

Matsumoto, Y., Park, I., Kohyama, K.	B cell epitope spreading is a critical step for the switch from C-protein-induced myocarditis to dilated cardiomyopathy	Am J Pathol	170	43-51	2007
Okura, Y., Matsumoto, Y	Anti-Abeta vaccination as a promising therapy for Alzheimer's disease	Current Topics in Neuro-immunology		139-145	2007
Sugihara, T., Sekine, C., Nakae, T., Kohyama, K., Harigai, M., Iwakura, Y., Matsumoto, Y., Miyasaka, N., Kohsaka, H.	A new murine model to define the critical pathologic and therapeutic mediators of polymyositis	Arthritis and Rheumatism	56,	1304-1314	2007
Kim, H., Moon, C., Ahn, M., Lee, Y., Kim, S., Matsumoto, Y., Koh, C., Kim, M., Shin, T.	Increased phosphorylation of cyclic AMP response element-binding protein in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis.	Brain Res	1162	113-120	2007
Matsumoto, Y., Sakuma, H., Kohyama, K., Park, I.	Paralysis of CD4+CD25+ regulatory T cell response in chronic autoimmune encephalomyelitis.	J Neuroimmunol	187	44-54	2007
Katsumata, Y., Harigai, M., Sugiura, T., Kawamoto, M., Kawaguchi, Y., Matsumoto, Y., Kohyama, K., Soejima, M., Kamatani, N., Hara, M.	Attenuation of experimental autoimmune myositis by blocking ICOS-ICOS ligand interaction	J Immunol	179	3772-3779	2007
Okura, Y., Miyakoshi, A., Kohyama, K., Staufenbiel, M., Matsumoto, Y.	Non-viral A beta DNA vaccine therapy against Alzheimer's disease: Long term-effects and safety	Proc Natl Acad Sci USA	103	9619-9624	2006
大倉良夫、松本陽	Alzheimer 病の新ワクチン療法	医学のあゆみ	219	164-165	2006
Matsumoto, Y., Matsuo, H., Sakuma, H., Park, I., Tsukada, Y., Kohyama, K., Kondo, T., Kotorii, S., Shibuya, N.	CDR3 spectratyping analysis of the TCR repertoire in myasthenia gravis	J Immunol	176	5100-5107	2006
Kim, H., Ahn, M., Lee J., Moon, C., Matsumoto, Y., Koh, C., Shin, T.	Increased phosphorylation of caveolin-1 in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis	Neurosci Lett,	402	76-80	2006

Sakuma, H., Park, I, Kohyama, K., Feng, D., Matsumoto, Y.	Quantitation of myelin oligodendrocyte glycoprotein and myelin basic protein in the thymus and central nervous system and its relationship to the clinicopathological features of autoimmune encephalomyelitis	J Neurosci Res	84	606-613	2006
Tanuma, N., Sakuma, H., Sasaki, A., Matsumoto, Y.	Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis	Acta Neuropathol	112	195-204	2006
Kim, H., Ahn, M., Moon, C., Matsumoto, Y., Koh, C., Shin, T.	Immunohistochemical study of flotillin-1 in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis	Brain Res,	1114	204-211	2006
Ahn, M., Kim, H. , Matsumoto, Y., Shin, T	Increased expression of caveolin-1 and -2 in the heart of Lewis rats with experimental autoimmune myocarditis	Autoimmunity	39	489-495	2006
Warabi, Y., Yagi, K., Hayashi, H., Matsumoto, Y.	Characterization of the T cell receptor repertoire in the Japanese neuromyelitis optica: T cell activity is up-regulated compared to multiple sclerosis	J Neurol Sci.	249	145-152	2006

### III. 研究成果の刊行物・別刷

# Matrix Metalloproteinase (MMP)-9, but Not MMP-2, Is Involved in the Development and Progression of C Protein-Induced Myocarditis and Subsequent Dilated Cardiomyopathy<sup>1</sup>

Yoh Matsumoto,<sup>2</sup> Il-Kwon Park, and Kuniko Kohyama

Repeated or continuous inflammation of the heart is one of the initiation factors for dilated cardiomyopathy (DCM). In previous studies, we established a DCM animal model by immunizing rats with cardiac C protein. In the present study, we analyze the role of matrix metalloproteinases (MMPs) in experimental autoimmune carditis (EAC) and subsequent DCM to elucidate the pathomechanisms of this disease. In this model, inflammation begins ~9 days after immunization. At that time, MMP activities were detected by *in situ* zymography. Real-time PCR analysis revealed continuous up-regulation of MMP-2 mRNA from 2 wk and thereafter. MMP-9 mRNA, however, had only a transient increase at 2 wk. Double staining with *in situ* zymography and cell markers demonstrated that gelatinase (MMP-2 and MMP-9)-expressing cells are infiltrating macrophages during the early stage and cardiomyocytes at later stages. Minocycline, which inhibits MMP-9 activities more strongly than MMP-2, significantly suppressed EAC, but an MMP-2-specific inhibitor, TISAM, did not affect the course of the disease. Furthermore, immunohistochemical examination revealed that minocycline treatment suppressed T cell and macrophage infiltration strongly, whereas TISAM did not. These findings indicate that MMP-9, but not MMP-2, is involved in the pathogenesis of the acute phase of EAC, and further suggest that MMP-9 inhibitors, minocycline and its derivatives, may be useful therapies for EAC and DCM. *The Journal of Immunology*, 2009, 183: 4773–4781.

**D**ilated cardiomyopathy (DCM)<sup>3</sup> is a progressive disease of the myocardium that is characterized by dilatation and impaired contraction of either the left or both ventricles of the heart (1). The causes of DCM are multifactorial, but a viral or immune mechanism is suspected in some cases (2) because examination of heart tissues obtained during surgery has revealed inflammatory lesions in many patients with DCM (3). On the basis of these findings, several immunosuppressive therapies, including steroid and azathioprine administration (4, 5), immunoadsorption (6), and Ig therapy (2), have been attempted. Although the general outcome of these treatments was disappointing, immunoadsorption therapy increased the survival rate for a subset of patients by 5 years after treatment (7). Because the pathogenesis of DCM is still poorly understood, finding effective therapies has been a difficult challenge. The establishment of an animal model that mimics human DCM provides useful information on the pathogenesis of DCM and the development of effective therapies.

In previous studies, we have demonstrated that cardiac C protein localizes in thick filaments of cardiac muscles (8) and has a strong

carditis-inducing ability (9, 10). Experimental autoimmune carditis (EAC) that is induced in Lewis rats by immunization with C protein is a T cell-mediated disease, but various types of autoantibodies are involved for the shift from EAC to DCM (10). In addition, it was shown that several chemokines play an important role in disease progression (9).

Matrix metalloproteinases (MMPs) form a family of enzymes that mediate various functions in tissue destruction, remodeling, and immune responses by hydrolyzing components of the extracellular matrix under physiological and pathological conditions (11). In the heart, MMPs localize in the sarcomere within cardiomyocytes and become activated by acute cardiac injury (reviewed in Ref. 12). Therefore, it is essential to investigate the role of MMPs produced by exogenous infiltrating inflammatory cells and endogenous cardiomyocytes during the development and progression of DCM. Unfortunately, there is little information on MMP production in the heart during autoimmune processes.

In the present study, we investigated the role of MMPs in the development and progression of EAC. We determined when active MMP-producing cells appear in the heart and whether they are inflammatory cells, cardiomyocytes, or both. The relationship between the MMP expression and inflammatory lesions was also examined. The results suggest that MMP-2 and MMP-9 play important, but different roles in lesion formation of EAC and DCM. Furthermore, treatments with an MMP-9 inhibitor, but not with an MMP-2 inhibitor, ameliorated the pathology of EAC. Thus, immunotherapies to EAC, and subsequent DCM, should be designed on the basis of the diseases mechanism.

## Materials and Methods

Unless otherwise indicated, all reagents and apparatuses used in the present study were obtained in Tokyo, Japan.

### Animals

Lewis rats were purchased from SLC Japan and were bred in our animal facility. Male and female rats were used at 8–12 wk of age. All animal

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<sup>3</sup> Abbreviations used in this paper: DCM, dilated cardiomyopathy; CC2, cardiac C protein fragment 2; EAC, experimental autoimmune carditis; FIZ, film *in situ* zymography; ISZ, *in situ* zymography; MMP, matrix metalloproteinase; PI, postimmunization.

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experiments were approved by the institute ethics committee and performed in accordance with institutional guidelines.

#### Preparation of recombinant C protein fragments and synthetic peptides

The preparation of recombinant C protein was precisely described previously (9). PCR products corresponding to cardiac C protein fragment 2 (CC2) were inserted into a cloning vector, pCR4 Blunt-TOPO, in the Zero Blunt TOPO Kit (Invitrogen), and clones with correct sequences were subcloned into the pQE30 expression vector (Qiagen). Recombinant CC2 produced in transformed *Escherichia coli* was isolated under denaturing conditions and purified using Ni-NTA agarose (Qiagen).

#### EAC induction and tissue sampling

Lewis rats were immunized once on day 0 with CC2 emulsified in CFA (2.5 mg/ml *Mycobacterium tuberculosis*) in the hind footpads. In some experiments, rats received an i.p. injection of pertussis toxin (Seikagaku Kogyo) at the time of immunization. Histological and immunohistochemical examinations were performed at the indicated time points using frozen and paraffin-embedded sections of the heart. Although evaluation of EAC and DCM was mainly based on histological examinations (see below), the clinical score was also recorded, as follows: grade 1, dyspnea; grade 2, dyspnea plus ruffling of fur; and grade 3, moribund condition or dead.

#### Histological grading of inflammatory lesions and immunohistochemistry

EAC inflammatory lesions were evaluated using H&E-stained sections according to the following criteria: grade 1, rare focal inflammatory lesions; grade 2, multiple isolated foci of inflammation; grade 3, diffuse inflammation involving the outer layer of the muscle; grade 4, grade 3 plus focal transmural inflammation; and grade 5, diffuse inflammation. When dense fibrosis made it difficult to estimate the grade of inflammation using H&E-stained sections, ED1-stained sections were used for grading. The extent of fibrosis revealed by Azan staining was graded into five categories, as follows: grade 1, rare scattered foci of fibrosis; grade 2, multiple isolated foci of fibrosis; grade 3, fibrosis involving the outer layer of the muscle; grade 4, grade 3 plus partial transmural fibrosis; and grade 5, diffuse fibrosis. The perivascular connective tissue staining found in the normal heart was not included in this scoring system.

Single immunoperoxidase staining was performed, as described previously, using mAbs against TCR- $\alpha\beta$  (R73) and macrophages (ED1), as described previously (13). Briefly, frozen sections were air dried and fixed in ether for 10 min. After incubation with normal sheep serum, sections were reacted with mAb, biotinylated horse anti-mouse IgG (Vector Laboratories), and HRP-labeled VECTSTAIN Elite ABC Kit (Vector Laboratories). HRP binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide.

#### Film in situ zymography (FIZ), in situ zymography (ISZ), and gelatin zymography

To localize the activity of gelatinases (MMP-2, -9), zymography with gelatin-coated films (FIZ) was performed, according to the manufacturer's instructions. Frozen sections (4  $\mu\text{m}$ ) were cut and mounted onto MMP in situ Zymo-Films (WAKO). The films were incubated in a moist chamber at 37°C overnight. After being air dried, the films were stained with Biebrich scarlet stain solution (WAKO) for 4 min, followed by a wash with distilled water. Areas possessing gelatinolytic activities were stained white, as shown in Fig. 2, and were quantitated using NIH Image software. The films were first scanned with a Canoscan 8000F scanner (Canon), and the images were saved as 300-kb files. Using inverted images, the areas showing gelatinase activities were calculated as pixels. In 2007, WAKO stopped supplying gelatin-coated films, so additional FIZ experiments were not performed.

ISZ with FITC-labeled gelatin was performed using DQ gelatin (Invitrogen), according to Oh et al. (14). Briefly, 8- $\mu\text{m}$  frozen sections were incubated with reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, and 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (pH 7.6)) containing 40  $\mu\text{g}/\text{ml}$  DQ gelatin at 37°C overnight. Sections were washed with PBS before observation. The fluorescent intensity of ISZ sections was semiquantitatively analyzed using the Image J software (National Institutes of Health). All sections for analysis were stained, and microphotographs of each were obtained under the same conditions with a confocal microscope (TCS-SP; Leica Microsystems). Bright areas (above threshold, 115) were calculated and evaluated statistically.

To determine the type of cells showing gelatinolytic activities, sections after ISZ were fixed with 4% paraformaldehyde, washed, and incubated

with R73 (anti-T cell), ED1 (anti-macrophage), anti-mast cell tryptase (sc-32473, anti-mast cell; Santa Cruz Biotechnology), anti- $\alpha$ -actinin (EA-53; anti-cardiocyte), or anti-vimentin (SP20; Epitomics) for 1 h, followed by Cy3-labeled anti-mouse IgG (Chemicon International) for 1 h. After being mounted with Vectashield (Vector Laboratories), the sections were observed with a confocal microscope (Leica TCS NT; Leica Microsystems).

Gelatin zymography was performed using the Novex In-gel Zymography System (Invitrogen), according to the manufacturer's instructions. Heart tissues were homogenized in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. After centrifugation, supernatants were harvested and 100  $\mu\text{g}$  of protein was mixed with Novex Tris-glycine SDS sample buffer and run on Novex 10% Zymogram Gelatin Gel, followed by incubation with Zymogram Renaturing Buffer, and then with Zymogram Developing Buffer. After an overnight reaction, the gel was stained with SimplyBlue Safestain (Invitrogen).

#### Identification of mast cells

To identify mast cells, toluidine blue staining was performed. Frozen sections were fixed with ether, and paraffin-embedded sections were deparaffinized and rehydrated. They were then incubated with 0.05% w/v toluidine blue for 30 min, followed by counterstaining with 0.01% eosin for 1 min. Some sections were immunohistochemically stained with Abs against mast cell tryptase (Santa Cruz Biotechnology) to identify mast cells.

#### Real-time PCR

Total RNA was extracted, as described above, and first-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using random hexamer primers and ReverTra Ace (Toyobo). SYBR Green real-time PCR were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems) in a total volume of 25  $\mu\text{l}$  using the SYBR Premix Ex Taq (Takara Bio). Each reaction was performed in duplicate using the following thermocycler conditions: 95°C for 10 min for one cycle, followed by 50 cycles of 95°C for 15 s and 58°C for 1 min. All primers were designed over an intron-exon junction to prevent the coamplification of genomic DNA. The relative quantification of mRNA was performed using the standard curve method. MMP and cytokine mRNA were normalized to GAPDH for each sample (15). The absence of nonspecific amplification was confirmed by dissociation curve analysis.

#### Treatment of EAC with MMP inhibitors

Minocycline treatment of EAC was performed using a protocol applied for experimental autoimmune encephalomyelitis, with a few modifications (16, 17). Minocycline hydrochloride (Sigma-Aldrich) was dissolved in PBS and administered daily by i.p. injection at a dose of 50 mg/kg body weight for 3 wk. On day 21 postimmunization (PI), rats were sacrificed under deep anesthesia, and the effects of minocycline were evaluated histologically.

TISAM, an *N*-sulfonylamino acid derivative (18), was provided by Discovery Research Laboratories of Shionogi. Rats were orally fed with TISAM suspended in 0.5% w/v methylcellulose 400 solution (WAKO) at a dosage of 5 mg/kg/day, according to a previous protocol (19). Rats were sacrificed on day 21 under deep anesthesia, and hearts were removed and analyzed histologically.

