mock + CTLA4Ig started developing severe nephritis, as diagnosed by persistent proteinuria of >300 mg/dl. By 30 wk of age, 89% of the PBS control group, 88% of the mock + IG group, 63% of the AN3 + IG group, and 75% of the mock + CTLA4Ig group of mice had developed severe proteinuria, whereas none of the AN3 + CTLA4Ig mice showed excess proteinuria (Fig. 4*B*). However, the AN3 + CTLA4Ig-transferred mice started to develop severe proteinuria at 32 wk of age. Splenomegaly and an increase in the CD4:CD8 ratio, usually observed in aged NZB/W F_1 mice, were suppressed in AN3 + CTLA4Ig-injected mice (data not shown).

The kidneys from the controls and AN3 + CTLA4Ig-injected mice were examined at 30 wk of age (Fig. 5, *A–F*). Control mice had severe glomerulonephritis with mesangial proliferation and thickening of the capillary walls with marked deposition of IgG and complement. AN3 + CTLA4Ig-injected mice had mild glomerular lesions and deposition of IgG and complement was only restricted to the mesangial area. Although mock + CTLA4Ig-transferred mice showed formation of a number of large follicles with T cell invasion in the spleen, AN3 + CTLA4Ig-transferred mice showed only a limited number of small follicles (Fig. 5, *G* and *H*).

AN3 + CTLA4Ig-treated mice exhibited the normal humoral immune response upon active immunization

We next examined the T cell-dependent humoral immune response to active immunization of OVA. Mice transferred with the engineered T cells at 10 wk of age were immunized with OVA (100 μg) with CFA at 14 wk of age and boosted with OVA with IFA at 16 wk of age. The level of anti-OVA IgG Ab titer from 17-wk-old mice treated with AN3 + CTLA4Ig was not significantly different from those of the control mice (Fig. 4*C*). AN3 + CTLA4Ig transferred mice, but not other experimental groups, had low but detectable levels of serum CTLA4Ig (13.4 ± 10.1 μg/ml) (Fig. 4*D*), findings consistent with in vitro data shown in Fig. 3*C*. These results suggest that the engineered regulatory cells are sufficient to suppress autoimmune disease. However, they are not enough to induce general immunosuppression, because of the low serum level of CTLA4Ig in AN3 + CTLA4Ig-transferred mice.

Discussion

In this study, we demonstrated T cell hyperresponsiveness and the possibility of nucleosomal hyperpresentation of splenic DCs in NZB/W F_1 mice. In addition to the involvement of T cell hyperresponsiveness in Ab-mediated autoimmune disease (30), our results strongly suggest that the autoantigen hyperpresentation of DCs could contribute to the initiation and propagation of the response to the autoantigen, thereby resulting in florid autoimmune disease. This observation is consistent with those from previous reports indicating that mice with T cell hyperresponsiveness develop only a mild form of lupus-like symptoms (31, 32). Since hyperpresentation was not observed in the case of an exogenous Ag, OVA (peptides and whole protein), it is possible that the autoantigen hyperpresentation of splenic DCs was not due to the general hyperpresentation, e.g., excessive costimulatory signals, but rather to some Ag-restricted phenomenon. These features may be nucleosome specific, as reported in a previous study demonstrating that lupus-prone B6.NZMc1 mice showed nucleosome reactivity of T cells without generalized immunological deficits of B cells and T cells (33).

Although disease-related increases in the number of splenic DCs and chemokine production by myeloid DCs have been reported (34), these abnormalities have been observed in aged lupus-prone mice. Our finding of autoantigen hyperpresentation in the splenic DCs of young mice (10 wk) suggests the significance of the autoantigen hyperpresentation of splenic DCs in the pathogenesis of lupus.

Autoreactive response of nucleosome-specific T cells was much more prominent in the spleen than in the LNs. Although the mixed I-A haplotype of Aβz/Aαd molecules in NZB/W F_1 mice (35) may be associated with autoreactive response of AN3 infectant, the absence of the autoreactivity to B cells and DCs from peripheral LNs strongly suggests the requirement of an autoantigen for the autoreactivity. Differences between the splenic DCs and DCs from other peripheral lymphoid organs have been reported, including differences in the expression of chemokines (36) and chemokine receptors (37). Otherwise, localization of tissue-specific autoantigen among secondary lymphoid organs may be one explanation. For example, although DCs in the gastric LNs are known to exhibit constitutive presentation of gastric parietal cell-specific H^+/K^+-ATPase, peripheral or mesenteric DCs do not (38). Thus, the spleen could be one of the main sources of nucleosomes. Increased frequency of splenic apoptosis in SNF1 lupus mice has also been reported (23). Moreover, an insufficient complement system may allow apoptotic waste material to accumulate in the spleen (i.e., the "waste disposal" hypothesis) (39).

