree of amyloid deposition in the spleen was significantly decreased in an FK506 dose-dependent manner (Figure 2). The degree of amyloid deposition in the liver was too small to be counted, and amyloid deposition was not detected in kidneys in this model.

Changes in serum SAA and proinflammatory cytokine levels. Serum SAA levels did not change in AA amyloid model mice after administration of different doses of FK506 (2–200 μg/day) or PBS (Figure 3A). Serum IL-6 and IL-1ß levels showed a similar tendency after administration of FK506 (200 μg/day) or PBS (Figures 3B, 3C). Serum TNF-α levels in the mice were below detectable levels at 0, 1, 2, and 6 days (data not shown).

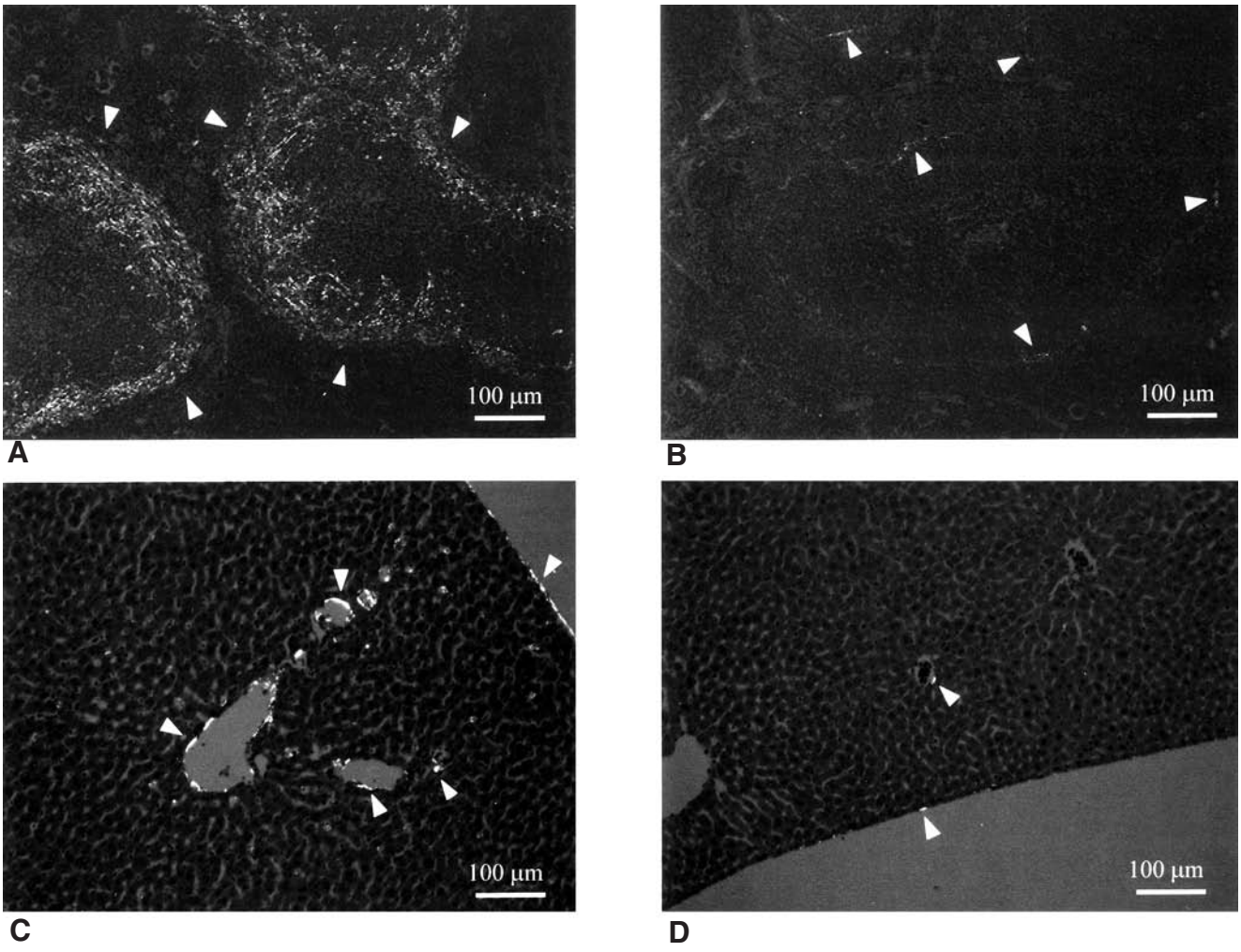

Figure 1. Effect of FK506 on amyloid formation in the murine model of AA amyloidosis. Amyloidosis was induced by injection of $AgNO_3$ and AEF in C3H/HeN mice. Splenic (A) and hepatic (C) samples of mice injected with PBS daily were stained with Congo red viewed under polarized light. Splenic (B) and hepatic (D) samples of mice injected with 200 μg FK506 daily from 3 days before to Day 6 after administration of AEF and $AgNO_3$ were stained with Congo red viewed under polarized light. Arrowheads show areas of amyloid deposition (original magnifications ×100).

Effect of FK506 injection schedule on amyloid formation. FK506 administration for 3 days, from the day of $AgNO_3$ and AEF administration to Day 2, reduced the amount of splenic amyloid deposition in comparison with that in PBS-treated control mice (Figure 4). In contrast, FK506 administrations for 3 days, from 3 days to 1 day before $AgNO_3$ and AEF administration, and from 3 to 5 days after $AgNO_3$ and AEF administration, did not significantly reduce the amount of splenic amyloid deposition (Figure 4).