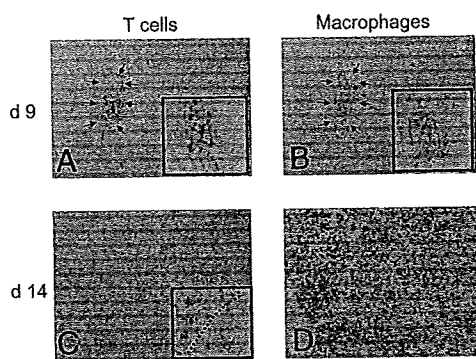
#### Statistical analysis

Data were analyzed by Student's *t* test or Mann-Whitney's *U* test. Values of *p* less than 0.05 were considered significant.

## Results

### Early immunopathological events and the switch from inflammation to fibrosis in the heart with C protein-induced EAC

One of the advantages of using C protein-induced EAC for analysis is that the onset and progression of the disease are rather uniform (9, 10). Therefore, this model is suitable for examining early immunopathological events occurring in the heart. We performed immunohistochemical staining for T cells with R73 mAb and macrophages with ED1 mAb using frozen sections of hearts taken from unimmunized and immunized rats at 0, 7, 9, 11, and 14 days after immunization. Consistent with our previous work, very few T cells might be trapped in blood vessels and macrophages residing in the hearts of control animals (10). On day 7, T cells and

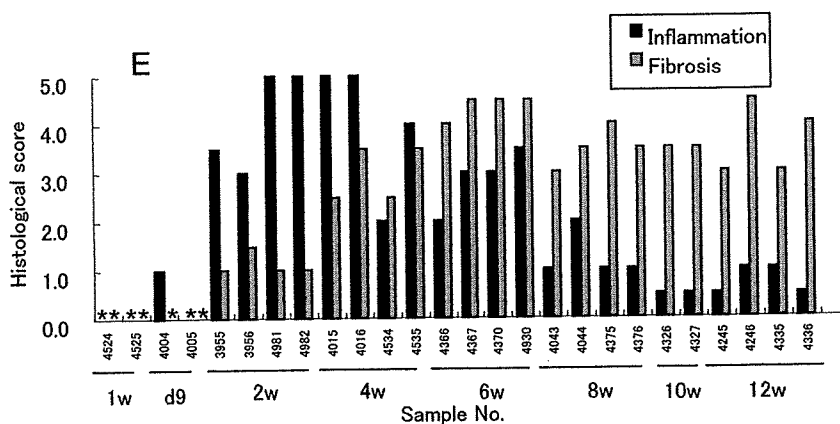
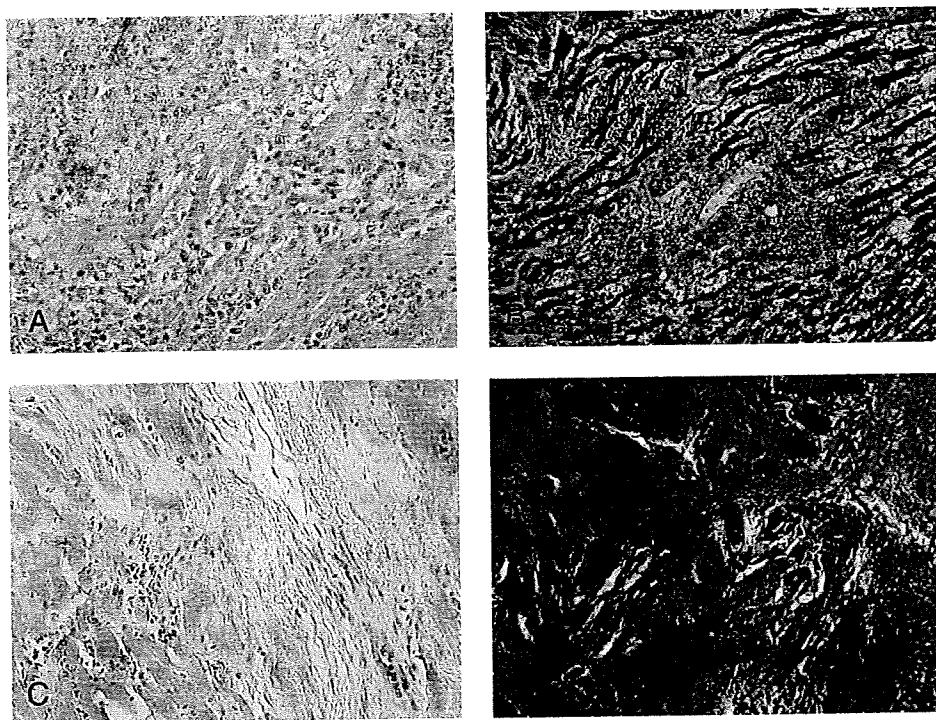


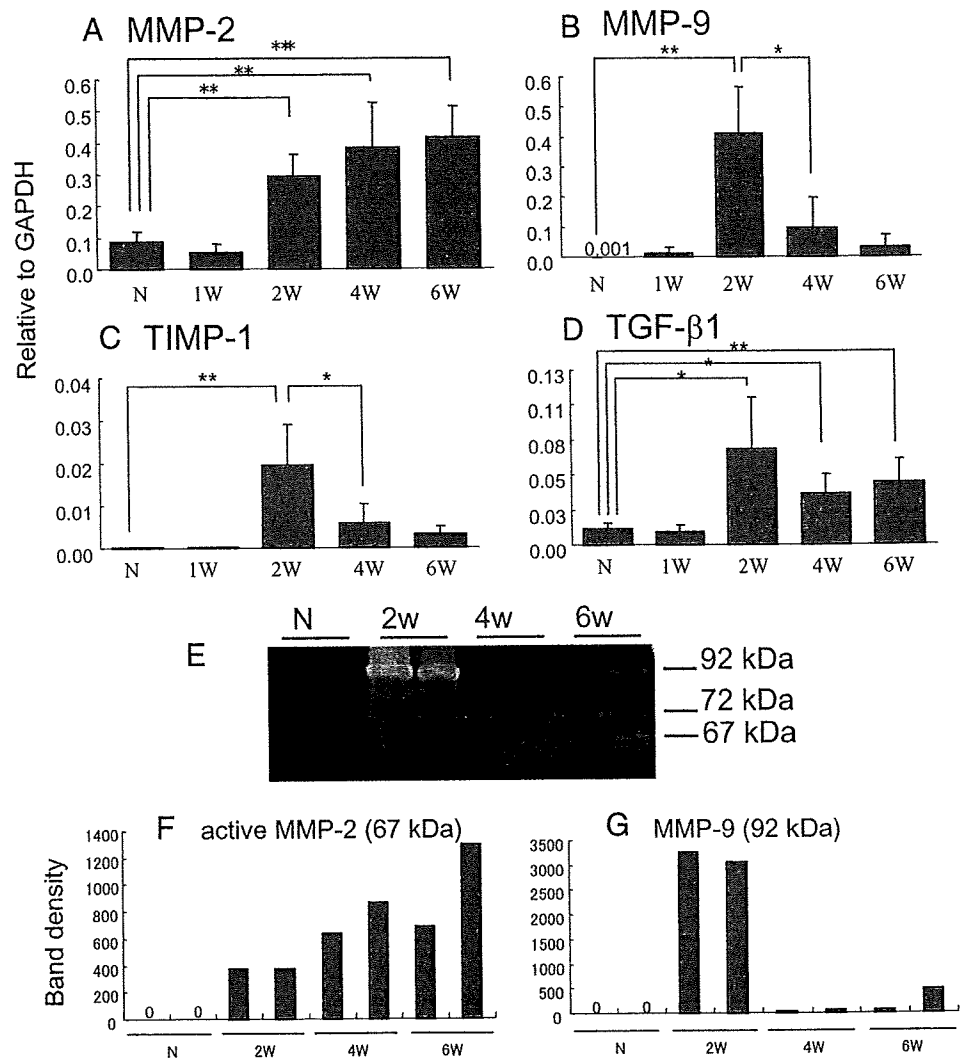
**FIGURE 1.** A–D, Immunohistochemical staining for T cells (A and C) and macrophages (B and D) of hearts taken on days 9 (A and B) and 14 (C and D). T cells and macrophages are colocalized in a small inflammatory focus in serial sections (A and B). At the peak of inflammation, T cells are distributed diffusely, and a much larger number of macrophages infiltrate the parenchyma. The same field of serial sections was photographed. An inset of C demonstrates that in some areas of lesions, T cells are almost absent (below the dotted line). A and C, R73 staining; B and D, ED1 staining. A–D, Original magnification  $\times 75$ ; insets  $\times 150$ .

macrophages increased in number, but they did not form inflammatory foci (data not shown). Small inflammatory foci appeared on day 9. As shown in Fig. 1, A and B, the foci were composed of T cells and macrophages (indicated by arrows). On day 14, the number and size of inflammatory foci increased considerably (Fig. 1, C and D). When the disease peaked (2–4 wk), diffuse T cell and dense macrophage infiltration was noted, as shown in Fig. 1, C and D. However, in some lesions, T cells were sparsely distributed in the cell cluster (inset of Fig. 1C). These findings indicated that definite lesion formation starts on day 9 and suggested that subtle inflammatory processes began on day 7.

Using H&E- and Azan-stained sections, inflammation and fibrosis were scored according to the grading system described under *Materials and Methods*. Representative features at early (2 wk; Fig. 2, A and B) and late (12 wk; Fig. 2, C and D) stages are shown. During the early stage, there was extensive inflammatory cell infiltration in the parenchyma of some rats (Fig. 2A), whereas fibrosis shown in Azan staining was minimal (Fig. 2B). In sharp contrast, a very small number of inflammatory cells was present at the later stage (Fig. 2C), but dense fibrosis was seen by Azan staining (Fig. 2D). Consistent with these findings, inflammation was not obvious on days 7 and 9 (Fig. 2E). However, inflammation was

**FIGURE 2.** Estimation of inflammation and fibrosis using H&E- and Azan-stained sections. Representative feature at early (2 wk; A and B) and late (12 wk; C and D) stages is shown. During the early stage, there was extensive inflammatory cell infiltration in the parenchyma of some rats (A), whereas fibrosis shown in Azan staining was minimal (B). In sharp contrast, very small number of inflammatory cells was present at the later stage (C). Instead, dense fibrosis was recognized in Azan staining (D). A–D, Original magnification  $\times 180$ . E, Longitudinal study of inflammation and fibrosis in the hearts during autoimmune carditis and subsequent dilated cardiomyopathy. Inflammation and fibrosis were scored according to the grading system provided under *Materials and Methods*. There was severe inflammation between 2 and 6 wk, and fibrosis was significantly beginning at 4 wk. Between 4 and 6 wk, inflammation and fibrosis were colocalized in diseased hearts. Asterisks indicate score 0 for inflammation and fibrosis.





**FIGURE 3.** A–D, Quantitative analysis of MMP-2, MMP-9, TIMP-1, and TGF- $\beta$ 1 mRNA by real-time PCR. Real-time PCR was performed using heart tissues from normal and immunized rats at 1, 2, 4, and 6 wk to determine the levels of MMP-2 (A), MMP-9 (B), TIMP-1 (C), and TGF- $\beta$ 1 (D) mRNA. Each group consisted of four rats. The y-axis represents values normalized with GAPDH. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . N, Normal. E–G, Gelatin zymography (E) and its density analysis (F and G) revealed essentially the same findings as obtained by real-time PCR.

prominent after 2 wk (the early inflammation phase) and lasted until week 6. In contrast, marked fibrosis appeared after 4 wk and became predominant after 8 wk (the late fibrosis phase). Between weeks 4 and 6, inflammation and fibrosis were colocalized in diseased hearts (Fig. 2E).

#### Kinetics of MMP-2, MMP-9, TIMP-1, and TGF- $\beta$ 1 mRNA in the heart

We quantitated the mRNA and protein concentration of inflammation- and fibrosis-related molecules in the hearts of normal and immunized animals by real-time PCR and gelatin zymography. As shown in Fig. 3, A and B, MMP-2 and MMP-9 mRNA had different kinetics. MMP-2 mRNA increased gradually until the chronic phase, with significant increase at 2 and 4 wk compared with normal hearts (Fig. 3A). In sharp contrast, MMP-9 mRNA increased abruptly at 2 wk and decreased thereafter (Fig. 3B). Interestingly, TIMP-1 mRNA had essentially the same kinetics as MMP-9 mRNA (Fig. 3C). TGF- $\beta$ 1 mRNA increased at 2 wk, and expression remained high during the chronic phase (Fig. 3D).

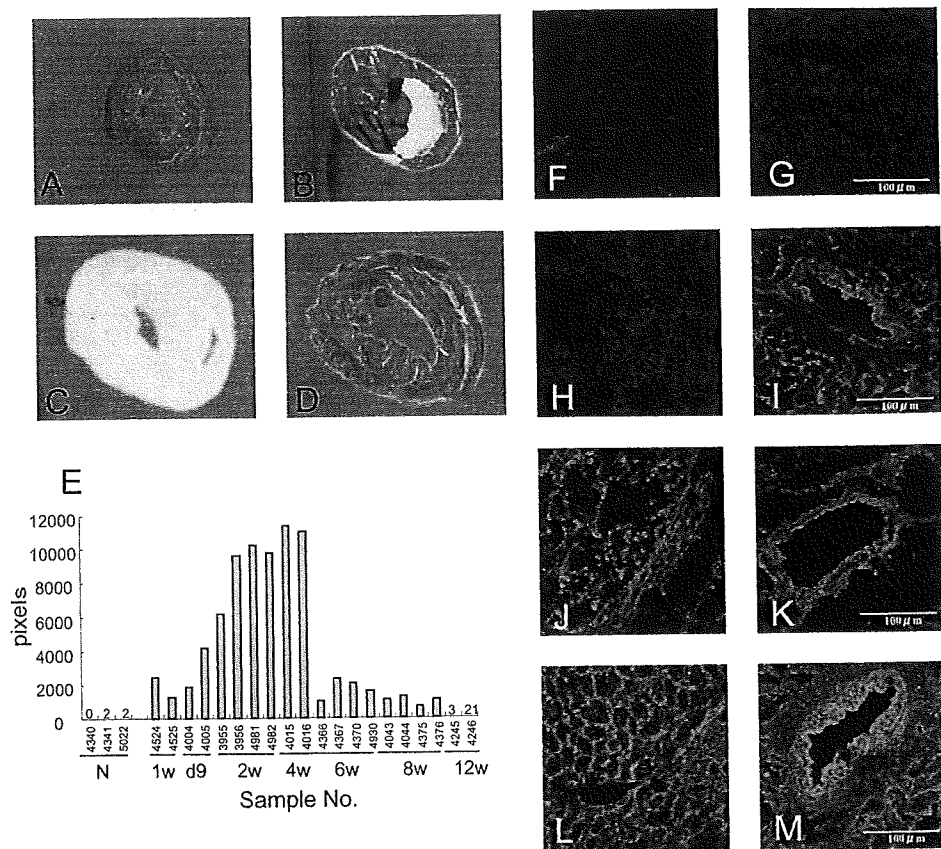
Gelatin zymography (Fig. 3E) demonstrated that the MMP activity correlated well with mRNA expression revealed by real-time PCR analysis. MMP-9 increased abruptly at 2 wk PI (Fig. 3G), whereas both active (Fig. 3F) and latent (data not shown) MMP-2 showed continuous up-regulation during the inflammation and fibrosis phases.

#### Kinetics of gelatinase activity in the heart during EAC

To quantitatively estimate gelatinase activity, we performed FIZ. Representative FIZ figures at different time points (Fig. 4, A–D) and a summary of the results (Fig. 4E) are shown in Fig. 4. Under normal conditions, gelatinase activities were not detected in the heart (Fig. 4A). Gelatinase activity was seen in part of the heart on day 9 when small inflammatory foci were formed, and in the entire heart at 4 wk when all inflammatory parameters peaked (Fig. 4C). It should be noted that at both time points, the areas showing gelatinase activity were larger than those of inflammation shown in Figs. 1 and 2. Gelatinase activity dropped quickly at 6 wk and was almost absent at 12 wk (Fig. 4, D and E). A summary of the FIZ analysis (Fig. 4E) revealed two interesting findings. First, consistent with immunohistochemical findings, weak, but definite gelatinase activity was seen in the hearts as early as day 7. Second, gelatinase activity as detected by FIZ analysis dropped down quickly at 6 wk as fibrosis became prominent. The latter finding, along with previous reports (20), suggests that an imbalance of the MMP/TIMP ratio may facilitate cardiac fibrosis.