In our study, the therapeutic effect with minimal systemic immunosuppression was archived by the use of nucleosome-specific T cells secreting CTLA4Ig. Although elevation of CTLA4Ig protein was detected in the serum of AN3 + CTLA4Ig mice, the

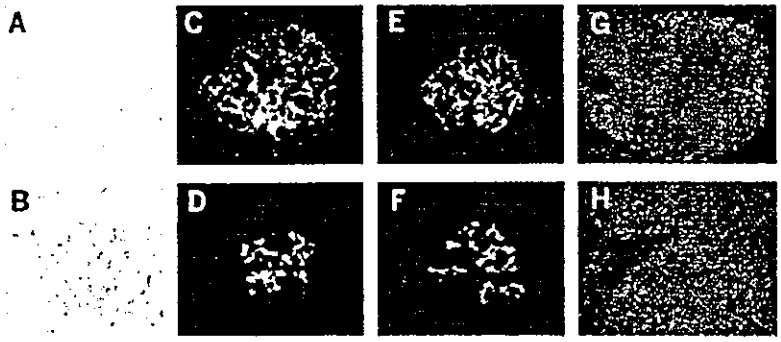

FIGURE 5. Histological examination from the AN3 + CTLA4Ig-treated mice compared with control mice. Sections of kidney from mock + IG-injected mice (*A*, *C*, and *E*) and AN3 + CTLA4Ig-injected mice (*B*, *D*, and *F*) subjected to staining with periodic-acid-Schiff solution (*A* and *B*) or to immunofluorescence staining with anti-IgG (*C* and *D*) or anti-C3 (*E* and *F*). Immunofluorescence staining of sections from the spleen, from mock + CTLA4Ig-injected mice (*G*), and from AN3 + CTLA4Ig-injected mice (*H*) with Abs to B220 (green), CD4 and CD8 (red), and with peanut agglutinin (blue). A section from one representative mouse from the indicated group is shown.

average concentration of CTLA4Ig in AN3 + CTLA4Ig mice is less than one-tenth of the level of previous systemic CTLA4Ig treatment with 5×10^8 PFU of adenovirus (27). Although the systemic adenoviral-CTLA4Ig (5×10^8 PFU) treatment exhibited a therapeutic effect equivalent to that of our experiment, the systemic treatment was accompanied with generalized immunosuppression. Since autoantigen-specific CTLA4Ig-secreting T cells showed normal Ab production on active immunization, this treatment may be superior to systemic CTLA4Ig administration. However, a systemic effect of a very low level of CTLA4Ig cannot be excluded and should be investigated further.

It is not surprising that 10^6 AN3 + mock cells did not aggravate the disease, since as many as 4×10^7 original L3A clone cells were needed to accelerate lupus nephritis in young lupus-prone mice (40). Thus, a relatively small amount of Ag-specific and potentially pathogenic T cells could be used for the immunotherapy. Foxp3, a member of the transcription factor family, has been identified as a key molecule for the development of $CD4^+CD25^+$ regulatory T cells (41). Retroviral transfer of Foxp3 confers regulatory function on $CD4^+CD25^-$ T cells. The introduction of such regulatory molecules with TCR could possibly generate Ag-specific regulatory T cells.

In a preliminary analysis of the persistence of the transferred genes in the spleen and LNs from 30-wk-old mice with RT-PCR, expression of AN3α gene was detected in the spleens from two of two AN3 + IG^- and AN3 + CTLA4Ig$^-$-injected mice (data not shown). These results may suggest the persistence of introduced genes at 20 wk after the transfer in the spleen.

Although several models of adoptive cell gene therapy have been reported using T cell hybridomas or lines (42, 43), our method has the advantage of using autologous lymphocytes for gene recipients. However, TCR-transduced recipient T cells could gain heterodimeric TCR consisting of endogenous and exogenous chains. If such an unexpected TCR recognizes a certain unrelated self-derived molecule, the transduced T cells may be harmful. We did not observe evident autoreactivity in single AN3α or AN3β genes transferred into $CD4^+$ T cells (data not shown), and the renal disease of AN3 TCR-transferred mice was not accelerated (Fig. 5*B*). There was a recent report of tumor rejection mediated by retrovirally reconstituted Ag-specific T cells without any significant autoimmune pathology (44, 45). However, the possibility of developing autoimmunity should be carefully investigated further in application of TCR gene transfer.

In the present study, the efficacy of triple gene transfer in peripheral T cells was demonstrated for the first time. Although several improvements of the present method are still necessary, these findings suggest that the direct engineering of Ag-specific functional cells with multiple gene transfer is a powerful technique for the development of future Ag-specific therapies.

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Dissection of the role of MHC class II A and E genes in autoimmune susceptibility in murine lupus models with intragenic recombination

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Systemic lupus erythematosus (SLE) is a multigenic autoimmune disease, and the major histocompatibility complex (MHC) class II polymorphism serves as a key genetic element. In SLE-prone (NZB × NZW)F_1 mice, the MHC H-$2^{d/z}$ heterozygosity (H-2^d of NZB and H-2^z of NZW) has a strong impact on disease; thus, congenic H-$2^{d/d}$ homozygous F_1 mice do not develop severe disease. In this study, we used *Ea*-deficient intra-H-2 recombination to establish $A^{d/d}$-congenic (NZB × NZW)F_1 mice, with or without E molecule expression, and dissected the role of class II A and E molecules. Here we found that $A^{d/d}$ homozygous F_1 mice lacking E molecules developed severe SLE similar to that seen in wild-type F1 mice, including lupus nephritis, autoantibody production, and spontaneously occurring T cell activation. Additional evidence revealed that E molecules prevent the disease in a dose-dependent manner; however, the effect is greatly influenced by the haplotype of A molecules, because wild-type H-$2^{d/z}$ F_1 mice develop SLE, despite E molecule expression. Studies on the potential of dendritic cells to present a self-antigen chromatin indicated that dendritic cells from wild-type F_1 mice induced a greater response of chromatin-specific T cells than did those from $A^{d/d}$ F_1 mice, irrespective of the presence or absence of E molecules, suggesting that the self-antigen presentation is mediated by A, but not by E, molecules. Our mouse models are useful for analyzing the molecular mechanisms by which MHC class II regions regulate the process of autoimmune responses.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the appearance of autoantibodies to several nuclear components. The deposition of formed immune complexes mediates the disease in a wide variety of tissues and organs, including the kidney and the vascular system. There is evidence that the development of SLE is under the control of multiple susceptibility genes (1). Among these, genes in the major histocompatibility complex (MHC) have been implicated as a key genetic element. Because SLE is an autoantibody-mediated disease, MHC class II polymorphisms are probably involved in the pathogenesis. However, because of the complex multifactorial inheritance and heterogeneity of SLE, and because of the linkage disequilibrium that exists among the class I, II, and III genes within the MHC complex, the absolute contribution of individual MHC class II loci has been difficult to dissect. Thus, our knowledge of the molecular mechanism of MHC class II contribution to SLE remains incomplete.