Amyloid formation in immunodeficient mouse strains. After simultaneous injection of $AgNO_3$ and AEF, SCID mice showed resistance to AA amyloid formation compared with CB-17 mice (Figures 5A, 5B, 5C; Table 1). Serum SAA levels were similar in these 2 strains of mice (Figure 5D). In addition, BALB/cA-nude (*nu*/*nu*) mice also showed resistance to AA amyloid formation compared with heterozygous BALB/cA (*nu*/+) mice (Figures 6A, 6B, 6C; Table 1). Serum SAA levels of nude mice were also elevated, but were lower than those of heterozygous BALB/cA (*nu*/+) mice at 2 days after the administration (Figure 6D).

Effect of FK506 on amyloid formation and serum SAA levels in amyloidosis induced without AEF. We induced amyloidosis in ICR mice by injection of FCA and *M. butyricum* as described. Histopathologic analyses revealed severe amyloid deposits in various tissues (Figure 7). FK506 (200 μg/day) significantly inhibited the incidence of amyloidosis in various tissues (Table 2). With only 14 days' injection of FK506 from Day 7, 14, and 21, FK506 reduced the incidence of amyloidosis (Table 2). However, FK506 injection from Day 0 for 14 days did not reduce the incidence of amyloidosis (Table 2). FK506-treated mice did not differ from PBS-treated control mice in serum SAA levels at 7, 14, 21, and 28 days after the first injection of FCA and *M. butyricum* (Figure 8A).

Incidence of amyloid deposition in mice injected with FCA

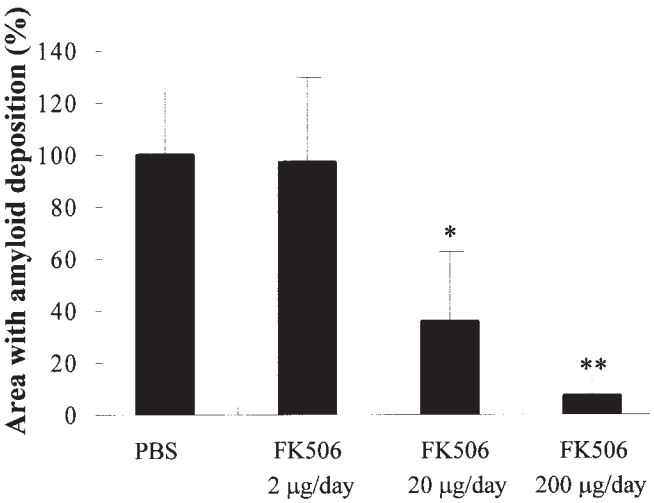

Figure 2. Degree of amyloid formation with different doses of FK506. Splenic lesions with amyloid deposition in the AA amyloid model mice given FK506 or PBS were assessed quantitatively. C3H/HeN mice were injected intraperitoneally with FK506 (2–200 μg/day; 3 treatment groups, each group n = 8) or PBS (control; n = 5) daily, beginning 3 days before AEF and $AgNO_3$ administration to the last day of the experiments. The average of amyloid-positive areas in the control group was established as 100%. *$p < 0.05$ for mice given 20 μg/day FK506 vs PBS-treated mice; **$p < 0.01$ for mice given 200 μg/day FK506 vs PBS-treated mice.

and M. butyricum. We also induced amyloidosis in CB-17, CB-17-SCID, heterozygous BALB/cA (*nu*/+), and BALB/cA-nude (*nu*/*nu*) mice by injections of FCA and *M. butyricum*. SCID and nude mice did not develop amyloidosis, whereas like the ICR mice, heterozygous BALB/cA (*nu*/+) mice and CB-17 mice developed amyloidosis in various tissues (Table 3). In this type of amyloid induction, after injections of FCA and *M. butyricum*, serum SAA levels were elevated in SCID and nude mice. In contrast, serum SAA levels in these mice were lower than those of heterozygous BALB/cA (*nu*/+) mice and CB-17 mice (Figures 8B, 8C).

DISCUSSION

We observed that administration of FK506 effectively suppressed deposition of amyloid in murine AA amyloidosis models. Histopathologic studies revealed that administration of FK506 reduced the amount of amyloid deposit in a dose-dependent manner (Figures 1 and 2). FK506 is an immunosuppressive agent that specifically inhibits T cell activation[25,26]. FK506 binds to an intracellular protein called FK506-binding protein (FKBP). The FK506-FKBP complex inhibits activity of a calcineurin that dephosphorylates the nuclear factor of activated T cell proteins, leading to their nuclear entry and gene transcriptions for synthesis of cytokines in T cells[26]. The agent has been widely used to prevent immunologic rejection of transplanted organs and has been proposed as a therapeutic drug in RA[32-34]. To our knowledge, this is the first study in which the effect of FK506 on AA amyloidogenesis was examined precisely; one clinical case report suggests that 0.1% FK506 ointment may provide effective therapy for lichen amyloidosis, another localized type of amyloidosis[35]. The FK506 dose we injected was 2–200 μg/day (0.08–8 μg/kg). As shown in Figure 2, administration of FK506 20 to 200 mg/day significantly reduced the degree of amyloid deposition. Although these doses are 10 to 100-fold higher than those given in human diseases, inflammation induced in the mice was much more severe than that in human disease, such as RA. The findings suggest that FK506 therapy may be applicable to human disease.