We next determined the localization of active gelatinase by ISZ (Fig. 4, F–M). Under normal conditions (Fig. 4, F and G) and before inflammation (data not shown), gelatinase activity was not detected in the parenchyma or blood vessels. However, on day 9 when hearts were at an early stage of inflammation (Fig. 1), some

**FIGURE 4.** A–E, FIZ of normal and inflamed hearts. FIZ was performed at various time points. Representative figures taken from a normal rat (A) and rats at day 9 (B), 4 wk (C), and 12 wk (D). A–D, Original magnification  $\times 1$ . Quantitative analysis of individual rats is illustrated in E. The y-axis represents pixels determined by image analysis. F–M, ISZ for gelatinase using unfixed frozen heart sections. Sections from normal and immunized rats at 1, 2, 4, and 6 wk were incubated with DQ gelatin (FITC-labeled gelatin) overnight. Gelatinase activities were visualized by cleaving gelatin. F, H, J, and L, Show activities in the parenchyma; G, I, K, and M, show activities in the perivascular region in hearts of normal (F and G) and immunized (H–M) rats. Scale bar = 100  $\mu\text{m}$ .



vessel walls and the perivascular region were positive for gelatinase (Fig. 4, H and I). At the peak of inflammation (2 wk), vascular and parenchymal staining was strong and diffuse (Fig. 4, J and K). Thereafter, inflammation was gradually replaced by fibrosis (Fig. 2E), but gelatinase activity, as detected by ISZ, was maintained throughout the observation period (Fig. 4, L and M). Considering that the level of MMP-9, but not MMP-2, mRNA dropped very rapidly at 4 wk (Fig. 3), we concluded that the gelatinase activity at 6 wk is mainly due to MMP-2.

#### Identifying cells with gelatinase activities in the heart

Using ISZ and immunohistochemistry, we identified which cells expressed gelatinase in the heart by using markers for T cells (R73), macrophages (ED1), mast cells (anti-mast cell tryptase), cardiomyocytes (anti- $\alpha$ -actinin), and myofibroblasts (anti-vimentin). The results are illustrated in Fig. 5. At 2 wk (Fig. 5, A–L), T cells (Fig. 5, A–C), and mast cells (Fig. 5, G–I) did not express gelatinase activity (arrows in Fig. 5, C and I). The majority of gelatinase-expressing cells were infiltrating macrophages (Fig. 5, D and E, and arrowheads in F). Cardiomyocytes showed diffuse and faint staining (Fig. 5, J–L). As clearly seen in Fig. 5L, cells showing strong gelatinase activity were negative for  $\alpha$ -actinin (arrowheads in Fig. 5L), suggesting that they are infiltrating inflammatory cells. At 6 wk (Fig. 5, P–X), T and mast cells were also negative for gelatinase (data not shown). Macrophages and cardiomyocytes had different staining patterns. At 6 wk, macrophages were negative for gelatinase (Fig. 5, P and Q, and arrows in R), and cardiomyocytes stained strongly for gelatinase (Fig. 5, S–U). Vimentin, one of myofibroblast markers, was positive for a few cells at 2 wk (Fig. 5N), and vimentin-positive cells increased in number considerably at 6 wk (Fig. 5W). At both time points, it was clearly

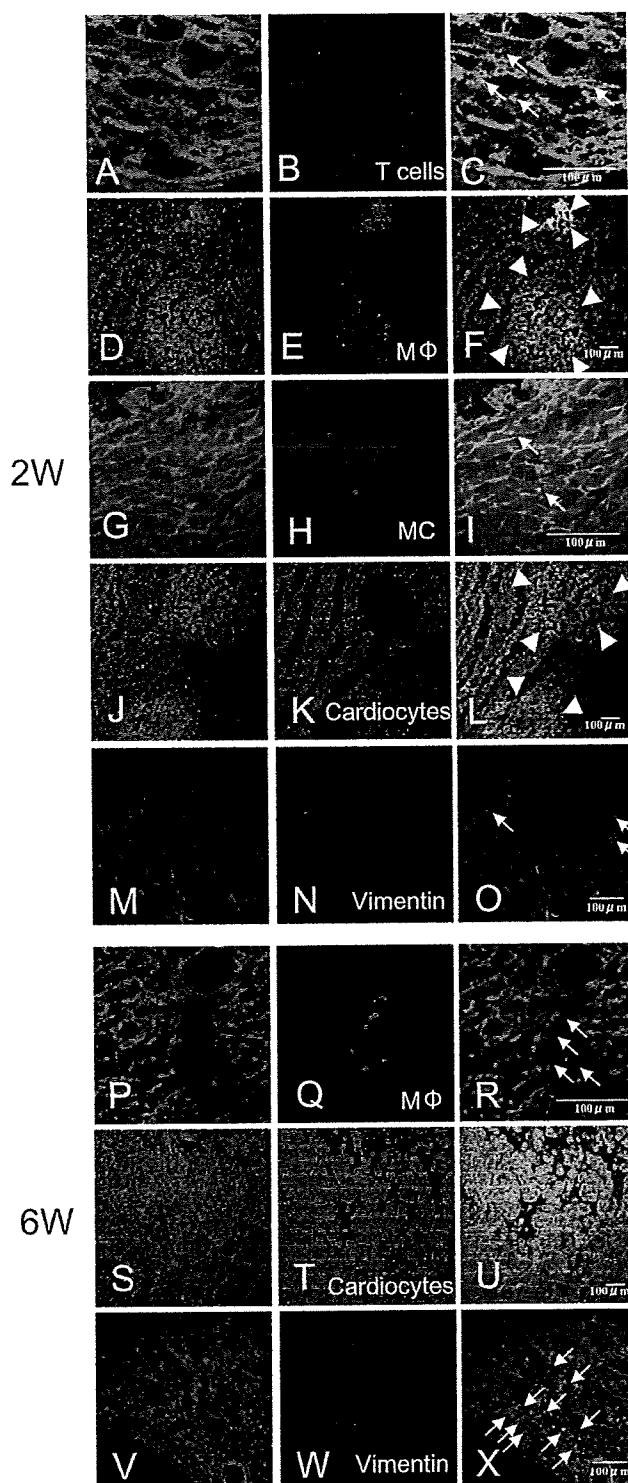
demonstrated that vimentin-positive cells were negative for gelatinase (arrows in Fig. 5, O and X).

#### Treatment of CC2-immunized rats with MMP inhibitors

Finally, we treated CC2-immunized rats with two types of MMP inhibitors to examine whether the suppression of MMPs could modulate EAC development (Figs. 6 and 7). Minocycline, which was reported to be an inhibitor of MMP-2 and MMP-9, was administered at a dose of 50 mg/kg for 3 wk from the day of immunization. Hearts were examined both macroscopically and microscopically on day 21 (Fig. 6). Minocycline treatment significantly ameliorated the histological severity of EAC (Fig. 6A). Although minocycline is considered as an inhibitor of both MMP-2 and MMP-9, real-time PCR analysis of heart tissues revealed that MMP-9, but not MMP-2, mRNA was almost completely suppressed by minocycline (Fig. 6, C and D). Gelatin zymography demonstrated that the treatment resulted in complete inhibition of MMP-9 and moderate suppression of active MMP-2 (Fig. 6B). The findings obtained by in vivo treatments were consistent with those obtained by in vitro studies, showing that, especially at low concentrations, minocycline severely inhibits MMP-9 activity, but has less effects on MMP-2 (16, 21). Collectively, these results indicate that minocycline treatment predominantly suppressed MMP-9 production to ameliorate the severity of C protein-induced EAC. Minocycline treatment also affected the survival rate. Approximately 10% of untreated immunized rats died at this time point (9), whereas there were no dead rats in the treated group. These findings suggest that MMP-9 plays an important role in the development and progression of a certain type of EAC and DCM.

We next treated immunized animals with the MMP-2-specific inhibitor, TISAM (an *N*-sulfonylamino acid derivative) (22), for 3





**FIGURE 5.** Double staining for gelatinase activity and markers of T cells (A–C), macrophages (D–F), mast cells (G–I), cardiocytes (J–L), and myofibroblasts (M–O and V–X) at 2 wk (A–O) and 6 wk (P–X). At 2 wk, T cells and mast cells were negative for gelatinase (arrows in C and I, respectively). Infiltrating macrophages were strongly positive for gelatinase (D–F and arrowheads in F), and cardiomyocytes exhibited weak gelatinase activity (J–L). Strong gelatinase activity was seen in  $\alpha$ -actinin-negative cells (arrowheads in L). At 6 wk, macrophages become negative for gelatinase (M–O and arrows in O), whereas cardiomyocytes had diffuse and strong gelatinase activity (P–R). Vimentin was positive for a few cells at 2 wk (N), and vimentin-positive cells increased in number considerably at 6 wk (W). At both time points, vimentin-positive cells were negative for gelatinase (arrows in O and X). Scale bar = 100  $\mu$ m.

wk and evaluated the histological severity of TISAM-treated and vehicle-treated rats on day 21. TISAM treatment caused no significant difference in pathology between the treated and control groups (Fig. 7A). Then, we determined the mRNA levels of MMP-2 and MMP-9. Real-time PCR analysis revealed that in vivo administration of TISAM did not suppress the level of MMP-2 mRNA (Fig. 7B). Similarly, gelatin zymography revealed no difference in the band density of MMP-2 between the TISAM-treated and vehicle-treated groups (Fig. 7C). Because MMP-2 activities could reappear if the TISAM-MMP-2 complex dissociated during electrophoresis, we performed ISZ to evaluate in situ enzymatic activity. Gelatinolytic activity, mainly by MMP-2 produced by cardiomyocytes (Fig. 5), was diminished in both the parenchyma and the blood vessels compared with controls (Fig. 7D). Semi-quantitative analysis revealed that gelatinolytic activity was significantly suppressed in the TISAM-treated groups compared with the control group. These findings are consistent with previous studies (19), demonstrating that TISAM specifically inhibited MMP-2 activities in a cell-free assay. These findings indicate that, although TISAM treatment inhibited MMP-2 activities in a contact-dependent manner, it does not modulate the development and progression of EAC.

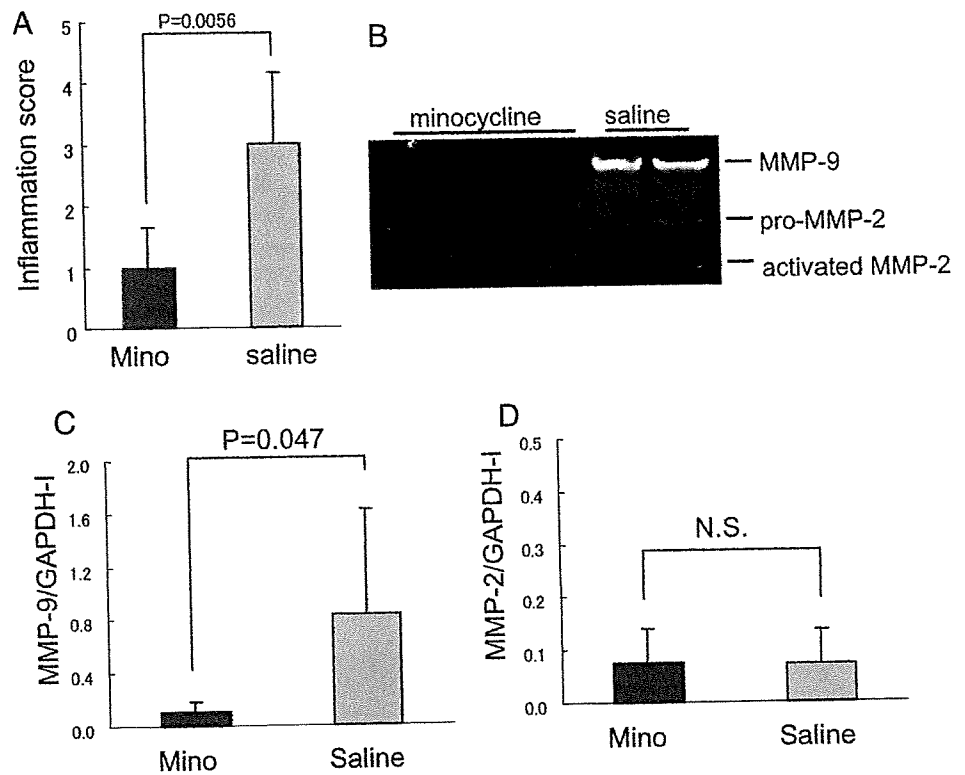
We also performed immunohistochemical examination of hearts of rats treated with minocycline (Fig. 8, A and B) and TISAM (Fig. 8, C and D), and untreated controls at 2 wk (Fig. 8, E and F) and 6 wk (Fig. 8, G and H). Compared with untreated controls (Fig. 8E), infiltrating T cells decreased in number drastically in the minocycline-treated groups (Fig. 8A). TISAM treatment did not suppress T cell infiltration (Fig. 8C). At 6 wk, T cells were sparsely distributed in the heart parenchyma in the untreated controls (Fig. 8G). Macrophage staining showed a similar finding, but the number of macrophages was higher than that of T cells (Fig. 8, B, D, F, and H).

## Discussion

DCM is a serious problem for patients with heart failure because the disease progresses irreversibly and often causes death. To develop effective therapies, it is essential to elucidate the pathomechanism of DCM development. Unfortunately, there are few sufficient experimental models for DCM. In previous studies, we succeeded in inducing severe EAC by immunizing Lewis rats with cardiac C protein (9, 10). The animals presented characteristics of EAC with a high rate of fatality, and the survivors developed DCM. This animal model is useful not only to understand the mechanism of DCM development from viral and immune induction, but also to develop effective immunotherapies (9). Furthermore, as shown in the present study, the disease course consistently exhibits early inflammatory and late fibrosis phases with a transitional phase between them. This uniform lesion formation enables the precise examinations of disease development and progression. In the present study, we demonstrate the important role of MMPs in the development of EAC and DCM to identify a successful, mechanism-based therapy.