Substantial progress in research in this area has been achieved through studies using SLE-prone mice with genetic recombination and manipulation of MHC (H-2) genes. In (NZB × NZW)F_1 mice that spontaneously develop disease closely resembling human SLE, the disease is strongly associated with H-2 haplotypes from both parents (H-2^d from NZB and H-2^z from NZW) (2–6). Genetic dissection by producing H-2-congenic mice revealed that an early onset of severe SLE occurs in only heterozygous H-$2^{d/z}$ mice and not in homozygous H-$2^{d/d}$ and H-$2^{z/z}$ mice (2, 3, 5). Although both A and E class II molecules may be involved, evidence has suggested that mixed haplotype class II $A\alpha^d\beta^z$ molecules are responsible for the pathogenesis by promoting the production of pathogenic high-affinity IgG autoantibodies to nuclear components (7, 8).

In contrast to class II A molecules, E molecules are suggested to be a suppressive genetic element for SLE. This notion was based mainly on the results obtained by using a transgene technique. A BXSB strain of mouse, another spontaneous SLE model, carries H-2^b haplotype and expresses A^b, but not E, molecules, because of a defect in the *Ea* gene (9). The development of BXSB disease is closely associated with the H-2^b haplotype (10) and is almost completely prevented by a transgene encoding $E\alpha^d$ chains (11). Similar findings were noted in the nonobese diabetic (NOD) mouse, a model of spontaneous autoimmune diabetes. NOD mice express class II A^{g7}, but not E, molecules, because of a defect in the *Ea* gene (12). Evidence indicated that whereas the class II A^{g7} gene is critical for the disease susceptibility (13), the transgenic introduction of $E\alpha^d$ or $E\alpha^k$ does prevent the disease (14–16). Thus, A and E molecules seem to provide the susceptible and protective genetic elements for autoimmune diseases, respectively, at least in these mouse models.

Nevertheless, the conclusion awaits further studies, because the transgene possibly induces unexpected improper effects on immune cells. For example, unpaired or mispaired transgene-derived class II molecules can be toxic to B cell maturation (17). Furthermore, there are reports suggesting that excessively generated transgenic $E\alpha^d$ molecules bind to A molecules, thereby decreasing the availability of A molecules for antigen presentation (18), and that overexpression of E molecules suppresses expression levels of endogenously encoded A molecules (19). In the (NZB × NZW)F_1 model, severe SLE occurs despite the presence of intact E molecules. To examine the role of A and E molecules in (NZB × NZW)F_1 lupus, we generated several kinds of congenic (NZB × NZW)F_1 mice with intra-MHC recombination at the *E* subregion, taking advantage of natural recombinants, including those we found among ≈3,000 meiosis in crosses of NZB strains.

Materials and Methods

Mice. NZB (H-2^d) and NZW (H-2^z) mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were maintained in our animal facility. The H-2-congenic

Abbreviations: DC, dendritic cell; NOD, nonobese diabetic; SLE, systemic lupus erythematosus; TCR, T cell receptor.

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Table 1. H-2 haplotypes of established MHC-congenic and intra-MHC recombinant-congenic New Zealand strains of mice

Strains	H-2 haplotype	*K*	*Ab*	*Aa*	*Eb*		*Ea*		*Tnfa*	*D*
NZB	d	d	d	d	d		d		d	d
NZB.GD	g2	d	d	d	d	//	b		b	b
NZW.GD	g2	d	d	d	d	//	b		b	b
NZB.GDr	g2r	d	d	d	d		d	//	b	b
NZW.H-2^d	d	d	d	d	d		d		d	d
(NZB × NZW.H-2^d)F$_1$	d/d	d	d	d	d		d		d	d
(NZB × NZW.GD)F$_1$	d/g2	d	d	d	d		d/b		d/b	d/b
(NZB.GDr × NZW.GD)F$_1$	g2r/g2	d	d	d	d		d/b		b	b
(NZB.GD × NZW.GD)F$_1$	g2/g2	d	d	d	d	//	b		b	b

//, Intra-H-2 recombination site between d and b haplotype.