It is generally believed that one of the most important factors for preventing AA amyloidosis is to keep serum SAA levels low[7,10]. However, one interesting finding was that in mice treated with FK506, serum SAA levels did not differ from those in mice treated without FK506 (Figure 3A), even though FK506 administration reduced the amount of amyloid deposition in the tissues (Figures 1 and 2). FK506 specifically inhibits T cell activation by suppression of gene transcription for synthesis of cytokines, especially IL-2, in T cells[25,26]. Similarly to SAA levels, serum levels of IL-6 and IL-1ß, which stimulate SAA expression, remained unchanged compared with those in PBS-treated mice (Figures 3B, 3C). It has also been reported that FK506 did not suppress secretion of IL-1ß, IL-6, and TNF-α from lipopolysaccharide-stimulated macrophages and monocytes *in vitro*[36,37]. These reports strongly support our findings. FK506 may act mainly in tissues in which amyloid deposition is developed, but not at the site of inflammation, to prevent amyloid formation.

FK506 administration for 3 days, from the day of $AgNO_3$ and AEF injection to Day 2, reduced the amount of splenic amyloid deposits, while the administrations for 3 days, from 3 days before and from 3 days after the day of $AgNO_3$ and AEF injection, did not significantly reduce the amount of amyloid deposition (Figure 4). This result suggests that FK506 may act mainly on the early phase of amyloid formation induced by $AgNO_3$ and AEF.

It is also interesting that the degree of amyloid deposition was markedly reduced in immunodeficient mice injected with AEF and $AgNO_3$. SCID mice lack functional T and B cells as the result of mutation of the *scid* gene; this gene has its main influence on rearrangement of genes during maturation of T and B cells[38]. In mice, however, macrophage activation and antigen-presenting functions remain unimpaired, as does natural killer cell activity[39,40]. Inasmuch as the *scid* mutation occurs in the CB-17 strain and mice homozygous for the *scid* mutation are designated SCID mice, we used CB-17 mice as the controls in experiments. As expected, SCID mice were resistant to amyloid induction (Figures 5A, 5B, 5C). Serum SAA levels of SCID mice were similar to those of CB-17 mice (Figure 5D). It should be noted that AA amyloid formation was inhibited in nude mice (Figures 6A, 6B, 6C), which lack a thymus gland as the result of a mutation of the *nu* gene and cannot generate mature T cells[41]. However, the functions of B cells and the IgM response to thymus-independent antigens,