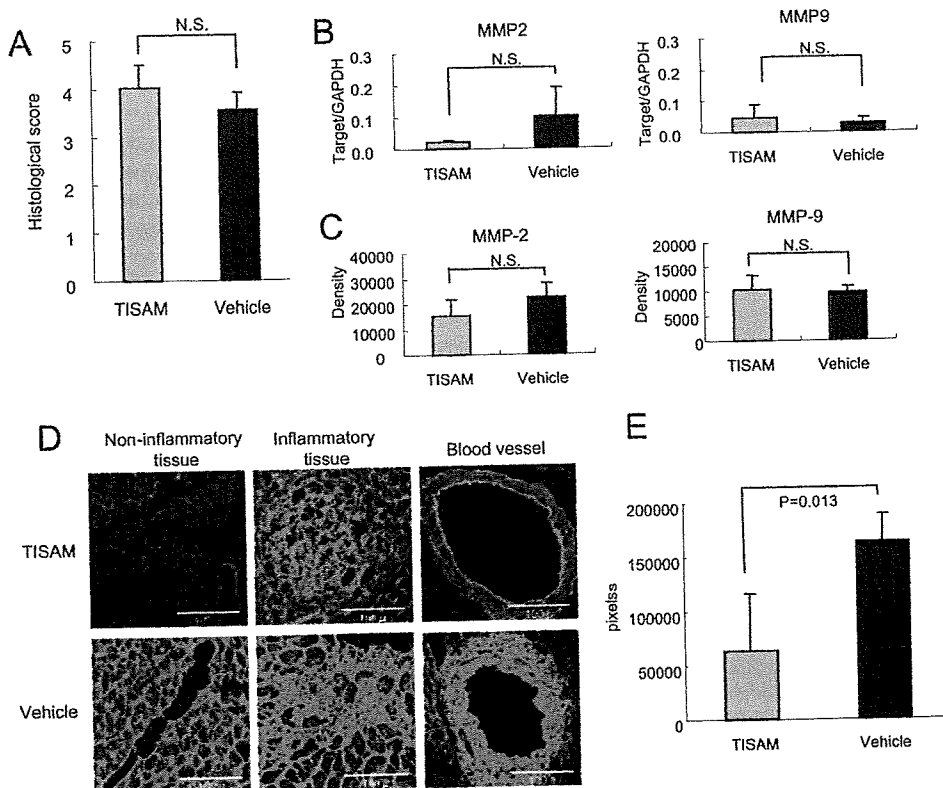
MMPs form a family of enzymes that mediate various functions in tissue destruction and immune responses in autoimmune diseases. In the heart, it was demonstrated that MMPs play a pivotal role in acute cardiac injury and chronic remodeling (19, 23, 24). However, virtually no reports have shown longitudinal examinations of MMPs at either the RNA or protein level. In the present study, we correlate MMP expression and enzymatic activity with the degree and extent of inflammation and fibrosis in the heart over a 12-wk period. In our model, inflammation peaked after 2 wk, and then fibrosis developed. MMP-2, MMP-9, TIMP-1, and TGF- $\beta$ 1 mRNA all showed peak levels at 2 wk, and gelatinase activity was seen in both the parenchyma and the perivascular space. At 6 wk,

**FIGURE 6.** Minocycline treatment ameliorates EAC and down-regulates MMP-9, but not MMP-2, activity. Minocycline hydrochloride was dissolved in PBS and administered daily by i.p. injection at a dose of 50 mg/kg body weight for 3 wk. On day 21, rats were sacrificed under deep anesthesia, and the effects of minocycline were evaluated histologically. *A*, Inflammation score; *B*, gelatin zymography; *C* and *D*, real-time PCR.

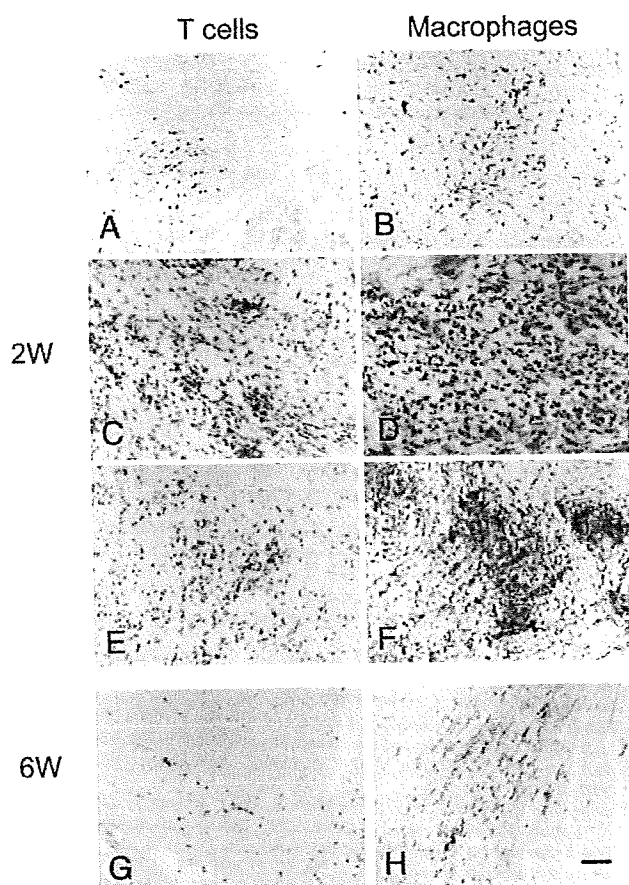


every parameter shifted to the fibrosis phase. Whereas MMP-2 and TGF- $\beta$ 1 mRNA were maintained at a high level, MMP-9 and TIMP-1 mRNA levels dropped after week 2. As shown in the present study, FIZ and ISZ had distinct patterns during the chronic phase. Whereas FIZ activity dropped down rapidly after week 6, ISZ activity remained at a high level during this period. The results

obtained by gelatin zymography clearly demonstrated that at 2 wk PI, MMP-9 and, to a lesser degree, MMP-2 represented the gelatinase activity. However, at the 4- and 6-wk time point, MMP-2 was the major gelatinase present in the heart. These findings strongly suggested that FIZ and ISZ mainly represent MMP-9 and MMP-2 activities, respectively.



**FIGURE 7.** TISAM treatment suppresses MMP-2 activities, but does not modulate the severities of EAC. TISAM, suspended in 0.5% w/v methylcellulose 400 solutions, was orally fed at a dosage of 5 mg/kg/day. Rats received TISAM daily for 3 wk after immunization with CC2. Control rats received the vehicle. Rats were sacrificed on day 21 under deep anesthesia, and hearts were removed and analyzed histologically (*A*). In parallel, real-time PCR (*B*) and gelatin zymography (*C*) were performed using heart tissues. Cryostat sections were stained with ISZ (*D*) and evaluated semiquantitatively (*E*).



**FIGURE 8.** Immunohistochemical examination of hearts of rats treated with minocycline (A and B) and TISAM (C and D), and untreated controls at 2 wk (E and F) and 6 wk (G and H). Frozen sections were stained for T cells (A, C, E, and G) and macrophages (B, D, F, and H). Compared with untreated controls (E), infiltrating T cells decreased in number drastically in the minocycline-treated groups (A). TISAM treatment did not suppress T cell infiltration (C). At 6 wk, T cells were sparsely distributed in the heart parenchyma in the untreated controls (G). Macrophage staining showed a similar finding, but the number of macrophages was much higher than that of T cells (B, D, F, and H). A, C, E, and G, R73 staining for T cells; B, D, F, and H, ED1 staining for macrophages. All of the microphotographs were taken under the same magnification. A bar in H indicates 100  $\mu\text{m}$ .

Examinations of ISZ and immunohistochemistry clearly demonstrated that gelatinase (MMP-2 and MMP-9)-expressing cells in the early stage consist of infiltrating macrophages, whereas those in the later stage are cardiomyocytes. Gelatin zymography and real-time PCR analysis suggest that infiltrating macrophages produce both MMP-2 and MMP-9 during the inflammation phase, whereas cardiomyocytes produce mainly MMP-2 at later stages. Although it is well known that MMPs, especially MMP-2, are expressed in cardiomyocytes under various pathological conditions (25–28), the present study clearly demonstrates that MMP-2 expression by cardiomyocytes takes place during later stages, but not during early inflammation. It should be noted that T cells and mast cells, which are reported to secrete MMPs (29, 30), were negative for gelatinase activity throughout the observation period. These findings suggest that, although MMPs can be expressed in a variety of cells, they are limited to certain cell types under individual pathological conditions.

It is interesting to note that MMP-2 mRNA was at a high level from 2 wk onward throughout the observation period, whereas MMP-9 mRNA was up-regulated at week 2 and then dropped at

week 4. These time points corresponded with the switch from inflammation to fibrosis in the diseased hearts. Although both MMP-2 and MMP-9 belong to the gelatinase family, their role in autoimmune inflammation is substantially different. Morphologic and enzymatic analyses suggested that MMP-9 promotes disease progression during the early inflammatory phase and that MMP-2 is active during the chronic fibrosis phase. To test these hypotheses, we treated rats with MMP inhibitors. Administration of minocycline significantly reduced inflammation (Fig. 6) (16, 21). The effective use of minocycline in brain diseases such as encephalomyelitis (16, 31) and stroke (21) has been demonstrated, but this is the first report to demonstrate the effectiveness of minocycline in heart inflammation. In addition to down-regulation of MMPs, minocycline has several functions, including antiapoptotic and antioxidant actions (32, 33). Therefore, it is possible that cardioprotection may be also exerted by these mechanisms other than MMP suppression. These findings suggest that minocycline and its derivatives could be potential drugs for EAC and DCM therapy.

The role of MMP-2 in various disease conditions remains to be controversial. MMP-2-deficient mice exhibited more severe forms of autoimmune inflammation, such as arthritis (34), encephalomyelitis (35), myocarditis (36), and colitis (37), suggesting that MMP-2 plays a suppressive role in the development of autoimmune diseases. However, targeted disruption of MMP-2 ameliorated myocardial remodeling after myocardial infarction (19, 38) and chronic pressure overload (24). The precise mechanism of MMP-2's differential effects under various disease conditions remains unclear; there are several explanations. Esparza et al. (35) demonstrated a compensatory increase in MMP-9 in MMP-2-deficient mice with encephalomyelitis, which may be an aggravation factor of the disease. In addition, MMP-2 may suppress inflammation by cleaving MCP-3 (39) and IL-1 (40). In contrast, in situation of cardiac remodeling without inflammation, disruption or inhibition of MMP-2 could improve disease conditions. In the present study, we treated immunized animals with a specific MMP-2 inhibitor, TISAM, to evaluate whether MMP-2 inhibition modulates the disease course. TISAM treatment during the early inflammatory phase neither ameliorated nor aggravated the disease, although the treatment did suppress MMP-2 activity (Fig. 7). These findings suggest that MMP-2 has a small role in early lesion formation.

The involvement of MMP-2 and MMP-9 in fibrosis formation during the late stage is controversial. Although some groups insisted that MMP-2 is necessary and sufficient for fibrosis formation (41, 42), other groups reported that both MMP-2 and MMP-9 are involved (43, 44). In contrast, there are several reports suggesting that MMPs play no or little role in fibrosis formation (45–48). Because there are so many differences in the types of diseases, experimental condition, and clinical observations, it is difficult to draw conclusions at the moment. It should be elucidated in the near future.

In summary, we have demonstrated that during the course of EAC and subsequent DCM, different types of cells produced MMPs in a manner that corresponds to the disease phase. During the early inflammation phase, infiltrating macrophages mainly produced MMP-9, and cardiomyocytes produced MMP-2 in later stages. Inhibition of MMP-9, but not of MMP-2, ameliorated the disease status. Thus, immunotherapy against EAC should be performed based on an understanding of the disease development mechanism.

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#### Disclosures

The authors have no financial conflict of interest.