NZW.H-2^d (2, 3, 5) strain was established by selective backcrossing of (NZB × NZW)F$_1$ to NZW for 15 generations. NZB.GD (H-2^{g2}) (20) and NZW.GD strains were established by selective backcrossing of (B10.GD × NZB)F$_1$ and (B10.GD × NZW)F$_1$ with NZB and with NZW mice, respectively, for 15 generations. The NZB strain with an intragenic recombination between *Ea* of NZB and *Tnfa* of NZB.GD was obtained in crosses of NZB and NZB.GD strains and was tentatively designated NZB.GDr. Alleles at the H-2 loci in established H-2-congenic and recombinant H-2-congenic New Zealand mice are shown in Table 1. These mice were crossed to produce (NZB × NZW)F$_1$ hybrids with the same d haplotype of the upstream region of the *Eb* gene but with different haplotypes of downstream regions of the *Ea* gene (Table 1), and the disease severity was compared among these female F$_1$ mice.

Typing of H-2 Haplotype. Peripheral blood was obtained from the periorbital sinus, followed by lysis of red blood cells with ammonium chloride. Aliquots of 5×10^5 to 10×10^5 cells were incubated with anti-A^d (K24–199) (21); anti-E (ISCR, which reacts with a common determinant of the E molecule) (a kind gift from Dr. N. Shinohara, Kitasato University, Kanagawa, Japan); anti-D^d (T19–191); and anti-D^b (H141–30) mAbs, followed by FITC-labeled anti-mouse polyclonal IgG antibodies (ICN). Incubations were run for 30 min at 4°C, and the stained cells were analyzed by using FACSTAR and CELLQUEST software (Becton Dickinson).

Microsatellite DNA Polymorphism in the *Tnfa* Promoter. *Tnfa* promoter was shown to have microsatellite polymorphism, and different tumor necrosis factor alleles have different lengths of microsatellites (22, 23). To determine the tumor necrosis factor alleles of each mouse strain, PCRs were performed with genomic DNAs, using 5′ primer (5′-GGACAGAGAAGAAATGGGTTTC-3′) and 3′ primer (5′-TCGAATCTGGGGCCAATCAGGAGGG-3′) (22), and differences in lengths of PCR products were determined by using electrophoresis of PCR products on 7% denaturing polyacrylamide gels, as described in ref. 24.

Measurement of Proteinuria. The onset of renal disease was monitored by biweekly testing for proteinuria, as described in ref. 25. Mice with a proteinuria of 111 mg/ml or more in repeated tests were regarded as being positive.

Measurements of Anti-DNA and Anti-Chromatin Antibodies. Serum levels of IgG autoantibodies to DNA and chromatin were determined by ELISA, using peroxidase-conjugated polyclonal anti-mouse IgG antibodies (ICN). The DNA- and chromatin-binding activities were expressed in units, referring to a standard curve obtained by serial dilutions of a standard serum pool from 7- to 9-month-old (NZB × NZW)F$_1$ mice, containing 1,000 units/ml (5). DNA was obtained from calf thymus (Sigma). Chromatin was prepared as described in ref. 26. Briefly, nucleosomes were isolated by solubilizing chromatin from purified chicken erythrocyte nuclei with micrococcal nuclease. The solubilized chromatin was fractionated into sucrose gradients that were analyzed for monomers by using electrophoresis, and the appropriate fractions were dialyzed and pooled.

Analysis of T Cell Activation and T Cell Receptor (TCR) V$_\beta$ Repertoires. To examine the activation states of CD4$^+$ T cells, aliquots of 10^6 spleen cells were double-stained with FITC-conjugated anti-CD4 mAb and phycoerythrin-conjugated anti-CD69 mAb. To analyze TCR V$_\beta$ repertoires, spleen cells were stained with biotin-labeled mAbs to each TCR V$_\beta$ repertoire (Pharmingen), followed by incubation with avidin-phycoerythrin and FITC-conjugated anti-CD4 mAb. All incubations were run for 30 min at 4°C, and the frequency of CD4$^+$ T cells with each TCR V$_\beta$ repertoire per total CD4$^+$ T cells was calculated by using FACSTAR and CELLQUEST software.

Preparation of Retroviral Construct with Chromatin-Specific TCR Genes and Transduction to Splenocytes. Chromatin-specific TCR V$_\alpha$ and V$_\beta$ DNA fragments were synthesized, using PCR based on the published sequences of the nucleosome-specific T cell line derived from the lupus-prone (SWR × NZB)F$_1$ mouse (27, 28), as described in ref. 29. These fragments were cloned into a pMXW retroviral vector (30) and transfected into PLAT-E packaging cell lines (31) by using FuGENE6 transfection reagent (Roche Diagnostics). The viral supernatant of transfected cells were placed on fibronectin-coated 24-well plates, and total spleen cells from 2-month-old (NZB × NZW)F$_1$ mice prestimulated for 48 h with Con A (10 μg/ml) and interleukin 2 (50 ng/ml) were added to the wells (1×10^6 cells per well). Cells were cultured further for 36 h to allow infection to occur.

Purification and Functional Analysis of Dendritic Cells (DCs). Spleen cells were treated with collagenase type IV (Sigma) and DNase I, and CD11c$^+$ cells were positively collected by passing spleen cells twice through MACS CD11c microbeads and magnetic separation columns. The purity (85% in average) of DCs was determined by flow cytometry with anti-CD11c-biotin, followed by streptavidin-phycoerythrin.

To analyze the potential of chromatin presentation, CD4$^+$ T cells were purified by negative selection, using MACS microbeads with anti-CD19, -CD11c, and -CD8 mAbs 24 h postinfection, and 2×10^4 cells per well of T cells were cocultured with 1×10^5 cells per well of irradiated CD11c$^+$ DCs in 96-well flat-bottom plates with 1 μg/ml chromatin. After 24 h of culture, the cells were pulse-labeled with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per well (NEN) for 15 h, and the [^{3}H]thymidine incorporation was determined.