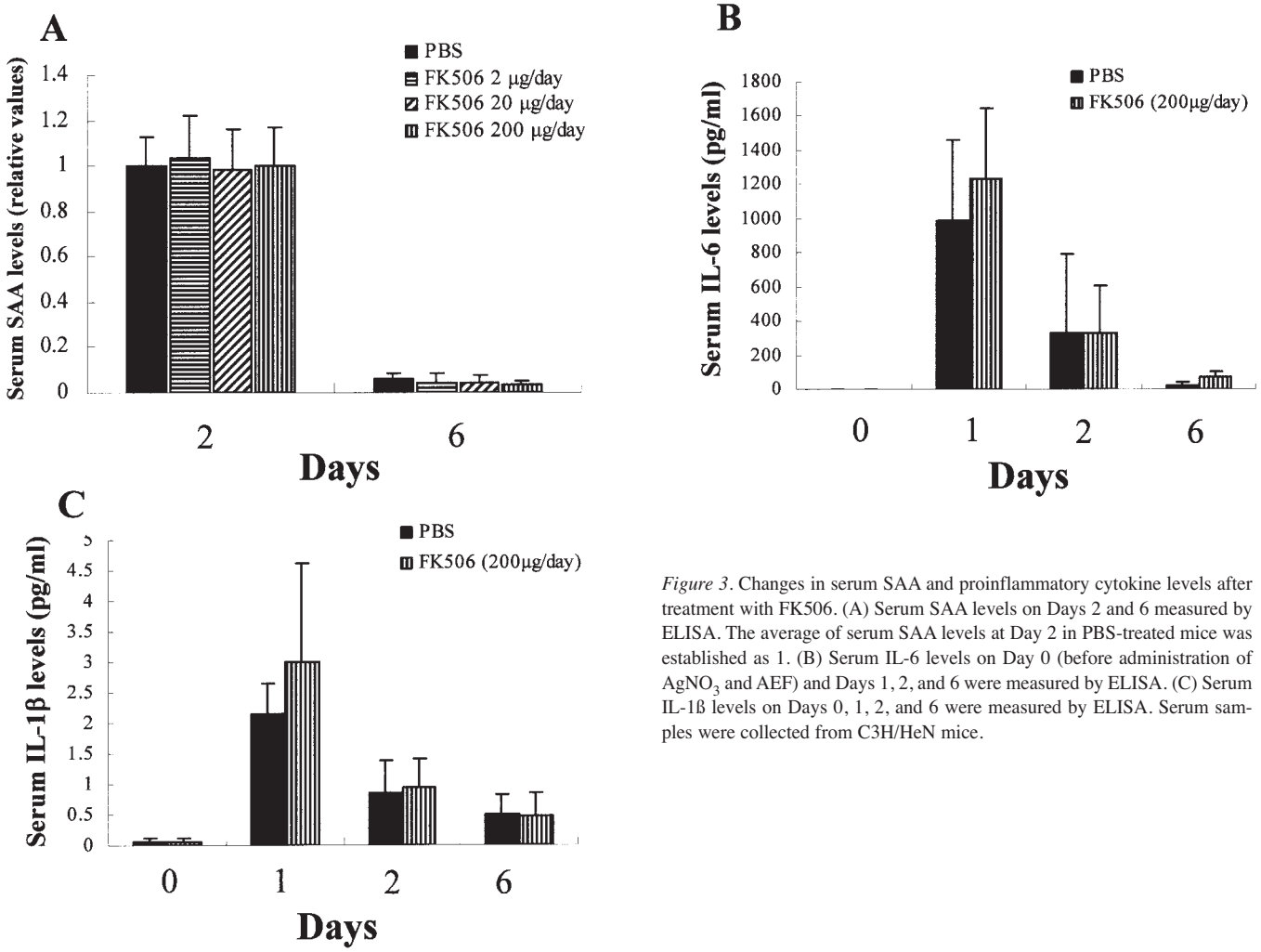

Figure 3. Changes in serum SAA and proinflammatory cytokine levels after treatment with FK506. (A) Serum SAA levels on Days 2 and 6 measured by ELISA. The average of serum SAA levels at Day 2 in PBS-treated mice was established as 1. (B) Serum IL-6 levels on Day 0 (before administration of $AgNO_3$ and AEF) and Days 1, 2, and 6 were measured by ELISA. (C) Serum IL-1ß levels on Days 0, 1, 2, and 6 were measured by ELISA. Serum samples were collected from C3H/HeN mice.

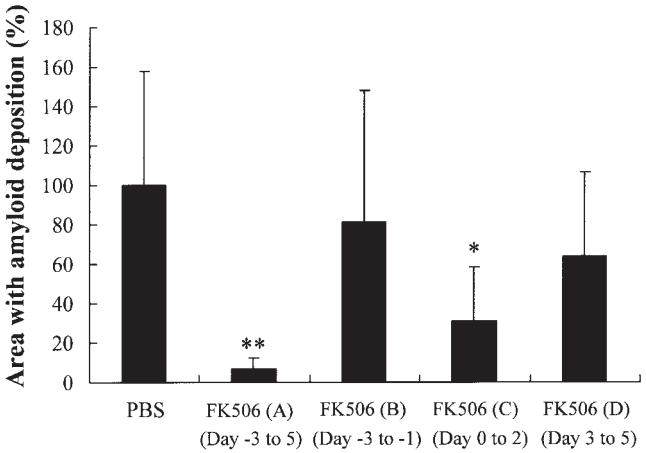

Figure 4. Effect of FK506 on amyloidogenesis in different periods of administration. Splenic lesions with amyloid deposition in mice given FK506 or PBS were assessed quantitatively. (A): mice were injected intraperitoneally with FK506 (200 μg/day) for 9 days, beginning 3 days before to 5 days after AEF and $AgNO_3$ administration. Mice were injected intraperitoneally with FK506 (200 μg/day) for 3 days (B: from Day –3 to –1; C: from Day 0 to 2; D: from Day 3 to 5). The average of amyloid-positive areas in PBS-treated mice was established as 100%. *$p < 0.05$ for mice given 200 μg/day FK506 from Day 0 to 2 (C) vs PBS-treated mice; **$p < 0.01$ for mice given 200 μg/day FK506 from Day –3 to 5 (A) vs PBS-treated mice.