## References

- Dec, G. W., and V. Fuster. 1994. Idiopathic dilated cardiomyopathy. *N. Engl. J. Med.* 331: 1564–1575.
- McNamara, D. M., R. Holubkov, R. C. Starling, G. W. Dec, E. Loh, G. Torre-Amione, A. Gass, K. Janosko, T. Tokarczyk, P. Kessler, et al. 2001. Controlled trial of intravenous immune globulin in recent-onset dilated cardiomyopathy. *Circulation* 103: 2254–2259.
- Kanzaki, Y., F. Terasaki, M. Okabe, T. Hayashi, H. Toko, H. Shimomura, S. Fujioka, Y. Kitaura, K. Kawamura, Y. Horii, et al. 2001. Myocardial inflammatory cell infiltrates in cases of dilated cardiomyopathy as a determinant of outcome following partial left ventriculectomy. *Jpn. Circ. J.* 65: 797–802.
- Parrillo, J. E., R. E. Cunnion, S. E. Epstein, M. M. Parker, A. F. Suffredini, M. Brenner, G. L. Schaer, S. T. Palmeri, R. O. Cannon, D. Alling, et al. 1989. A prospective, randomized, controlled trial of prednisone for dilated cardiomyopathy. *N. Engl. J. Med.* 321: 1061–1068.
- Wojnicz, R., E. Nowalany-Kozielecka, C. Wojciechowska, G. Glanowska, P. Wilczewski, T. Niklewski, M. Zembala, L. Polonske, M. M. Rozek, and J. Wodniecki. 2001. Randomized, placebo-controlled study for immunosuppressive treatment of inflammatory dilated cardiomyopathy. *Circulation* 104: 39–45.
- Staudt, A., F. Schaper, V. Stangl, A. Plagemann, M. Bohm, K. Merkel, G. Wallukat, K. D. Wernecke, K. Stangl, G. Baumann, and S. B. Felix. 2001. Immunohistological changes in dilated cardiomyopathy induced by immunoadsorption therapy and subsequent immunoglobulin substitution. *Circulation* 103: 2681–2686.
- Hessel, F. P., C. Wegner, J. Mueller, C. Glaveris, and J. Wasem. 2004. Economic evaluation and survival analysis of immunoglobulin adsorption in patients with idiopathic dilated cardiomyopathy. *Eur. J. Health Econ.* 5: 58–63.
- Dennis, J. E., T. Shimizu, F. C. Reinach, and D. A. Fischman. 1984. Localization of C-protein isoforms in chicken skeletal muscle: ultrastructural detection using monoclonal antibodies. *J. Cell Biol.* 98: 1514–1522.
- Matsumoto, Y., Y. Tsukada, A. Miyakoshi, H. Sakuma, and K. Kohyama. 2004. C-protein-induced myocarditis and subsequent dilated cardiomyopathy: rescue from death and prevention of dilated cardiomyopathy by chemokine receptor DNA therapy. *J. Immunol.* 173: 3535–3541.
- Matsumoto, Y., I. K. Park, and K. Kohyama. 2007. B-cell epitope spreading is a critical step for the switch from C-protein-induced myocarditis to dilated cardiomyopathy. *Am. J. Pathol.* 170: 43–51.
- Visse, R., and H. Nagase. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* 92: 827–839.
- Schulz, R. 2007. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. *Annu. Rev. Pharmacol. Toxicol.* 47: 211–242.
- Ohmori, K., Y. Hong, M. Fujiwara, and Y. Matsumoto. 1992. In situ demonstration of proliferating cells in the rat central nervous system during experimental autoimmune encephalomyelitis: evidence suggesting that most infiltrating T cells do not proliferate in the target organ. *Lab. Invest.* 66: 54–62.
- Oh, L. Y., P. H. Larsen, C. A. Krekoski, D. R. Edwards, F. Donovan, Z. Werb, and V. W. Yong. 1999. Matrix metalloproteinase-9/gelatinase B is required for process outgrowth by oligodendrocytes. *J. Neurosci.* 19: 8464–8475.
- Bas, A., G. Forsberg, S. Hammarstrom, and M. L. Hammarstrom. 2004. Utility of the housekeeping genes 18S rRNA,  $\beta$ -actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand. J. Immunol.* 59: 566–573.
- Brundula, V., N. B. Rewcastle, L. M. Metz, C. C. Bernard, and V. W. Yong. 2002. Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* 125: 1297–1308.
- Maier, K., D. Merkler, J. Gerber, N. Taheri, A. V. Kuhnert, S. K. Williams, C. Neusch, M. Bahr, and R. Diem. 2007. Multiple neuroprotective mechanisms of minocycline in autoimmune CNS inflammation. *Neurobiol. Dis.* 25: 514–525.
- Oba, K., H. Konno, T. Tanaka, M. Baba, K. Kamiya, M. Ohta, T. Kaneko, T. Shouji, A. Igarashi, and S. Nakamura. 2002. Prevention of liver metastasis of human colon cancer by selective matrix metalloproteinase inhibitor MMI-166. *Cancer Lett.* 175: 45–51.
- Matsumura, S., S. Iwanaga, S. Mochizuki, H. Okamoto, S. Ogawa, and Y. Okada. 2005. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. *J. Clin. Invest.* 115: 599–609.
- Heymans, S., F. Lupu, S. Terclavers, B. Vanwetswinkel, J. M. Herbert, A. Baker, D. Collen, P. Carmeliet, and L. Moons. 2005. Loss or inhibition of uPA or MMP-9 attenuates LV remodeling and dysfunction after acute pressure overload in mice. *Am. J. Pathol.* 166: 15–25.
- Machado, L. S., A. Kozak, A. Ergul, D. C. Hess, C. V. Borlongan, and S. C. Fagan. 2006. Delayed minocycline inhibits ischemia-activated matrix metalloproteinases 2 and 9 after experimental stroke. *BMC Neurosci.* 7: 56.
- Maekawa, R., H. Maki, T. Wada, H. Yoshida, K. Nishida-Nishimoto, H. Okamoto, Y. Matsumoto, H. Tsuzuki, and T. Yoshioka. 2000. Anti-metastatic efficacy and safety of MMI-166, a selective matrix metalloproteinase inhibitor. *Clin. Exp. Metastasis* 18: 61–66.
- Spinale, F. G. 2002. Matrix metalloproteinases: regulation and dysregulation in the failing heart. *Circ. Res.* 90: 520–530.
- Matsusaka, H., T. Ide, S. Matsushima, M. Ikeuchi, T. Kubota, K. Sunagawa, S. Kinugawa, and H. Tsutsui. 2006. Targeted deletion of p53 prevents cardiac rupture after myocardial infarction in mice. *Cardiovasc. Res.* 70: 457–465.
- Coker, M. L., M. A. Doscher, C. V. Thomas, Z. S. Galis, and F. G. Spinale. 1999. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. *Am. J. Physiol.* 277: H777–H787.
- Coker, M. L., J. R. Jolly, C. Joffs, T. Etoh, J. R. Holder, B. R. Bond, and F. G. Spinale. 2001. Matrix metalloproteinase expression and activity in isolated myocytes after neurohormonal stimulation. *Am. J. Physiol.* 281: H543–H551.
- Wang, W., C. J. Schulze, W. L. Suarez-Pinzon, J. R. Dyck, G. Sawicki, and R. Schulz. 2002. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 106: 1543–1549.
- Wang, W., C. J. Schulze, W. Wang, H. Leon, M. Sariahmetoglu, M. Sung, J. Sawicka, D. E. Sims, G. Sawicki, and R. Schulz. 2004. Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly(ADP-ribose) polymerase (PARP) in vitro. *FASEB J.* 18: 690–692.
- Di Girolamo, N., I. Indoh, N. Jackson, D. Wakefield, H. P. McNeil, W. Yan, C. Geczy, J. P. Arm, and N. Tedla. 2006. Human mast cell-derived gelatinase B (matrix metalloproteinase-9) is regulated by inflammatory cytokines: role in cell migration. *J. Immunol.* 177: 2638–2650.
- Abraham, M., S. Shapiro, A. Karni, H. L. Weiner, and A. Miller. 2005. Gelatinases (MMP-2 and MMP-9) are preferentially expressed by Th1 vs. Th2 cells. *J. Neuroimmunol.* 163: 157–164.
- Popovic, N., A. Schubart, B. D. Goetz, S. C. Zhang, C. Linington, and I. D. Duncan. 2002. Inhibition of autoimmune encephalomyelitis by a tetracycline. *Ann. Neurol.* 51: 215–223.
- Romero-Perez, D., E. Fricovsky, K. G. Yamasaki, M. Griffin, M. Barraza-Hidalgo, W. Dillmann, and F. Villareal. 2008. Cardiac uptake of minocycline and mechanisms for in vivo cardioprotection. *J. Am. Coll. Cardiol.* 52: 1086–1094.
- Buller, K. M., M. L. Carty, H. E. Reinebrant, and J. A. Wixey. 2009. Minocycline: a neuroprotective agent for hypoxic-ischemic brain injury in the neonate. *J. Neurosci. Res.* 87: 599–608.
- Itoh, T., H. Matsuda, M. Tanioka, K. Kuwabara, S. Itoharu, and R. Suzuki. 2002. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J. Immunol.* 169: 2643–2647.
- Esparza, J., M. Kruse, J. Lee, M. Michaud, and J. A. Madri. 2004. MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity. *FASEB J.* 18: 1682–1691.
- Matsusaka, H., M. Ikeuchi, S. Matsushima, T. Ide, T. Kubota, A. M. Feldman, A. Takeshita, K. Sunagawa, and H. Tsutsui. 2005. Selective disruption of MMP-2 gene exacerbates myocardial inflammation and dysfunction in mice with cytokine-induced cardiomyopathy. *Am. J. Physiol.* 289: H1858–H1864.
- Garg, P., M. Rojas, A. Ravi, K. Bockbrader, S. Epstein, M. Vijay-Kumar, A. T. Gewirtz, D. Merlin, and S. V. Sitaraman. 2006. Selective ablation of matrix metalloproteinase-2 exacerbates experimental colitis: contrasting role of gelatinases in the pathogenesis of colitis. *J. Immunol.* 177: 4103–4112.
- Hayashidani, S., H. Tsutsui, M. Ikeuchi, T. Shiomi, H. Matsusaka, T. Kubota, K. Imanaka-Yoshida, T. Itoh, and A. Takeshita. 2003. Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction. *Am. J. Physiol.* 285: H1229–H1235.
- McQuibban, G. A., J. H. Gong, E. M. Tam, C. A. McCulloch, I. Clark-Lewis, and C. M. Overall. 2000. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 289: 1202–1206.
- Ito, A., A. Mukaiyama, Y. Itoh, H. Nagase, I. B. Thøgersen, J. J. Enghild, Y. Sasaguri, and Y. Mori. 1996. Degradation of interleukin 1 $\beta$  by matrix metalloproteinases. *J. Biol. Chem.* 271: 14657–14660.
- Cheng, S., and D. H. Lovett. 2003. Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation. *Am. J. Pathol.* 162: 1937–1949.
- Bergman, M. R., J. R. Teerlink, R. Mahimkar, L. Li, B. Q. Zhu, A. Nguyen, S. Dahi, J. S. Karliner, and D. H. Lovett. 2007. Cardiac matrix metalloproteinase-2 expression independently induces marked ventricular remodeling and systolic dysfunction. *Am. J. Physiol.* 292: H1847–H1860.
- Lim, D. H., J. Y. Cho, M. Miller, K. McElwain, S. McElwain, and D. H. Broide. 2006. Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice. *Am. J. Physiol.* 291: L265–L271.
- Dwivedi, D., G. Pino, A. Banh, Z. Nathu, D. Howchin, P. Margettes, J. Sivak, and J. West-Mays. 2006. Matrix metalloproteinase inhibitors suppress transforming growth factor- $\beta$ -induced subcapsular cataract formation. *Am. J. Pathol.* 168: 69–79.
- Zhou, X., C. J. Hovell, S. Pawley, M. I. Hutchings, M. J. Arthur, J. P. Iredale, and R. C. Benyon. 2004. Expression of matrix metalloproteinase-2 and -14 persists during early resolution of experimental liver fibrosis and might contribute to fibrolysis. *Liver Int.* 24: 492–501.
- Heymans, S., B. Schroen, P. Vermeersch, H. Milting, F. Gao, A. Kassner, H. Gillijns, P. Herijgers, W. Flameng, P. Carmeliet, et al. 2005. Increased cardiac expression of tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 is related to cardiac fibrosis and dysfunction in the chronic pressure-overloaded human heart. *Circulation* 112: 1136–1144.
- Nishida, M., Y. Okumura, S. Ozawa, I. Shiraishi, T. Itoi, and K. Hamaoka. 2007. MMP-2 inhibition reduces renal macrophage infiltration with increased fibrosis in UUO. *Biochem. Biophys. Res. Commun.* 354: 133–139.
- Reif, S., R. Somech, E. Brazovskii, R. Reich, A. Belson, F. M. Konikoff, and A. Kessler. 2005. Matrix metalloproteinases 2 and 9 are markers of inflammation but not of the degree of fibrosis in chronic hepatitis C. *Digestion* 71: 124–130.

## Review

# Recent advance in immunotherapies for alzheimer's disease

With special reference to DNA vaccination

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**Key words:** immunotherapy, alzheimer's disease, DNA vaccine, non-viral vector, clinical application

Alzheimer's disease (AD) is the most common cause of dementia characterized by progressive neurodegeneration. Based on the amyloid cascade hypothesis, several immunotherapies for AD have been developed as curative treatment. In 1999, Schenk et al. reported for the first time that amyloid beta (A $\beta$ ) deposits in AD model mice could be reduced by active vaccination with A $\beta$  peptide. Although clinical trials with the A $\beta$  peptide were halted due to the development of meningoencephalitis in some treated patients, the vaccine therapy was judged to be effective on the basis of clinical and pathological analyses. Passive immunization using humanized anti-A $\beta$  monoclonal antibodies is also under clinical trials; however they have some problems to be solved. As other strategies, DNA vaccines have been developed as immunotherapies for AD, which is simple, easily modified and can be administered without adjuvant. DNA vaccines were developed by several groups including our laboratory, which induced A $\beta$  reduction in AD model mice without side effects. DNA vaccination may be open up new avenue of vaccine therapies for AD in the near future.

## Introduction

Alzheimer's disease (AD) is the most common cause of age-related cognitive decline, affecting more than 12 million people in worldwide. The disease is characterized by progressive memory impairment, cognitive decline, altered behavior and language deficit. Later, patients show global amnesia and slowing of motor function, and finally died.<sup>1</sup> Therefore, development of therapies against AD is extremely important from medical, social and economical points of view.

It is generally believed that accumulation of amyloid beta (A $\beta$ ) is the first event in the pathogenesis of AD. In other words, A $\beta$  deposition is an upstream event of tau phosphorylation, tangle formation and neuronal death (amyloid cascade hypothesis).<sup>2,3</sup> Therefore, already deposited or depositing A $\beta$  should be the first target of AD. Recently, several immunotherapies have been developed as curative

treatments of AD by targeting the underlying cause. In this article, we review conventional vaccine therapies and further introduce DNA vaccines which are now promising as an alternative therapy for human AD.

## Development of Anti-A $\beta$ Immunotherapy and Human Clinical Trials

In 1999, Schenk and his colleagues demonstrated that monthly inoculation with synthetic A $\beta$  in adjuvants (complete Freund adjuvant for the first time and incomplete Freund adjuvant thereafter) could lead to high anti-A $\beta$  antibody titers and dramatic reductions of A $\beta$  deposition in PDAPP transgenic mice.<sup>4</sup> The vaccine was able to slow or reverse amyloid deposit formation, even if administered after A $\beta$  deposition occurred. Subsequent studies demonstrated that clearance of A $\beta$  deposits following immunization protected APP-transgenic (Tg) mice from developing memory deficits.<sup>5,6</sup> Approximately 50% reduction in dense-cored A $\beta$  plaques is sufficient to ameliorate cognition.<sup>7</sup> Based on the promising results using model mice, clinical trials were started for AD patients. Phase I trial showed that active immunization to mild-to-moderate AD patients with Betabloc (AN-1792; Elan Pharmaceuticals, Dublin, Ireland), synthetic A $\beta$ 1-42 in adjuvant (QS-21) was safe and well-tolerated.<sup>8</sup> However, subsequent phase II-A study was halted in January 2002 due to the development of meningoencephalitis in 18 of 298 patients (6%) who received the vaccine.<sup>9</sup> Later, autopsy revealed T cell infiltration mainly comprising CD4<sup>+</sup> (CD3<sup>+</sup> and CD45RO<sup>-</sup>) T cells.<sup>10</sup> CD8<sup>+</sup> T cells were very few were and B lymphocytes were not present. Since examinations of viruses that may cause encephalitis were negative, the pathogenesis of this encephalitis was thought to be similar to autoimmune encephalomyelitis. Although the precise mechanisms remain to be obscure, the addition of new emulsifier, polysorbate 80, to the formulation used in phase II study might induce a different T-cell response.<sup>11</sup>

## Lessons from Clinical Studies with an A $\beta$ Peptide Vaccine

Although the clinical trial was halted due to the presence of the adverse effect, histological examinations of autopsied brains of treated patients revealed benefits of the vaccine. The first autopsy case of human AD after the trial was reported in 2003.<sup>10</sup> A 72 years-old woman with a 5-year history of Alzheimer's disease was immunized with AN-1792. After fifth injection, she was suffered from meningoencephalitis and died of pulmonary embolism 20 months after the first

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injection. Histological examination revealed that there were extensive areas of the neocortex with very few A $\beta$  plaques. Those areas of the cortex that were devoid of A $\beta$  plaques contained neurofibrillary tangles, neuropil threads and cerebral amyloid angiopathy (CAA) similar to untreated AD, but lacked plaque-associated dystrophic neurites and astrocyte clusters. These findings were very similar to those obtained in mouse models of AD after A $\beta$  immunotherapies and suggested that the immune response generated against the peptide accelerated A $\beta$  clearance.<sup>12</sup> Essentially the same findings were found in subsequent two autopsy cases.<sup>13,14</sup>

The effects of A $\beta$  immunotherapies on cognitive functions are controversial. Hock et al. followed thirty patients (24 patients received 2 immunizations and 6 received placebo) in Zurich University for 1 year after halt of the AN-1792 trial.<sup>15</sup> Twenty patients generated antibodies against A $\beta$ , as determined by tissue amyloid plaque immunoreactivity assay. AD patients who generated antibodies performed markedly better on the Mini Mental State Examination (MMSE) scores after the immunization compared to control patients. As compared to baseline, patients who generated antibodies against A $\beta$  remained unchanged after 1 year. In contrast, patients in the control group worsened significantly.<sup>15</sup> Later, Gilman et al. reported the results of large-scale phase II-A studies that did not include Hock's data.<sup>16</sup> Of the 300 AN1792 (QS-21)-treated patients, 59 (19.7%) developed the antibody response. Complete analysis of all the treated patients demonstrated that no significant differences were found in 11 of 14 cognitive disability global change score between antibody responder and placebo groups. However, antibody responders had an improvement in the Wechsler Memory Verbal-Delayed (WMVer-D) scale. Furthermore, nine-component composite NTB z-score and all memory composite z-score ( $p = 0.033$ ) indicate less worsening at 12 month from the first analysis. CSF examinations performed in a small number of subjects revealed that CSF tau was decreased in antibody responders ( $n = 11$ ) compared with placebo-given patients ( $n = 10$ ;  $p < 0.001$ ).<sup>16</sup> Recently, long-term (6years) effects of 80 patients in phase I clinical trial, were reported.<sup>17</sup> This report contains both expected and unexpected results. Expectedly, cortical A $\beta$  loads were lowered in treated patients than in the control group. Furthermore, in patients with higher antibody titers showed more extensive A $\beta$  removal. Unexpectedly however, there was no statistically significant evidence for improvement in cognitive function or survival, even in patients with high antibody titers. These results suggest that plaque removal is not enough to halt progressive neurodegeneration in AD.

There are a number of possible mechanisms to explain the differences in the outcome. First, A $\beta$  plaque formation might be necessary to initiate pathological processes, but not to progressive neurodegeneration. This hypothesis explains well why there is a poor correlation between A $\beta$  plaque load and the degree of dementia.<sup>18</sup> Second, plaque removal occurs progressively over a period of months.<sup>19</sup> Therefore, it is easily assumed that the improvement of cognitive functions after A $\beta$  removal by the treatment takes a considerable time. Follow-up period may be too short to elucidate the relationship between A $\beta$  clearance and the cognitive function. Third, it is well known that A $\beta$  oligomers are critical factor to induce synaptic dysfunction in AD patients.<sup>20,21</sup> The degree of oligomeric A $\beta$  removal by the treatment should be estimated, which was not performed in these studies. Fourth, the human clinical studies were performed on aged patients with mild to moderate AD.<sup>8</sup> Obviously, anti-A $\beta$  immuno-

therapies should be initiated on younger patients with mild signs and symptoms before irreversible AD-related brain damages occur.<sup>22</sup> Taken together, further studies are necessary to evaluate the effects of anti-A $\beta$  immunotherapies for AD patients.