Statistical Analysis. Statistical analysis was performed by using Student's t test and the χ^2 test. $P < 5\%$ was considered to have a statistical significance.

Results

Establishment of Intra-MHC Recombinant-Congenic New Zealand Mice. By selective backcrossing, we first introduced the H-2^{g2} haplotype derived from the B10.GD strain into NZB (H-2^d) and NZW (H-2^z) and established H-2-congenic NZB.GD (20) and NZW.GD strains. H-2^{g2} has an intragenic recombination between d and b haplotype in the E gene and the H-2 haplotype is $K^dAb^dAa^dEb^dEa^bTnfa^bD^b$ (Table 1) (32). Because the Ea^b gene is defective, H-2^{g2} mice do not express E molecules (20). To establish the strain that carries the H-2 haplotype of $K^dAb^dAa^dEb^dEa^dTnfa^bD^b$, we conducted a search for the mouse with a spontaneously occurring intragenic recombination between Ea and $Tnfa$ in the progeny of NZB and NZB.GD crosses. In ≈3,000 meiosis, there was a single mouse carrying this recombination, and the recombination-congenic NZB line, provisionally designated NZB.GDr (H-2^{g2r}), was generated. This haplotype is valid to evaluate the effect of E molecule expression on the same $Tnfa^bD^b$ background. Table 1 summarizes the haplotypes of the intra-MHC-congenic New Zealand mouse and the related strains.

E Molecule Expression Levels in (NZB × NZW)F_1 Mice with Different Intra-H-2 Haplotypes. Congenic and recombinant-congenic New Zealand mice were crossed to obtain (NZB × NZW)F_1 mice with four different combinations of the H-2 haplotype (Table 1). Fig. 1 shows flow cytometric analyses for expression profiles of A, E, and D molecules on peripheral blood lymphocytes. As expected, although levels of A^d expression were almost identical, levels of E molecules differed significantly among these F_1 mice, i.e., full expression levels in H-$2^{d/d}$ homozygous, approximately one-half of expression levels in H-$2^{d/g2}$ and H-$2^{g2r/g2}$ heterozygous, and no expression in H-$2^{g2/g2}$ homozygous F_1 mice. Profiles of the class I D molecule expression showed that lymphocytes from H-$2^{d/g2}$ F_1 mice were positive for both D^d and D^b, whereas those from H-$2^{g2r/g2}$ and H-$2^{g2/g2}$ F_1 mice were positive for D^b and negative for D^d. As also shown in Fig. 1, E molecules in wild-type H-$2^{d/z}$ heterozygous F_1 mice were fully expressed. Because these F_1 mice are heterozygous H-$2^{d/z}$, the level of A^d and D^d expression was approximately one-half of that seen in the H-$2^{d/d}$ homozygote.

Comparisons of Disease Features. Fig. 2A compares cumulative incidences of proteinuria in wild-type H-$2^{d/z}$ heterozygous (NZB × NZW)F_1 mice with intact E molecule expression and four kinds of H-2-congenic (NZB × NZW)F_1 (H-$2^{d/d}$, H-$2^{d/g2}$, H-$2^{g2r/g2}$, and H-$2^{g2/g2}$) carrying identical homozygous $A^{d/d}$ molecules but different levels of E molecule expression. Compared with findings in wild-type F_1 mice, the incidence was markedly reduced in homozygous $A^{d/d}$ F_1 mice with intact E molecule expression (H-$2^{d/d}$). In a striking contrast, homozygous $A^{d/d}$ F_1 mice deficient in E expression (H-$2^{g2/g2}$) showed an early onset and a high incidence of proteinuria comparable to those found in the wild-type F_1 mice. Findings in H-$2^{d/g2}$ and H-$2^{g2r/g2}$ with one-half of E expression levels were in between. Together with the finding that heterozygous H-$2^{g2r/g2}$ and homozygous H-$2^{g2/g2}$ F_1 mice share the same H-2 haplotype except for the Ea subregion (Table 1), it is strongly suggested that the development of lupus nephritis in $A^{d/d}$ F_1 mice is down-regulated by E molecules in a dose-dependent manner.

As shown in Fig. 2B, the decrease in survival rate was associated with an increase in the incidence of proteinuria in all groups of mice. Whereas all H-$2^{g2/g2}$ F_1 mice and 90% of wild-type F_1 mice died of disease by 12 months of age, 80% of H-$2^{d/d}$ F_1 mice and ≈50% of H-$2^{d/g2}$ and H-$2^{g2r/g2}$ F_1 mice