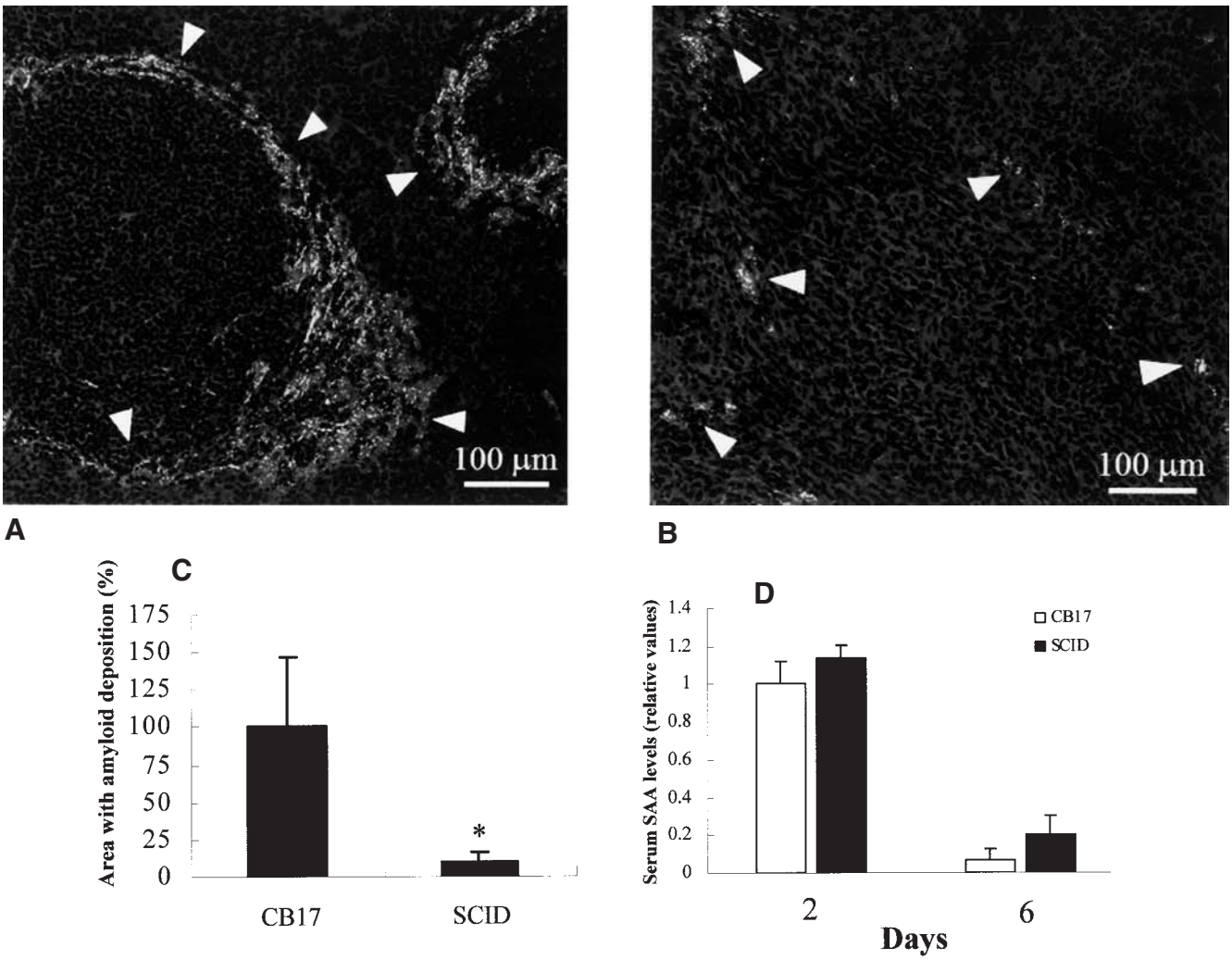

Figure 5. Changes in the amount of amyloid deposition and serum SAA levels in SCID mice. Amyloidosis was induced by injections of $AgNO_3$ and AEF in CB-17 (A), and CB-17-SCID mice (B). Splenic samples were stained with Congo red viewed under polarized light (A and B). Arrowheads indicate the areas of amyloid deposition (original magnifications ×100). (C) Areas of amyloid deposition in CB-17 mice and CB-17-SCID mice 6 days after administration of $AgNO_3$ and AEF. The average of the amyloid-positive areas in CB-17 mice was established as 100%. Number of mice in each group was 5. *$p < 0.01$ for CB-17 vs CB-17-SCID mice. (D) SAA concentrations, by ELISA, in mice described in (C), 2 and 6 days after administration of $AgNO_3$ and AEF. The average of serum SAA levels at Day 2 in CB-17 mice was established as 1.

macrophages, and natural killer cells are present in the nude mice[42,43]. These findings strongly suggest that T cells may play a role in AA amyloid formation.

Elevation of serum SAA levels was observed after injections of $AgNO_3$ and AEF, but serum SAA levels were lower than those in heterozygous BALB/cA (*nu*/+) mice at 2 days after injection (Figure 6D). The reason nude mice showed lower serum SAA levels could not be explained, while the levels were higher than those in mice without inflammation. Further investigation to elucidate this is needed.

To clarify longterm effects of FK506 and eliminate the possibility that FK506 reduced amyloid formation depending on AEF activity, we investigated the effect of FK506 on amyloid formation in the mice with amyloid induced without AEF.

Table 1. Incidence of amyloidosis in immunodeficient mice injected with $AgNO_3$ and amyloid-enhancing factor.

Mouse Strain	Incidence of Amyloidosis				
	Spleen	Liver	Kidney	Duodenum	Heart
CB-17 (+/+)	5/5	5/5	4/5	4/5	5/5
CB-17-SCID (scid/scid)	4/5	5/5	1/5	1/5	1/5
BALB/cA (nu/+)	5/5	5/5	3/5	4/5	5/5
BALB/cA-nude (nu/nu)	4/5	4/5	0/5	2/5	2/5