## Development of New A $\beta$ Peptide Vaccines

To control harmful T cell responses, new A $\beta$  vaccines are developing after the clinical trials with A $\beta$  peptide vaccines. Nasal vaccination with proteosome-based adjuvant plus glatiramer acetate cleared A $\beta$  plaques in Alzheimer model mice.<sup>23</sup> Fine epitope analysis with overlapping oligomers of the A $\beta$ 1-42, sequence identified the 1-15 region as a dominant B cell epitope.<sup>24</sup> A short A $\beta$  peptide (A $\beta$ 1-15), which contains antibody epitopes but lacks T-cell reactive sites, induced anti-A $\beta$  antibodies in the absence of T cell responses in wild-type mice<sup>25</sup> and significantly reduced A $\beta$  plaques in APP transgenic mice.<sup>26</sup> A $\beta$  epitope vaccine consists of self-B cell epitope of A $\beta$  and non-self T helper cell epitope produced high titers of anti-A $\beta$  antibodies in mice. In addition, the new approach to develop vaccine using virus-like particles (VLPs) was reported.<sup>27</sup> Due to their virus-like nature, VLPs are very immunogenic and their potential to be effective vaccines have long been recognized.<sup>28</sup> A $\beta$  conjugated to VLPs elicited anti-A $\beta$  antibody responses at low doses and without the use of adjuvants. The flexibility of these virus-based display systems allowed to induce antibodies against short A $\beta$ -derived peptides from the amino- and carboxyl-termini of the peptide.<sup>27</sup>

Recently, other clinical trials using second-generation peptide vaccines were started.<sup>29</sup> Wyeth and Elan Inc., developed ACC-001, which contains A $\beta$ 1-7 derivatives. The trial was suspended in April 2008 because a patient in a phase II trial was hospitalized with skin vasculitis. Vasculitis was considered as immune, or allergic, reactions. However, the patient was treated successfully and the trial was restarted 6 weeks later. Novartis Inc., developed CAD106 which consists of carrier Qb coupled with A $\beta$ 1-6. Phase I trial was finished without problems and Phase II trial was started in 2007 (all information was obtained from press-release of the indicated companies).

## Passive Immunization with Anti-A $\beta$ Antibodies

Passive transfer of anti-A $\beta$  antibodies is an alternative strategy, which is effective as well as active immunization in the mouse model of AD. Peripheral administration of antibodies against A $\beta$  peptide was sufficient to reduce amyloid burden. Despite relatively modest serum levels, passively administered antibodies were able to enter the central nervous system, decorated plaques and induced clearance of A $\beta$  deposits.<sup>30</sup> Passive immunization is more acceptable than active vaccination because it does not need an adjuvant and does not elicit hazardous cellular responses found in the clinical trial of active immunization. In addition, the dose can be controlled easily. However, administration of anti-A $\beta$  antibodies may cause micro-hemorrhages in old APP mice<sup>19</sup> and its incidence was significantly increased following 5 months of passive immunization.<sup>31</sup>

Phase II clinical trial of A $\beta$ -specific N-terminal directed humanized monoclonal antibodies, bapineuzumab (AAB-001, Elan and Wyeth), was completed in 2008.<sup>29,32</sup> On July 29, 2008, Elan and Wyeth Corporation reported in the press release that the treatment showed beneficial effects on the Apolipoprotein E4 (ApoE4) no-carrier population. However, vasogenic cerebral edema occurred dose-dependently in 12 out of 124 antibody-treated patients and

10 patients carried the ApoE4 allele. Moreover, eight of 12 patients belonged to the high-dosage group. As was mentioned above, it is also well known that a number of microhemorrhages occurred after administration of anti-A $\beta$  in old APP mice.<sup>31,33</sup> These findings suggest that the abrupt reduction of A $\beta$  deposits in the vessel wall may result in the functional change of the blood brain barrier, leading to these adverse effects. Another humanized monoclonal antibody, LY2062430 (Eli Lilly Co.), is now in the phase II trial.<sup>32</sup> The favorable outcome is expected for LY2062430 because this antibody directs to the central domain of the A $\beta$ 1-42 peptide and does not induce intracerebral hemorrhage or vascular pathology in the experiment.<sup>33</sup> However, there are still some problems to be solved in passive immunization. In vivo production of anti-idiotypic antibodies, which may neutralize the effects of anti-A $\beta$  antibodies, must be controlled to maintain the effect of the antibodies. Moreover, a serious disadvantage of passive immunization is the cost of the antibodies. The expense of weekly or monthly monoclonal infusion for decades could be extremely difficult from the economic point of view. Many patients cannot afford expensive medical costs which is estimated over several tens thousands dollars totally.

### Development of DNA Vaccines as Effective Drugs in the Next Generation

To compensate the disadvantage of conventional immunotherapies, DNA vaccination has been developed as a new therapy for AD, which is simple, easily modified and can be administered without adjuvants.<sup>34,35</sup> At the injection site, vaccines are taken up by muscle cells and the A $\beta$  peptide-protein complex is produced for a certain period.<sup>36</sup> Translated A $\beta$  complex stimulates immune responses in the host, and induced anti-A $\beta$  antibodies.<sup>37</sup> Importantly, immune responses of the host can be easily manipulated to obtain a Th2 type reaction.<sup>34,38,39</sup>

**DNA vaccines with viral vectors.** Viral DNA vaccines were developed using adeno-associated virus (AAV) vectors<sup>40,41</sup> or adenovirus vector.<sup>42</sup> The effects of viral DNA vaccines are expected to continue for several months after one administration because the viral vector has the high introduction efficiency. An AAV vaccine developed by Zhang et al. could express CB-A $\beta$ 1-42 (Cholea toxin B subunit and A $\beta$ 1-42 fusion protein) in vivo. A single administration of the AAV vaccine induced a prolonged (at least 12 month) production of A $\beta$ -specific serum IgG (1:4,096–1:16,384) in model mice and resulted in improved ability of memory and cognition, decreased A $\beta$  depositions and plaque-associated astrogliosis in the brain.<sup>43</sup> Hara et al. constructed AAV vaccines that express for A $\beta$ 1-43 or A $\beta$ 1-21, and administered to Tg2536 mice transorally. IgG antibodies were detected in the serum at 4 weeks, and existed in the body for more than 6 months. Quantitative analysis of the brain showed a significant decrease of A $\beta$  burden in all vaccinated mice.<sup>41</sup> Intranasal injection of an adenovirus vector encoding A $\beta$ 1-42 and granulocyte/macrophage-colony stimulating factor (GM-CSF) decreased the A $\beta$  load in Tg2536 mice.<sup>42</sup>

Although viral DNA vaccines are effective, it remains to be determined whether viral vectors are really safe. In 1999, an 18-year-old patient with an inherited enzyme deficiency died 4 days after gene therapy with a genetically altered adenovirus vector in Pennsylvania, USA.<sup>44</sup> In France, clinical trials of gene therapy with retrovirus vector to treat X-linked severe combined immunodeficiency disease (SCID)

were halted due to the development of leukemia.<sup>45</sup> Furthermore, significantly increased incidence of hepatocellular carcinomas and angiosarcomas was noted in rAAV-mediated gene therapy.<sup>46,47</sup> Thus, these problems should be overcome as soon as possible. In addition, limitations to scale up the AAV vector production severely restrict the commercialization and use of AAV-associated DNA vaccines.<sup>48</sup> Taken together, the clinical application of DNA vaccines using viral vectors seems to be difficult at present.

**Non-viral DNA vaccines.** Others and we have focused on the use of plasmid vectors for A $\beta$  DNA vaccines. Non-viral DNA vaccines have many advantages over those with virus vectors. Non-viral plasmid DNA vaccines can be easily mass-produced at a low cost<sup>34,35</sup> have no possibility of viral infection or transformation.<sup>49,50</sup> Non-viral DNA vaccine with A $\beta$  fused to mouse interleukin-4 (pA $\beta$  42-IL-4) as a molecular adjuvant generated anti-A $\beta$  antibodies in wild type B6 mice.<sup>51</sup> A $\beta$  DNA vaccine with a secretory signal, tissue-type plasminogen activator (tPA) reduces amyloid plaque in mice mode with simultaneous use of low dose A $\beta$  peptide. However, significant reductions of A $\beta$  deposition were not obtained only with these DNA vaccines.<sup>52</sup> In addition, other groups utilized gene-gun for the delivery of DNA vaccines, which has the advantage over peptide vaccination of higher efficacy in breaking self-tolerance and for inducing beneficial Th2-based immune responses<sup>53,54</sup> to reduce the possible adverse effects related to Th1 adverse responses seen with A $\beta$ 1-42 peptide vaccine. A $\beta$ 1-42 and A $\beta$ 1-16 gene were chemically fused and delivered to APP<sup>sw</sup>/PS1<sup>DE9</sup> transgenic mice with gene-gun.<sup>55</sup>

In our laboratory, three types of A $\beta$  DNA vaccines were prepared using a mammalian expression vector and reduce A $\beta$  plaque in AD model mice without side effects.<sup>56</sup> The first vaccine possessed the core 1–42 sequence inserted into plasmid vector (K-A $\beta$  vaccine). The second one possessed the immunoglobulin leader sequence at the N terminus (IgL-A $\beta$  vaccine) to increase the secretion of the A $\beta$  peptide. Furthermore, human immunoglobulin Fc portion was added to the third vaccine to stabilize the vaccine product (Fc-A $\beta$  vaccine). These leader sequences inserted into the plasmid were important for the A $\beta$  secretory property of the vaccines. Supernatants of cultured cells that had been transfected with IGL-A $\beta$  and A $\beta$ -Fc vaccines contained translated proteins, whereas K-A $\beta$ -transfected cells did not secrete the peptide into the extracellular space. K-A $\beta$  vaccine was less effective in A $\beta$  reduction than the former two and was not used in subsequent experiments. AD model mice received 6 weekly and subsequent biweekly injections of the vaccines. Prophylactic treatment, administration before the appearance of amyloid deposition, with A $\beta$ -Fc vaccine demonstrated that the final reduction rate of A $\beta$  burden in the cerebral cortex at 18 months of age was approximately 39% of untreated groups (Fig. 1). Therapeutic treatment, administration after the appearance of A $\beta$  deposition revealed almost equal A $\beta$  plaque reduction as prophylactic treatment (Figs. 1 and 2). Concerning the excessive immune reaction of our vaccine, T cell activation and proliferation, as measured by [<sup>3</sup>H]-thymidine incorporation of T cells from vaccinated mice, was negative in both wild type B6 and model mice (Fig. 3). Furthermore, pathological examinations using monoclonal antibodies, CD5 (anti-T cell) and Mac-3 (anti-macrophage), demonstrated no inflammatory lesion in the brain after long-term treatments. Subsequent analysis revealed that A $\beta$ -monomers and dimers were significantly reduced after DNA vaccination.<sup>57</sup> Recently, to make DNA vaccine more safe and

effective, DNA epitope vaccine was developed, which consists of 3 copies of the self-B cell epitope of A $\beta$ 1-11, a non-self T helper cell epitope (PADRA), and macrophage-derived cytokine (MDC/CCL22).<sup>58</sup> It generated high titer of anti-A $\beta$  antibody (200–1,000  $\mu$ g/ml) after 3–5 times vaccination and reduced A $\beta$  plaques in triple transgenic mice. The A $\beta$  oligomers were significantly reduced and behavioral deficit of model mice were improved in the vaccine mice.

As mentioned above, there are two types of DNA vaccines, viral and non-viral DNA vaccines. We believe that non-viral DNA vaccines are superior to viral DNA vaccines for several reasons. Non-viral DNA vaccines can be prepared at a large amount with standard technology. One can make the vaccines at a low cost and the safety has been established. The primary concern for non-viral DNA vaccines is their potential to integrate into the host cell genome. With an integration assay based on purification of high-molecular-weight genomic DNA away from free plasmid using gel electrophoresis, such that the genomic DNA can be assayed for integrated plasmid using a sensitive PCR method. The assay sensitivity was approximately 1 plasmid copy/ $\mu$ g DNA (representing approximately 150,000 diploid cells).<sup>59</sup> When integration occurred, the frequency was 1–8 integrations per 150,000 diploid cells, which would be below the spontaneous mutation rate.<sup>59</sup> Thus, the risk of mutation due to integration of plasmid DNA vaccines following intramuscular injection is negligible. In addition, if unwanted side effects occur, they can be easily controlled by stopping further administration of the vaccine because their half-life within the body is shorter than viral vaccines.<sup>60</sup> When considering clinical use, AD patients receive vaccines for life long and such advantages are quite important factors for the choice of the treatment. Non-viral plasmid vector is the best choice at present for the DNA vaccine treatment against Alzheimer's disease.

### Mechanisms of A $\beta$ Reduction with Vaccine Therapies

Immunotherapies against AD are effective not only in the mouse model<sup>4,7</sup> but also in human clinical trials.<sup>10</sup> However, the mechanisms of A $\beta$  reduction in the brain remain to be elucidated. There are at least three hypotheses. One possible mechanism is operated through microglial phagocytosis.<sup>61,62</sup> When examined in an *in vivo* assay with sections of PDAPP mice or AD brain tissue, antibodies against A $\beta$ -peptide triggered microglial cells to clear plaques through Fc receptor-mediated phagocytosis and subsequent peptide degradation.<sup>30</sup> However, recent reports suggest that other receptors also participate to clear A $\beta$  plaques. Immunization of Tg2576 mice crossed with Fc receptor gamma knockout mice were as efficient at clearing plaques as Tg2576 mice alone.<sup>63</sup> Murine microglia bind and internalize fibrillar A $\beta$  microaggregates via the type A scavenger receptor.<sup>64</sup> The second mechanism is direct binding of antibodies leads to dissolution of A $\beta$  peptides.<sup>65,66</sup> Anti-A $\beta$  antibodies arose from mice selectively directed against residues 4–10 of A $\beta$ 42, and that these antibodies inhibit both A $\beta$  fibrillogenesis and cytotoxicity without eliciting an inflammatory response.<sup>67</sup> Using *in vivo* multiphoton microscopy, FITC-labeled F(ab')<sub>2</sub> fragments of 3d6 (which lack the Fc region of the antibody) also led to clearance of 45% of the deposits within 3 days, similar to the results obtained with full-length 3d6 antibody.<sup>65</sup> The third mechanism is that blood-circulating antibodies increase the net efflux of A $\beta$  peptides from the brain to the circulation (the peripheral sink hypothesis).<sup>68,69</sup> The monoclonal antibody m266, having a very high affinity for soluble

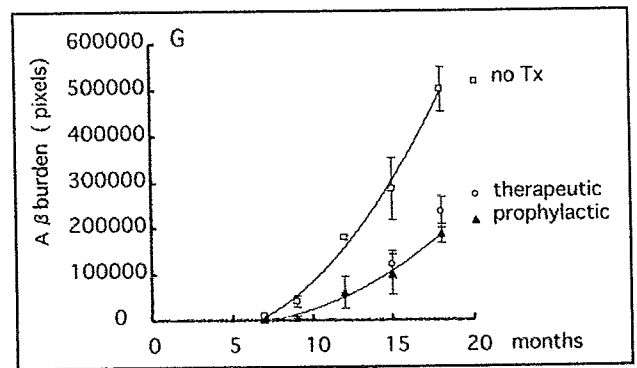


Figure 1. Reduction of A $\beta$  burden in APP23 mice after DNA vaccination. The overall quantitative analysis is depicted. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-A $\beta$  vaccine prevented the A $\beta$  deposition to 10–30% of that in untreated animals before 12 months of age and to 40–50% after 15 months (closed triangles). The effects of therapeutic administration were almost same as those of prophylactic administration (open circles). Each group consists of 4–6 mice.