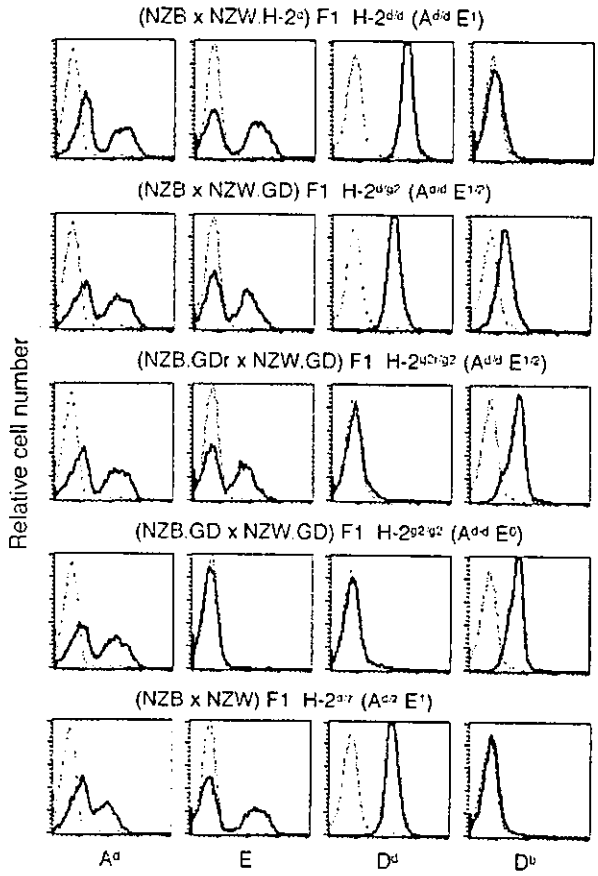

Fig. 1. Flow cytometry analysis for cell surface expression of A^d, E, D^d, and D^b molecules on peripheral lymphocytes in (NZB × NZW)F_1 mice with different H-2 haplotypes. The upper four groups of F_1 mice with homozygous $A^{d/d}$ showed the same expression level of A^d molecules, and the level in wild-type H-$2^{d/z}$ heterozygous F_1 mice was almost one-half of that seen in the former groups. When E molecule expression levels were examined with a mAb to a common determinant, H-$2^{d/g2}$ and H-$2^{g2r/g2}$ F_1 mice showed approximately one-half the level ($E^{1/2}$) of that seen in H-$2^{d/d}$ and wild-type H-$2^{d/z}$ F_1 mice (E^1). H-$2^{g2/g2}$ F_1 mice did not express E molecules (E^0). D^d expression levels in H-$2^{d/g2}$ and H-$2^{d/z}$ F_1 mice were approximately one-half of that seen in H-$2^{d/d}$ F_1 mice. D^b expression level in H-$2^{d/g2}$ F_1 mice was approximately one-half of that seen in H-$2^{g2r/g2}$ and H-$2^{g2/g2}$ F_1 mice.

remained alive. In Fig. 2 C and D, we compare serum levels of IgG autoantibodies to DNA and to chromatin, respectively, in 6-month-old homozygous $A^{d/d}$ F_1 mice with different levels of E expression. Whereas H-$2^{g2/g2}$ F_1 mice lacking E molecules showed high levels of both autoantibodies, comparable to those found in wild-type F_1 mice, the levels were greatly reduced in mice expressing E molecules.

Increase in Activated T Cells in E-Deficient Mice. Frequencies of $CD69^+$ activated $CD4^+$ T cells increase with age in (NZB × NZW)F_1 mice, as animals develop SLE (33). Fig. 3A compares frequencies of $CD69^+$ activated splenic $CD4^+$ T cells in total $CD4^+$ T cells among four groups of $A^{d/d}$ F_1 mice at 6 months of age. The frequency (mean ± SE) in E-negative H-$2^{g2/g2}$ F_1 mice (31.1 ± 8.6) was significantly higher than those found in three other groups of F_1 mice (11.0 ± 2.2 in H-$2^{d/d}$ F_1 mice, 14.2 ± 4.6 in H-$2^{d/g2}$ F_1 mice, and 17.2 ± 6.3 in H-$2^{g2r/g2}$ F_1 mice) ($P < 0.02$). Frequencies in H-$2^{d/g2}$ and H-$2^{g2r/g2}$ F_1 mice showed a tendency to be higher than those found in H-$2^{d/d}$ F_1 mice; however, there were no significant differences among the groups.

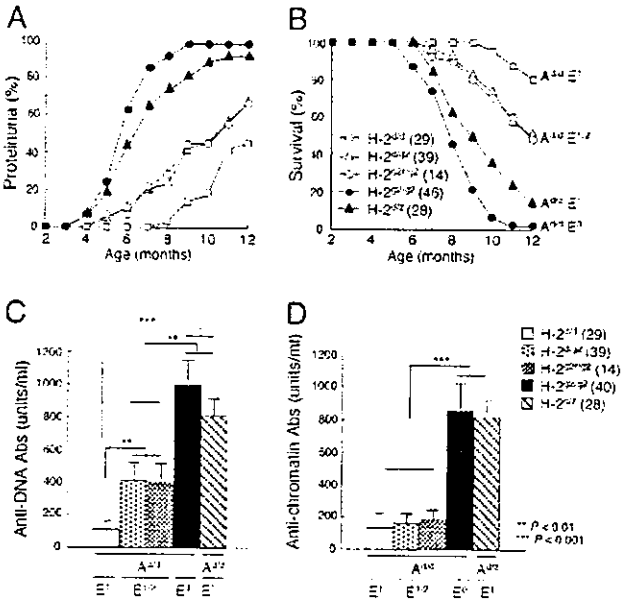

Fig. 2. Comparisons of the cumulative incidence of proteinuria (*A*), survival rate (*B*), and serum levels of IgG anti-DNA antibodies (*C*) and anti-chromatin antibodies (*D*) among F_1 mice with different H-2 haplotypes. The number of mice examined is shown in parentheses. H-$2^{g2/g2}$ F_1 showed a significantly higher incidence of proteinuria and a lower survival rate, as compared with three other $A^{d/d}$ F_1 strains ($P < 0.001$). Incidence of proteinuria and survival rate in H-$2^{d/d}$ F_1 mice were significantly reduced, as compared with H-$2^{d/g2}$ F_1 and H-$2^{g2r/g2}$ F_1 mice ($P < 0.01$). Compared with H-$2^{g2/g2}$ F_1 mice, wild-type H-$2^{d/z}$ F_1 mice showed a subtle but significantly lower incidence of proteinuria after 7 months of age ($P < 0.05$) and an improved survival rate after 9 months of age ($P < 0.05$). Serum levels of autoantibodies are compared at 6 months of age. Column and bar represent mean and SE, respectively. Asterisks indicate a significant difference.