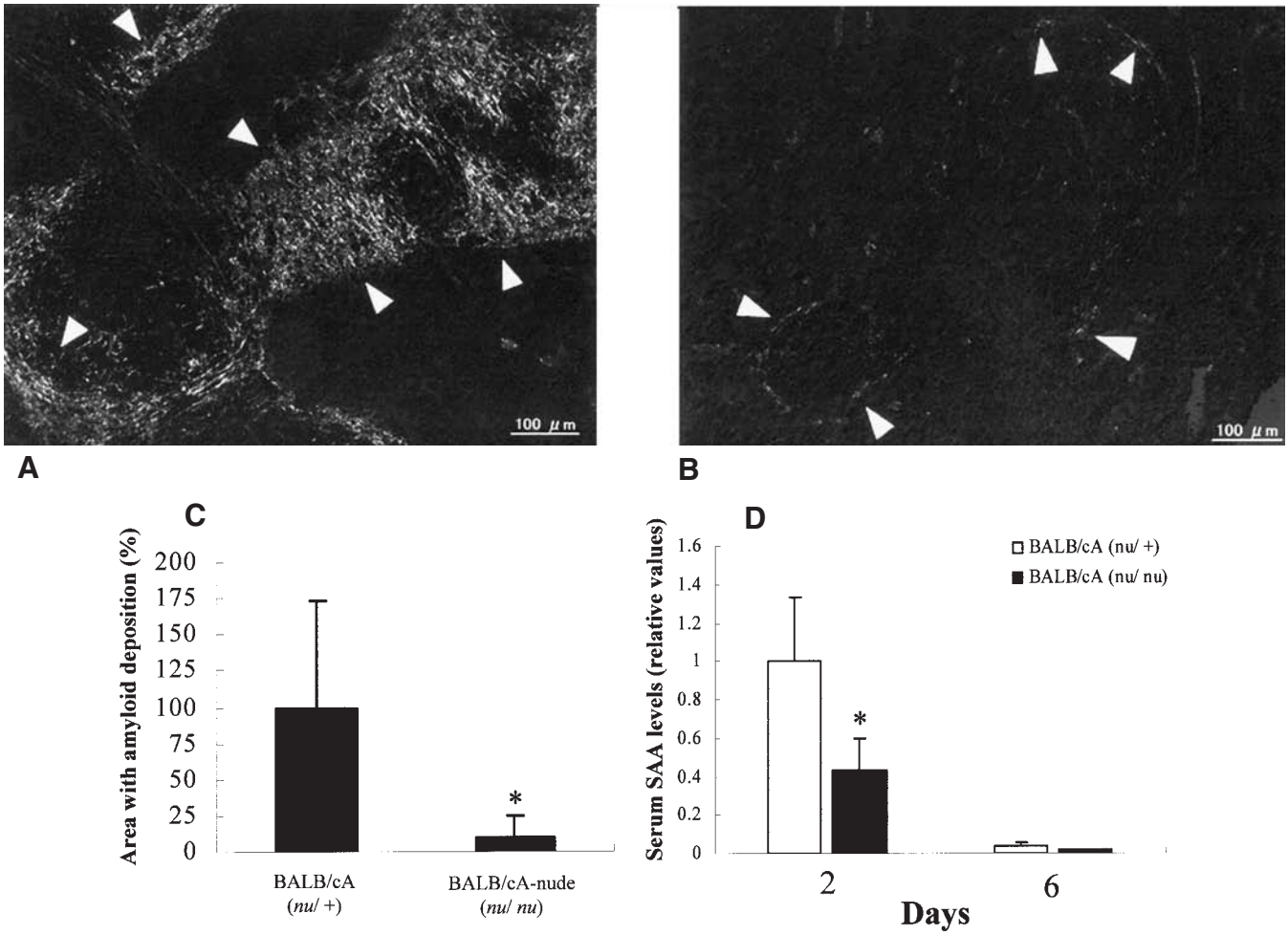

Figure 6. Changes in the amount of amyloid deposition and serum SAA levels in nude mice. Amyloidosis was induced by injections of $AgNO_3$ and AEF in heterozygous BALB/cA (*nu*/+) mice (A) and BALB/cA-nude mice (B). Splenic samples were stained with Congo red viewed under polarized light (A and B). Arrowheads indicate areas of amyloid deposition (original magnifications ×100). (C) Areas of amyloid deposition in heterozygous BALB/cA (*nu*/+) and BALB/cA-nude (*nu*/*nu*) mice 6 days after administration of $AgNO_3$ and AEF. The average of the amyloid-positive areas in heterozygous BALB/cA (*nu*/+) mice was established as 100%. Number of mice in each group was 5. *$p < 0.01$ for BALB/cA-nude (*nu*/*nu*) vs heterozygous BALB/cA (*nu*/+) mice. (D) SAA concentrations, by ELISA, in mice described in (C), 2 and 6 days after administration of $AgNO_3$ and AEF. The average of serum SAA levels at Day 2 in BALB/cA (*nu*/+) mice was established as 1.

The chemical structure of AEF has not been fully determined, but protofibrils or fibril-forming peptides are now believed to serve as a nidus for amyloid formation[44-47]. In ICR mice injected with FCA and *M. butyricum*, amyloid deposits were much more severe than those in C3H/HeN mice injected with $AgNO_3$ and AEF (Figure 7), and FK506 injections clearly reduced the incidence of amyloidosis (Table 2), without reducing serum SAA concentrations (Figure 8A). The action of FK506 in reducing amyloid formation was corroborated in mice in the AA amyloidosis model.

We also induced AA amyloidosis in SCID and nude mice by injections of FCA and *M. butyricum*. Neither strain developed amyloidosis in this model (Table 3). Resistance to amyloid formation in these immunodeficient strains was clearly demonstrated. It is likely that AA amyloidosis is reduced under conditions of inhibiting T cell activities. While the elevated SAA levels were higher than those in mice without inflammation, the reason why both mice showed lower serum SAA levels (Figures 8B and 8C) could not be explained. We cannot exclude the possibility that resistance to amyloid formation in these immunodeficient mice could be derived from the lower serum SAA levels. Further investigation to elucidate this is also needed.

Because both FK506-treated mice and immunosuppressed mice had resistance to amyloid deposition, T cells might play a role in AA amyloid formation. The spleen participates in immune responses against many types of pathogens, and it is the site in which amyloid deposition is initially observed and is most severe around the T cell zone in mouse models. Therefore, suppression of T cell activity may change pathologic conditions of the spleen, such as construction of extracellular matrix and adhesion molecules of spleen cells, and may alter the process of amyloid formation by eliminating scaffolding molecules.