A $\beta$ , may not require entering the brain, which can extract free brain A $\beta$  into blood circulation when given peripherally.<sup>68</sup> Peripheral administration of m266 to PDAPP transgenic mice results in a rapid 1,000-fold increase in plasma A $\beta$ ,<sup>68</sup> which can rapidly reverse memory deficits in both an object recognition task and a holeboard learning and memory task.<sup>69</sup>

We examined these possible A $\beta$  reduction mechanisms to determine which the major route of A $\beta$  clearance is in our DNA vaccination system.<sup>57</sup> Immunohistochemical examinations revealed that activated microglia significantly increased after DNA vaccination not only around plaques but also in areas remote from plaques. Furthermore, microglia in treated mice phagocytosed A $\beta$  debris more frequently than in untreated mice. Although microglia had an activated form, they did not produce a significant amount of TNF $\alpha$ . Amyloid plaque immunoreactivity and A $\beta$  concentration in plasma increased slightly in the treated group at 9, but not at 15, months of age. Collectively, these data indicate that phagocytosis of A $\beta$  deposits by microglia play a central role in A $\beta$  reduction after DNA vaccination. There are at least two explanations for these results. First, anti-A $\beta$  antibodies in plasma were mildly elevated after DNA vaccination,<sup>56</sup> compared with active immunization,<sup>4</sup> which is sufficient for A $\beta$  clearance by microglia but not for transition of A $\beta$  from the brain to the blood stream. Second, cerebral amyloid angiopathy may progress, especially in the late stage, and interfere with the perivascular drainage pathway of A $\beta$ .<sup>70</sup> The findings obtained in this study provide useful information for the development of new and more effective DNA vaccines against AD because DNA vaccines are easily reconstructed by adding or changing the sequence in the plasmid vector.

Recently, it is also known that A $\beta$  clearance is augmented by drugs via the antibody-independent and cell-mediated pathways. Nasal vaccination with a proteasome-based adjuvant that is well tolerated in humans plus glatiramer acetate, an FDA-approved synthetic copolymer used for treatment of multiple sclerosis, decreases A $\beta$  plaques in model mice.<sup>23</sup> This effect did not require the presence of anti-A $\beta$  antibodies in the brain because A $\beta$  reduction by the treatment was also observed in B cell-deficient (Ig mu-null) mice. Vaccinated



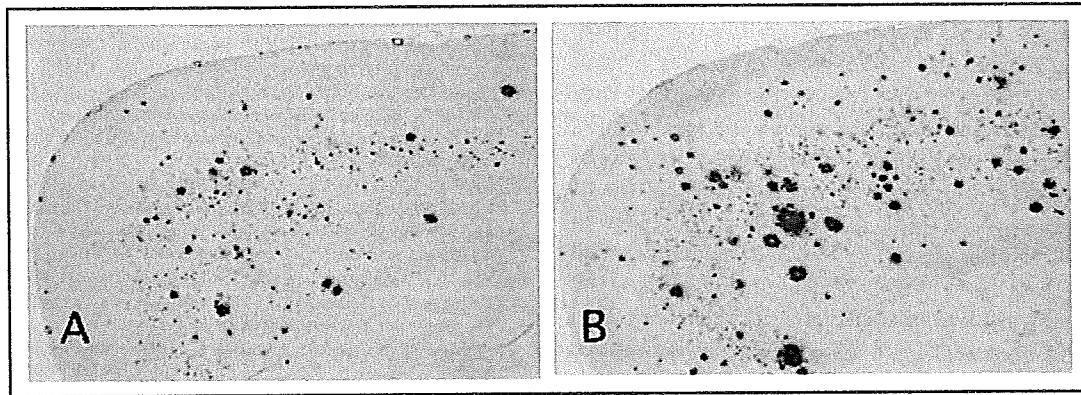


Figure 2. A $\beta$  burden reduction at 18 months of age after therapeutic treatment starting from 12 months. While large A $\beta$  deposits (>100  $\mu$ m) were observed in the frontal cortex of control mice at 18 months of age (B), significant reduction was observed after 6 month therapeutic administration of the IgL-A $\beta$  vaccine (A). Magnification (A and B),  $\times 27$ .

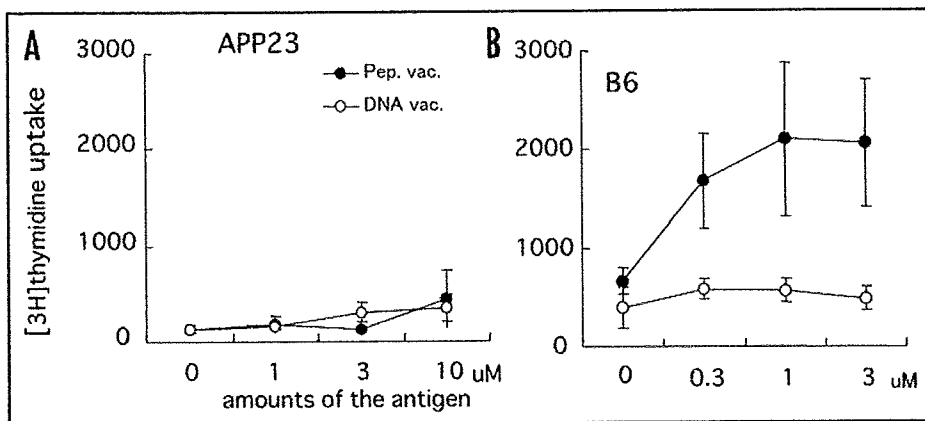


Figure 3. T cell responses in APP23 (A) and B6 (B) mice after immunization with A $\beta$  peptide/CFA or DNA vaccination. Lymphocytes isolated from two strains were incubated with A $\beta$  peptide (0–10  $\mu$ M) for 3 days. Incorporation of [ $^3$ H] thymidine was measured by liquid scintillation spectrometry. In APP23 mice, neither T cells from peptide-immunized mice nor those from DNA vaccinated mice were activated in the presence of A $\beta$ 1–42 ( $n = 3$ ) (A). In contrast, A $\beta$  peptide immunization, but not DNA vaccination, induced a significant T cell response in B6 mice ( $n = 3$ ) (B). All the data are the mean values  $\pm$  S.D. and the representative results from three different experiments are shown. Figures 1–3 are reproduced with permission from PNAS (ref. 56).

animals developed activated microglia that colocalized with A $\beta$  fibrils, and the extent of microglial activation correlated strongly with the decrease in A $\beta$  fibrils.<sup>23</sup>

### Role of Microglia in A $\beta$ Accumulation or Reduction

Microglia are resident brain cells and react with various pathological conditions including autoimmune inflammation, infection and degeneration in the central nervous system. Previously, microglia were thought to be harmful and toxic to neurons in the AD brain as there were sustained inflammatory responses including complement activation;<sup>71</sup> however it becomes consensus that microglia could play a neuroprotective role under a certain circumstance as well as pathogenic role in the disease processes.<sup>72–75</sup> It has been shown that microglia react with A $\beta$  plaques and phagocytose A $\beta$  deposits under various stimulations.<sup>76–79</sup> When activated, microglia express receptors involved in the clearance and phagocytosis of A $\beta$  (e.g., class A scavenger receptor, CD36 and receptor for advanced-glycosylation endproducts).<sup>80,81</sup> Soluble A $\beta$  can be directly bound by microglial

receptors such as heparin sulphate proteoglycans (HSPGs),<sup>82</sup> insulin receptors<sup>83</sup> and proteinase inhibitor serpin-enzyme complex receptor (SEC-R),<sup>84</sup> resulting in phagocytosis of soluble A $\beta$ . Activated microglia can degrade A $\beta$  by releasing A $\beta$  degrading enzymes including metalloproteases, insulin-degrading enzyme<sup>55,85</sup> and gelatinase A.<sup>86</sup> In addition, activation of microglia with toll-like receptors (TLRs), a family of pattern-recognition receptors in the innate immune system, markedly boosted ingestion of A $\beta$  in vitro.<sup>87</sup>

Conversely, dysfunction of microglia is related to the progression of AD. A large number of apoptotic microglia are present in AD brain.<sup>88–90</sup> Microglia from old PS1-APP mice showed decrease expression of the A $\beta$ -binding scavenger receptors A (SRA), CD36 and RAGE (receptor for advanced-glycosylation endproducts), and the A $\beta$ -degrading enzymes insulysin, neprilysin, and MMP9, compared with their littermate controls.<sup>91</sup> PS1-APP microglia had a 2.5-fold increase in the pro-inflammatory cytokines IL-1 $\beta$  (interleukin-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), suggesting that there is an inverse correlation between cytokine production and A $\beta$  clearance.<sup>91</sup> In addition, microglial accumulation plays a protective role in the early stages of AD by promoting A $\beta$  clearance. The deficiency of CC-chemokine receptor 2 (Ccr2),<sup>92</sup> a chemokine receptor expressed on microglia, which mediates the accumulation of mononuclear phagocytes at the site of inflammation, accelerates early disease progression and markedly impairs microglial accumulation in Tg2576 mice.<sup>93</sup> These findings suggest that A $\beta$  clearance by microglia is essential for the maintenance of normal neural function in the brain.

### Effect of A $\beta$ Vaccines in Non-Human Primates

Treatments of non-human primates with DNA vaccines provide useful information as an essential step before clinical trials. As non-human primates have the same A $\beta$  sequence as humans and develop A $\beta$  plaques with aging, certain kinds of monkeys provide

an attractive Alzheimer's disease animal model.<sup>94</sup> Investigators have been employed vervet monkeys for studies on brain injuries<sup>95,96</sup> and AD.<sup>97,98</sup> Lemere et al. immunized five aged vervets (16–25 yr) with A $\beta$  peptide over 10 months and examined plasma and CSF samples longitudinally. By day 42, immunized monkeys generated plasma A $\beta$  antibodies that labeled plaques in the human AD and model mouse brain. The antibody levels peaked at day 100 (1,677  $\mu$ g/ml in plasma, 2.1  $\mu$ g/ml in CSF) in the immunized animals and dropped to relatively steady levels by Day 301 (452 mg/ml in plasma, 0.2 mg/ml in CSF). Soluble A $\beta$  levels were elevated approximately 2- to 5-fold in plasma and decreased up to 64% in CSF in immunized vervets. Insoluble A $\beta$  was decreased by 66% in brain homogenates of the immunized animals compared to controls.<sup>99</sup> Since it is difficult to obtain vervet monkeys, rhesus monkeys (*Macaca mulatta*) were also examined extensively.<sup>100</sup> Senile plaques appear in variable numbers in animals >23 years but were not seen in monkeys <15 years of age.<sup>101</sup> A $\beta$  deposits were observed in the cerebral cortex of all aged animals.<sup>102</sup> Gandy et al. injected aggregated A $\beta$ 1-42 to rhesus monkeys (*Macaca mulatta*). Immunized monkeys developed anti-A $\beta$  titers exceeding 1:1,000, and their plasma A $\beta$  levels were 5–10-fold higher than the plasma A $\beta$  levels observed in monkeys vaccinated with aggregated amylin.<sup>103</sup>

Recently, we evaluated the effects of DNA vaccines on A $\beta$  reduction found in aged rhesus monkeys (15–18 years old) (manuscript in preparation). DNA vaccine and empty vector (control) at a dose of 3 mg were injected 15 times for 6 months ( $n = 3$  in each group). Quantitative analysis revealed a significant reduction of A $\beta$  in treated monkeys without obvious side effects. Previous two studies<sup>99,103</sup> and our examination clearly demonstrated A $\beta$  reduction in monkeys after vaccination. Analysis of oligomeric A $\beta$  and cognitive assessment will be necessary to determine whether such immunotherapies provide beneficial effects on behavioral disorders. Collectively, these findings suggest that DNA vaccines are also effective in A $\beta$  reduction in non-human primates.

## Conclusion

Current drug therapies for AD aim at slowing the cognitive decline and ameliorating the affective and behavioral signs associated with disease progression. Based on the results obtained in animal and human clinical studies, A $\beta$  vaccine therapy is a principal strategy for AD treatment. Active peptide immunotherapy and passive immunotherapy have some problems to be solved before clinical application. Newly developed DNA vaccines were effective in reducing A $\beta$  plaque on model mice and aged monkeys. Oligomeric A $\beta$ , which is most neurotoxic among A $\beta$  species, was also decreased in the mouse brain after vaccination. Although preclinical trials are under way, DNA vaccination will be a promising therapy for Alzheimer's disease in the near future.

## References

- Citron M. Alzheimer's disease: treatments in discovery and development. *Nat Neurosci* 2002; 5:1055-7.
- Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* 1991; 12:383-8.
- Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science* 1992; 256:184-5.
- Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandever C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 1999; 400:173-7.
- Janus C, D'Amelio S, Amitay O, Chishti MA, Strome R, Fraser P, Carlson GA, Roder JC, St. George-Hyslop P, Westaway D. Spatial learning in transgenic mice expressing human presenilin 1 (PS1) transgenes. *Neurobiol Aging* 2000; 21:541-9.
- Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW. A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 2000; 408:982-5.
- Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, St. George-Hyslop P, Westaway D. A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. *Nature* 2000; 408:979-82.
- Bayer AJ, Bullock R, Jones RW, Wilkinson D, Paterson KR, Jenkins L, Millais SB, Donoghue S. Evaluation of the safety and immunogenicity of synthetic Abeta42 (AN1792) in patients with AD. *Neurology* 2005; 64:94-101.
- Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. *Neurology* 2003; 61:46-54.
- Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nat Med* 2003; 9:448-52.
- Pride M, Seubert P, Grundman M, Hagen M, Eldridge J, Black RS. Progress in the active immunotherapeutic approach to Alzheimer's disease: clinical investigations into AN1792-associated meningoencephalitis. *Neurodegener Dis* 2008; 5:194-6.
- Bacskaï BJ, Kajdasz ST, Christie RH, Carter C, Games D, Seubert P, Schenk D, BT H. Imaging of amyloid-beta deposits in brains of living mice permits direct observation of clearance of plaques with immunotherapy. *Nat Med* 2001; 7:369-72.
- Ferrer I, Boada Rovira M, Sanchez Guerra ML, Rey MJ, Costa-Jussa F. Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol* 2004; 14:11-20.
- Masliah E, Hansen L, Adame A, Crews L, Bard F, Lee C, Seubert P, Games D, Kirby L, Schenk D. Abeta vaccination effects on plaque pathology in the absence of encephalitis in Alzheimer disease. *Neurology* 2005; 64:129-31.
- Hock C, Konietzko U, Streffer JR, Tracy J, Signorell A, Muller-Tillmanns B, Lemke U, Henke K, Moritz E, Garcia E, Wollmer MA, Umrbricht D, de Quervain DJ, Hofmann M, Maddalena A, Papassotiropoulos A, Nitsch RM. Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 2003; 38:547-54.
- Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F, Orgogozo JM. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. *Neurology* 2005; 64:1553-62.
- Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW, Zotova E, Nicoll JA. Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. *Lancet* 2008; 372:216-23.
- Davis DG, Schmitt FA, Wekstein DR, Markesbery WR. Alzheimer neuropathologic alterations in aged cognitively normal subjects. *J Neuropathol Exp Neurol* 1999; 58:376-88.
- Wilcock DM, Rojiani A, Rosenthal A, Subbarao S, Freeman MJ, Gordon MN, Morgan D. Passive immunotherapy against Abeta in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage. *J Neuroinflammation* 2004; 1:24.
- Patton RL, Kalback WM, Esh CL, Kokjohn TA, Van Vickle GD, Luehrs DC, Kuo YM, Lopez J, Brune D, Ferrer I, Masliah E, Newell AJ, Beach TG, Castano EM, Roher AE. Amyloid-beta peptide remnants in AN-1792-immunized Alzheimer's disease patients: a biochemical analysis. *Am J Pathol* 2006; 169:1048-63.
- Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol* 2007; 8:101-12.
- Boche D, Nicoll JA. The role of the immune system in clearance of Abeta from the brain. *Brain Pathol* 2008; 18:267-78.
- Frenkel D, Maron R, Burt DS, Weiner HL. Nasal vaccination with a proteasome-based adjuvant and glatiramer acetate clears beta-amyloid in a mouse model of Alzheimer disease. *J Clin Invest* 2005; 115:2423-33.
- Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, Babikyan D, Kessler B, Kieber-Emmons T, Cotman CW, Agadjanyan MG. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. *Int Immunol* 2003; 15:505-14.
- Maier M, Seabrook TJ, Lemere CA. Developing novel immunogens for an effective, safe Alzheimer's disease vaccine. *Neurodegener Dis* 2005; 2:267-72.
- Maier M, Seabrook TJ, Lazo ND, Jiang L, Das P, Janus C, Lemere CA. Short amyloid-beta (Abeta) immunogens reduce cerebral Abeta load and learning deficits in an Alzheimer's disease mouse model in the absence of an Abeta-specific cellular immune response. *J Neurosci* 2006; 26:4717-28.
- Chackerian B, Rangel M, Hunter Z, Peabody DS. Virus and virus-like particle-based immunogens for Alzheimer's disease induce antibody responses against amyloid-beta without concomitant T cell responses. *Vaccine* 2006; 24:6321-31.
- Jennings GT, Bachmann MF. The coming of age of virus-like particle vaccines. *Biol Chem* 2008; 389:521-36.
- Masters CL, Beyreuther K. Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. *Brain* 2006; 129:2823-39.

30. Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med* 2000; 6:916-9.
31. Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M. Cerebral hemorrhage after passive anti-Abeta immunotherapy. *Science* 2002; 298:1379.
32. Brody DL, Holtzman DM. Active and passive immunotherapy for neurodegenerative disorders. *Annu Rev Neurosci* 2008; 31:175-93.
33. Racke MM, Boone LI, Hepburn DL, Parsadanian M, Bryan MT, Ness DK, Piroozzi KS, Jordan WH, Brown DD, Hoffman WP, Holtzman DM, Bales KR, Gitter BD, May PC, Paul SM, DeMattos RB. Exacerbation of cerebral amyloid angiopathy-associated microhemorrhage in amyloid precursor protein transgenic mice by immunotherapy is dependent on antibody recognition of deposited forms of amyloid beta. *J Neurosci* 2005; 25:629-36.
34. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature* 1992; 356:152-4.
35. Barry MA, Lai WC, Johnston SA. Protection against mycoplasma infection using expression-library immunization. *Nature* 1995; 377:632-5.
36. Wolff JA, Malone RW, Williams R, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. *Science* 1990; 247:1465-8.
37. Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato AI, Boyer J, Williams WV, Weiner DB. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1993; 90:4156-60.
38. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dworki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993; 259:1745-9.
39. Hoffman SL, Doolan DL, Sedegah M, Gramzinski R, Wang H, Gowda K, Hobart P, Margalith M, Norman J, Hedstrom RC. Nucleic acid malaria vaccines. Current status and potential. *Ann N Y Acad Sci* 1995; 772:88-94.
40. Zhang Z, Yu CX. Effect of melatonin on learning and memory impairment induced by aluminum chloride and its mechanism. *Yao Xue Xue Bao* 2002; 37:682-6.
41. Hara H, Monsonego A, Yuasa K, Adachi K, Xiao X, Takeda S, Takahashi K, Weiner HL, Tabira T. Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. *J Alzheimers Dis* 2004; 6:483-8.
42. Kim HD, Kong FK, Cao Y, Lewis TL, Kim H, Tang DC, Fukuchi K. Immunization of Alzheimer model mice with adenovirus vectors encoding amyloid beta-protein and GM-CSF reduces amyloid load in the brain. *Neurosci Lett* 2004; 370:218-23.
43. Zhang J, Wu X, Qin C, Qi J, Ma S, Zhang H, Kong Q, Chen D, Ba D, He W. A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and beta-amyloid plaques in a mouse model of Alzheimer's disease. *Neurobiol Dis* 2003; 14:365-79.
44. Marshall E. Gene therapy. Second child in French trial is found to have leukemia. *Science* 2003; 299:320.
45. Check E. Gene therapy put on hold as third child develops cancer. *Nature* 2005; 433:561.
46. Donsante A, Vogler C, Muzyczka N, Crawford JM, Barker J, Flotte T, Campbell-Thompson M, Daly T, Sands MS. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther* 2001; 8:1343-6.
47. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007; 317:477.
48. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* 2002; 13:1935-43.
49. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther* 2001; 12:861-70.
50. Nishikawa M, Hashida M. Nonviral approaches satisfying various requirements for effective in vivo gene therapy. *Biol Pharm Bull* 2002; 25:275-83.
51. Ghochikyan A, Vasilevko V, Petrushina I, Movsesyan N, Babikyan D, Tian W, Sadzikava N, Ross TM, Head E, Cribbs DH, Agadjanyan MG. Generation and characterization of the human immune response to DNA immunization with a chimeric beta-amyloid-interleukin-4 minigene. *Eur J Immunol* 2003; 33:3232-41.
52. Schilz JG, Salzer U, Mohajeri MH, Franke D, Heinrich J, Pavlovic J, Wollmer MA, Nitsch RM, Moelling K. Antibodies from a DNA peptide vaccination decrease the brain amyloid burden in a mouse model of Alzheimer's disease. *J Mol Med* 2004; 82:706-14.
53. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997; 158:2278-84.
54. Weiss R, Scheibelhofer S, Freund J, Ferreira F, Livey I, Thalhammer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rides the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine* 2002; 20:3148-54.
55. Qu B, Boyer PJ, Johnston SA, Hynan LS, Rosenberg RN. Abeta42 gene vaccination reduces brain amyloid plaque burden in transgenic mice. *J Neurol Sci* 2006; 244:151-8.
56. Okura Y, Miyakoshi A, Kohyama K, Park IK, Staufenbiel M, Matsumoto Y. Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: long-term effects and safety. *Proc Natl Acad Sci USA* 2006; 103:9619-24.
57. Okura Y, Kohyama K, Park IK, Matsumoto Y. Nonviral DNA vaccination augments microglial phagocytosis of beta-amyloid deposits as a major clearance pathway in an Alzheimer disease mouse model. *J Neuropathol Exp Neurol* 2008; 67:1063-71.
58. Movsesyan N, Ghochikyan A, Mkrtrichyan M, Petrushina I, Davryan H, Olkhanud PB, Head E, Biragyn A, Cribbs DH, Agadjanyan MG. Reducing AD-like pathology in 3xTg-AD mouse model by DNA epitope vaccine—a novel immunotherapeutic strategy. *PLoS ONE* 2008; 3:2124.
59. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, Harper LB, Beare CM, Bagdon WJ, Nichols WW. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. *Intervirology* 2000; 43:258-72.
60. Song YK, Liu F, Chu S, Liu D. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Hum Gene Ther* 1997; 8:1585-94.
61. Bard F, Barbour R, Cannon C, Carretto R, Fox M, Games D, Guido T, Hoenow K, Hu K, Johnson-Wood K, Khan K, Kholodenko D, Lee C, Lee M, Motter R, Nguyen M, Reed A, Schenk D, Tang P, Vasquez N, Seubert P, Yednock T. Epitope and isotype specificities of antibodies to beta-amyloid peptide for protection against Alzheimer's disease-like neuropathology. *Proc Natl Acad Sci USA* 2003; 100:2023-8.
62. Wilcock DM, Munireddy SK, Rosenthal A, Ugen KE, Gordon MN, Morgan D. Microglial activation facilitates Abeta plaque removal following intracranial anti-Abeta antibody administration. *Neurobiol Dis* 2004; 15:11-20.
63. Das P, Howard V, Loosbrock N, Dickson D, Murphy MR, Golde TE. Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma<sup>-/-</sup> knock-out mice. *J Neurosci* 2003; 23:8532-8.
64. Brazil MI, Chung H, Maxfield FR. Effects of incorporation of immunoglobulin G and complement component C1q on uptake and degradation of Alzheimer's disease amyloid fibrils by microglia. *J Biol Chem* 2000; 275:16941-7.
65. Bacskaï BJ, Kajdasz ST, McLellan ME, Games D, Seubert P, Schenk D, Hyman BT. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. *J Neurosci* 2002; 22:7873-8.
66. Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer beta-amyloid by site-directed mAb. *Proc Natl Acad Sci USA* 1997; 94:4109-12.
67. McLaurin J, Cecal R, Kierstead ME, Tian X, Phinney AL, Manea M, French JE, Lambermon MH, Darabie AA, Brown ME, Janus C, Chishti MA, Horne P, Westaway D, Fraser PE, Mount HT, Przybylski M, St. George-Hyslop P. Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4-10 and inhibit cytotoxicity and fibrillogenesis. *Nat Med* 2002; 8:1263-9.
68. DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA* 2001; 98:8850-5.
69. Dodart JC, Bales KR, Gannon KS, Greene SJ, DeMattos RB, Mathis C, DeLong CA, Wu S, Wu X, Holtzman DM, Paul SM. Immunization reverses memory deficits without reducing brain A beta burden in Alzheimer's disease model. *Nat Neurosci* 2002; 5:452-7.
70. Weller RO, Massey A, Newman TA, Hutchings M, Kuo YM, Roher AE. Cerebral amyloid angiopathy: amyloid beta accumulates in putative interstitial fluid drainage pathways in Alzheimer's disease. *Am J Pathol* 1998; 153:725-33.
71. McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res Brain Res Rev* 1995; 21:195-218.
72. Town T, Tan J, Sansone N, Obregon D, Klein T, Mullan M. Characterization of murine immunoglobulin G antibodies against human amyloid-beta1-42. *Neurosci Lett* 2001; 307:101-4.
73. Schenk DB, Yednock T. The role of microglia in Alzheimer's disease: friend or foe? *Neurobiol Aging* 2002; 23:677-9.
74. Morgan D, Gordon MN, Tan J, Wilcock D, Rojiani AM. Dynamic complexity of the microglial activation response in transgenic models of amyloid deposition: implications for Alzheimer therapeutics. *J Neuropathol Exp Neurol* 2005; 64:743-53.
75. Walker DG, Lue LF. Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases. *J Neurosci Res* 2005; 81:412-25.
76. Wisniewski HM, Barcikowska M, Kida E. Phagocytosis of beta/A4 amyloid fibrils of the neuritic neocortical plaques. *Acta Neuropathol (Berl)* 1991; 81:588-90.
77. Rogers J, Strohmeyer R, Kovelowski CJ, Li R. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia* 2002; 40:260-9.
78. Herber DL, Roth LM, Wilson D, Wilson N, Mason JE, Morgan D, Gordon MN. Time-dependent reduction in A beta levels after intracranial LPS administration in APP transgenic mice. *Exp Neurol* 2004; 190:245-53.
79. Akiyama H, McGeer PL. Specificity of mechanisms for plaque removal after A beta immunotherapy for Alzheimer disease. *Nat Med* 2004; 10:117-8.
80. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature* 1996; 382:685-91.
81. El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC. Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. *Neurobiol Aging* 1998; 19:81-4.
82. Giulian D, Haverkamp LJ, Yu J, Karshin W, Tom D, Li J, Kazanskaia A, Kirkpatrick J, Roher AE. The HHQK domain of beta-amyloid provides a structural basis for the immunopathology of Alzheimer's disease. *J Biol Chem* 1998; 273:29719-26.
83. Xie L, Helmerhorst E, Taddei K, Plewright B, Van Bronswijk W, Martins R. Alzheimer's beta-amyloid peptides compete for insulin binding to the insulin receptor. *J Neurosci* 2002; 22:221.

84. Boland K, Behrens M, Choi D, Manias K, Perlmutter DH. The serpin-enzyme complex receptor recognizes soluble, nontoxic amyloid-beta peptide but not aggregated, cytotoxic amyloid-beta peptide. *J Biol Chem* 1996; 271:18032-44.
85. Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *J Biol Chem* 1998; 273:32730-8.
86. Yamada T, Miyazaki K, Koshikawa N, Takahashi M, Akatsu H, Yamamoto T. Selective localization of gelatinase A, an enzyme degrading beta-amyloid protein, in white matter microglia and in Schwann cells. *Acta Neuropathol* 1995; 89:199-203.
87. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 2006; 129:3006-19.
88. Yang F, Sun X, Beech W, Teter B, Wu S, Sigel J, Vinters HV, Frautschy SA, Cole GM. Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease. *Am J Pathol* 1998; 152:379-89.
89. Jellinger KA, Stadelmann CH. The enigma of cell death in neurodegenerative disorders. *J Neural Transm Suppl* 2000; 21-36.
90. Jellinger KA, Stadelmann C. Mechanisms of cell death in neurodegenerative disorders. *J Neural Transm Suppl* 2000; 59:95-114.
91. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci* 2008; 28:8354-60.
92. Boddeke EW, Meigel I, Frentzel S, Gourmala NG, Harrison JK, Buttini M, Spleiss O, Gebicke-Harter P. Cultured rat microglia express functional beta-chemokine receptors. *J Neuroimmunol* 1999; 98:176-84.
93. El Khoury J, Toft M, Hickman SE, Means TK, Terada K, Geula C, Luster AD. Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nat Med* 2007; 13:432-8.
94. Selkoe DJ, Bell DS, Podlisny MB, Price DL, Cork LC. Conservation of brain amyloid proteins in aged mammals and humans with Alzheimer's disease. *Science* 1987; 235:873-7.
95. Raleigh MJ, Steklis HD, Ervin FR, Kling AS, McGuire MT. The effects of orbitofrontal lesions on the aggressive behavior of vervet monkeys (*Cercopithecus aethiops sabaeus*). *Exp Neurol* 1979; 66:158-68.
96. Raleigh MJ, Brammer GL, McGuire MT, Pollack DB, Yuwiler A. Individual differences in basal cisternal cerebrospinal fluid 5-HIAA and HVA in monkeys. The effects of gender, age, physical characteristics and matrilineal influences. *Neuropsychopharmacology* 1992; 7:295-304.
97. Liberini P, Piro E, Maysinger D, Ervin FR, Cuello AC. Long-term protective effects of human recombinant nerve growth factor and monosialoganglioside GM1 treatment on primate nucleus basalis cholinergic neurons after neocortical infarction. *Neuroscience* 1993; 53:625-37.
98. Piro E, Maysinger D, Ervin FR, Desypris G, Cuello AC. Primate nucleus basalis of Meynert p75NGFR-containing cholinergic neurons are protected from retrograde degeneration by the ganglioside GM1. *Neuroscience* 1993; 53:49-56.
99. Lemere CA, Beierschmitt A, Iglesias M, Spooner ET, Bloom JK, Leverone JF, Zheng JB, Seabrook TJ, Louard D, Li D, Selkoe DJ, Palmour RM, Ervin FR. Alzheimer's disease abeta vaccine reduces central nervous system abeta levels in a non-human primate, the Caribbean vervet. *Am J Pathol* 2004; 165:283-97.
100. Uno H, Alsum PB, Dong S, Richardson R, Zimbric ML, Thieme CS, Houser WD. Cerebral amyloid angiopathy and plaques, and visceral amyloidosis in aged macaques. *Neurobiol Aging* 1996; 17:275-81.
101. Struble RG, Price DL Jr, Cork LC, Price DL. Senile plaques in cortex of aged normal monkeys. *Brain Res* 1985; 361:267-75.
102. Sani S, Traul D, Klink A, Niaraki N, Gonzalo-Ruiz A, Wu CK, Geula C. Distribution, progression and chemical composition of cortical amyloid-beta deposits in aged rhesus monkeys: similarities to the human. *Acta Neuropathol* 2003; 105:145-56.
103. Gandy S, DeMattos RB, Lemere CA, Heppner FL, Leverone J, Aguzzi A, Ershler WB, Dai J, Fraser P, St. George Hyslop P, Holtzman DM, Walker LC, Keller ET. Alzheimer's Abeta vaccination of rhesus monkeys (*Macaca mulatta*). *Mech Ageing Dev* 2004; 125:149-51.