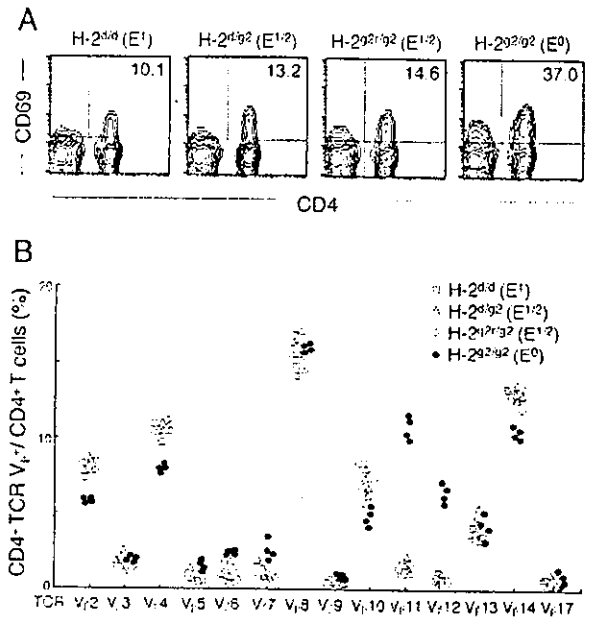

Fig. 3. Changes in splenic $CD4^+$ T cells among 6-month-old $A^{d/d}$ F_1 mice with different E molecule expression levels. (*A*) Representative flow cytometry profiles for the expression of activation marker CD69 on splenic $CD4^+$ T cells. Frequency of $CD69^+CD4^+$ T cells per total $CD4^+$ T cells is shown. (*B*) Comparisons of each TCR V_β repertoire frequency in splenic $CD4^+$ T cells. $V_\beta 11$ and $V_\beta 12$ repertoires were significantly higher in H-$2^{g2/g2}$ F_1 mice than those in other three F_1 mice ($P < 0.001$).

TCR V_β Repertoire Skewing in E-Deficient Mice. E molecule expression levels on thymic epithelial cells affect TCR V_β repertoire selection in the thymus (34–36). As shown in Fig. 3*B*, $V_\beta 11$ and $V_\beta 12$ repertoires in splenic $CD4^+$ T cells were negatively selected in E molecule-positive H-$2^{d/d}$, H-$2^{d/g2}$, and H-$2^{g2r/g2}$ F_1 mice, and there was no significant difference in these repertoire frequencies among the three groups. In contrast, significant proportions of $V_\beta 11$ (mean and SE, 10.8 ± 0.2%) and $V_\beta 12$ (6.0 ± 0.3%) repertoires were observed in E-negative H-$2^{g2/g2}$ F_1 mice.

Effects of E Molecule Expression on DC Function. Differences in haplotypes of class II molecules may affect autoimmune manifestations through the self-antigen presenting capacity of antigen-presenting cells. To determine whether the presence or absence of E molecules affects the potential for self-antigen presentation, we took advantage of a T cell proliferation assay against self-antigen. Splenic T cells from (NZB × NZW)F_1 mice were transfected *in vitro* with chromatin-specific TCR V_α and V_β, originally derived from a (SWR × NZB)F_1 mouse, which can recognize the immunodominant nucleosomal epitope (amino acids 71–94 in histone H4) in the context of $A^{d/d}$ (27, 28) and other A haplotype molecules (37). These T cells can reconstitute the specificity to the nucleosome (38). Such T cells and the T cells transfected with vector alone (mock) were cocultured with CD11c-positive splenic DCs obtained from (NZB × NZW)F_1 with different levels of E expression, in the presence of chromatin. As shown in Fig. 4, although DCs from $A^{d/d}$ F_1 mice with H-$2^{d/d}$, H-$2^{d/g2}$, and H-$2^{g2/g2}$ haplotypes induced significant levels of chromatin-specific T cell responses, there were no significant differences among the three groups of F_1 mice. Compared with findings in these $A^{d/d}$ F_1 mice, DCs from wild-type $A^{d/z}$ heterozygous (NZB × NZW)F_1 mice induced a significantly greater response of chromatin-specific T cells. Thus, it is suggested that, as compared with $A\alpha^d\beta^d$, $A^{d/z}$-unique $A\alpha^d\beta^z$ and/or $A\alpha^z\beta^d$ have an increased capacity of DCs to present chromatin and that the presence or absence of E molecules does not influence the potential of DCs for chromatin presentation.

Discussion

Our newly generated intra-MHC recombinant-congenic (NZB × NZW)F_1 mice make it feasible to examine the role of class II A and E molecules in the regulation of autoimmune disease, with disregard to the effect of other MHC genes such as *Tnfa* and class I D, which also have been implicated in autoimmune susceptibility (39, 40). The results clearly indicated that class II A and E serve as promoting and protective genetic elements, respectively.