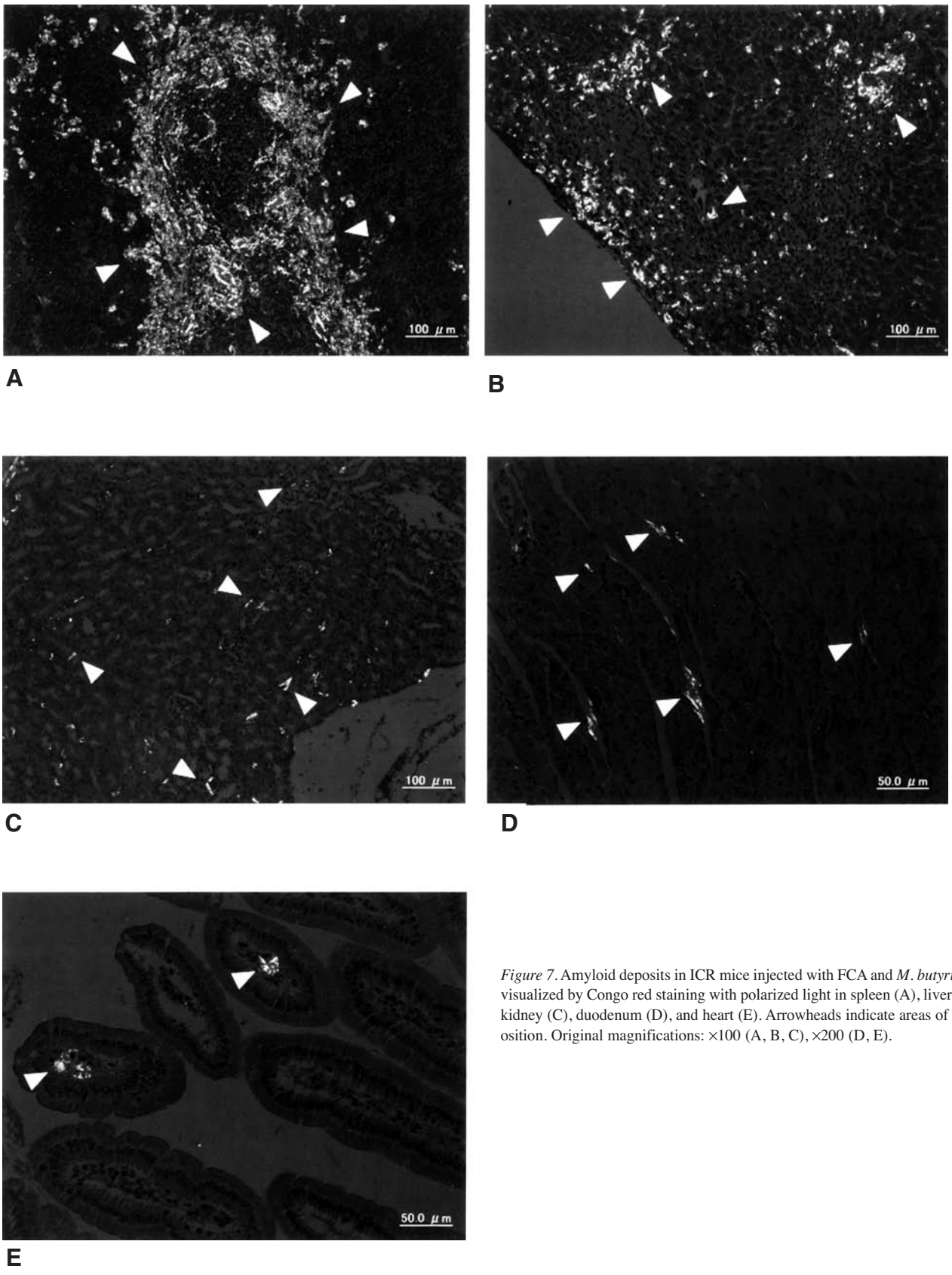

Figure 7. Amyloid deposits in ICR mice injected with FCA and *M. butyricum* visualized by Congo red staining with polarized light in spleen (A), liver (B), kidney (C), duodenum (D), and heart (E). Arrowheads indicate areas of deposition. Original magnifications: ×100 (A, B, C), ×200 (D, E).

Table 2. Incidence of amyloidosis with and without administration of FK506. ICR mice were injected intraperitoneally with FK506 (200 μg/day) or PBS.

Duration of FK506 Administrations	Incidence of Amyloidosis				
	Spleen	Liver	Kidney	Duodenum	Heart
PBS only (control group)	8/16	7/16	5/16	5/16	4/16
Day 0 to 28	0/10	0/10	0/10	0/10	0/10
Day 0 to 14	2/5	1/5	1/5	1/5	1/5
Day 7 to 21	0/5	0/5	0/5	0/5	0/5
Day 14 to 28	0/5	0/5	0/5	0/5	0/5

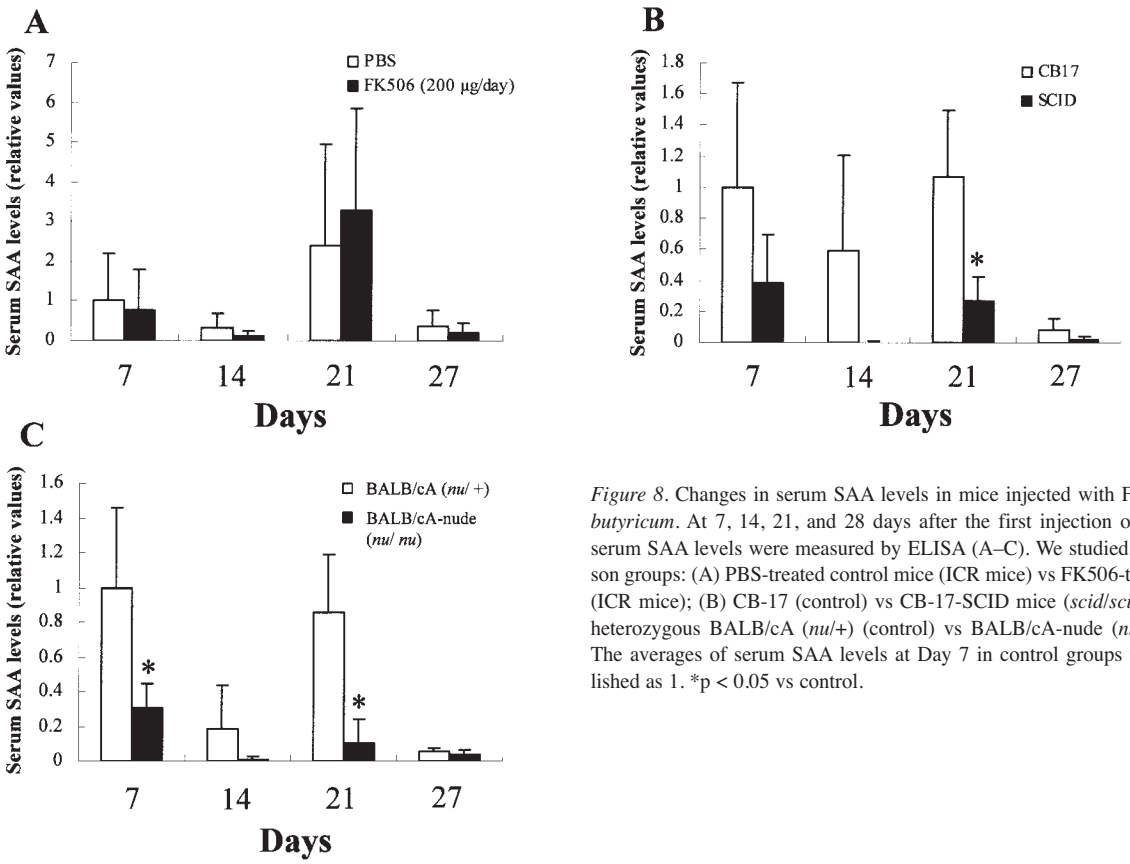

Figure 8. Changes in serum SAA levels in mice injected with FCA and *M. butyricum*. At 7, 14, 21, and 28 days after the first injection of emulsion, serum SAA levels were measured by ELISA (A–C). We studied 3 comparison groups: (A) PBS-treated control mice (ICR mice) vs FK506-treated mice (ICR mice); (B) CB-17 (control) vs CB-17-SCID mice (*scid*/*scid*); and (C) heterozygous BALB/cA (*nu*/+) (control) vs BALB/cA-nude (*nu*/*nu*) mice. The averages of serum SAA levels at Day 7 in control groups were established as 1. *$p < 0.05$ vs control.

Table 3. Incidence of amyloidosis in immunodeficient mice injected with Freund's complete adjuvant and *M. butyricum*.

Mouse Strain	Incidence of Amyloidosis				
	Spleen	Liver	Kidney	Duodenum	Heart
CB-17 (+/+)	3/5	3/5	3/5	3/5	3/5
CB-17-SCID (scid/scid)	0/4	0/4	0/4	0/4	0/4
BALB/cA (nu/+)	4/4	3/4	3/4	3/4	3/4
BALB/cA-nude (nu/nu)	0/5	0/5	0/5	0/5	0/5

Since macrophages colocalize with amyloid deposition in tissues and can degrade SAA to AA proteins in *in vitro* studies, macrophages have been considered to play the central role in AA amyloid formation[48,49]. Thus, it is speculated that in the process of AA amyloid formation, the interaction of macrophages and cytokines produced by T cells, such as IL-2, interferon-γ, and granulocyte-macrophage colony-stimulating factor, may be suppressed by FK506.

It has been observed that FK506 has many biological activities, such as inhibition of neuronal apoptosis and an effect on cerebral ischemia via inhibition of calcineurin activity, and that calcineurin and the nuclear factor of activated T cell proteins participate in the development and function of the immune, nervous, cardiovascular, and musculoskeletal systems[50]. However, the precise molecular mechanisms of those effects remain to be elucidated. Thus, it is speculated that FK506 might prevent amyloid formation via inhibition of calcineurin activity and/or those unknown FK506 functions.

In summary, FK506 administration significantly reduced the amount of amyloid deposition and the incidence of amyloidosis in murine AA amyloidosis models. FK506 inhibited AA amyloid formation without reducing serum SAA and proinflammatory cytokine levels in the mice. FK506 may act mainly on pathophysiologic changes of tissues that developed amyloid deposits, not in the site of inflammation. In addition, immunodeficient mice showed resistance to development of AA amyloidosis. These results suggest that T cells may play an important role in the mechanism of amyloid formation in AA amyloidosis. However, it is unclear whether T cells act on amyloid formation directly or indirectly via interaction with other types of cells. These data may lead to a new therapeutic strategy for amyloidosis. Our findings may partly explain why amyloid deposition is mainly in certain specific organs and sites in tissues, whereas SAA circulates in all tissues.

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