H-$2^{d/z}$ heterozygosity has a strong impact on SLE in (NZB × NZW)F_1 mice, because the disease is largely reduced in H-$2^{d/d}$ and H-$2^{z/z}$ homozygous mice (2, 3, 5). This observation means that a combination of H-2-linked genes from both parents play a role in an epistatic manner. In this context, we earlier speculated that the polymorphic class II α and β chain genes from both parents may form F_1-unique mixed haplotype α/β heterodimers, such as $A\alpha^d\beta^z$ and $A\alpha^z\beta^d$, either of which may serve as a restriction element for self-reactive T cells (5). Consistent was the finding in the present studies that, compared with DCs from $A^{d/d}$ homozygous F_1 mice, DCs from wild-type $A^{d/z}$ (NZB × NZW)F_1 mice showed a greater likelihood of chromatin presentation to T cells. Because the antigen-presenting capacity was not influenced by the presence or absence of E molecules, class II A molecules can be attributed to this event. The importance of mixed haplotype α/β heterodimers also was supported by findings of Gotoh *et al.* (7), in

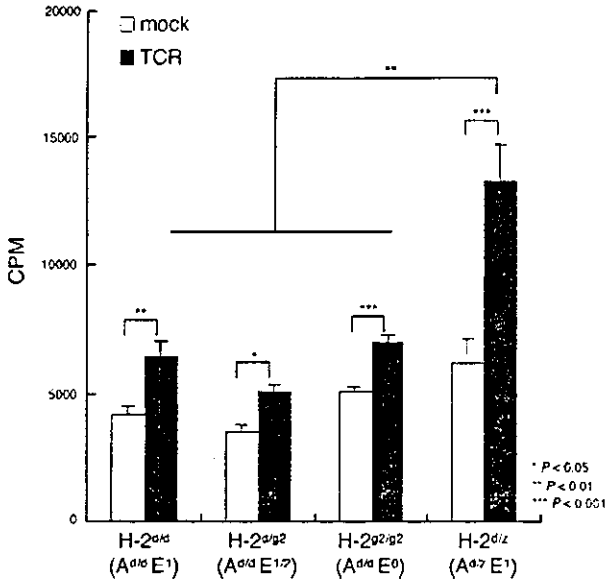

Fig. 4. The potential for chromatin presentation by CD11c-positive splenic cells from (NZB × NZW)F_1 mice with different H-2 haplotypes. Splenic T cells from (NZB × NZW)F_1 mice transfected with chromatin-specific TCR V_α and V_β or with vector alone (mock) were cocultured with CD11c-positive splenic DCs obtained from F_1 mice with different H-2 haplotypes, in the presence of chromatin, and T cell proliferative responses were compared. DCs from all these F_1 mice induced significant levels of chromatin-specific T cell responses; however, the potential of DCs for chromatin presentation was significantly higher in wild-type H-$2^{d/z}$ F_1 mice than that in other F_1 groups. Means and SE of three experiments are shown.

which the $A\alpha^d\beta^z$-restricted self-reactive T cell clone isolated from (NZB × NZW)F_1 mice had a potential to induce IgG anti-DNA antibodies *in vivo* (8). These F_1-unique mixed haplotype class II α/β heterodimers also may be involved in the aberrant selection of self-reactive T cells in the thymus. Based on studies by Braunstein and Germain (41), the assembly of $A\alpha$ and $A\beta$ chains of different haplotypes is under serious pairing restrictions. Thus, low expression levels of these heterodimers in the thymic epithelial cells may allow α/β heterodimer-restricted self-reactive T cells to escape from negative selection in the thymus.

In contrast to A molecules, the mixed-haplotype E molecules are not formed in most of the H-2 heterozygotes, because $E\alpha$ chains are usually nonpolymorphic (42). However, $E\alpha^z$ is unique among the $E\alpha$ chains of other haplotypes and has two amino acid substitutions in the α1 domain (43, 44). Thus, F_1 unique E molecules can be formed in H-$2^{d/z}$ F_1 mice. In this context, Nygard *et al.* (45) proposed the possible preferential formation of $E\alpha^d\beta^z$ mixed haplotype molecules and their involvement in the promotion of F_1 disease. However, evidence indicated that $E\alpha^d\beta^z$ molecules are protective rather than promoting (20).

The protective mechanism by E molecules remains elusive; however, several possibilities are suggested. First is the E-mediated clonal deletion model of self-reactive T cells in the thymus. It has been shown that T cells bearing TCR $V_\beta5$, $V_\beta11$, $V_\beta12$, and $V_\beta17a$ are eliminated in E-positive strains of mice (34–36), although the extent of negative selection is influenced by background genes (46–48). In this context, there are reports indicating that no clear-cut difference in the expression of TCR V_β repertoire was observed between E^+ and E^- transgenic NOD (16) as well as collagen-induced arthritis-susceptible B10.RQB3 mice (49), although the E transgene protected against the disease. Hence, the clonal deletion hypothesis for E-mediated protection of autoimmune disease has been dismissed in cases of these models. In the (NZB × NZW)F_1 genetic background, however, CD4 T cell repertoires bearing TCR $V_\beta11$ and $V_\beta12$ were significantly negatively selected in mice expressing E molecules. Thus, clonal deletion remains one possible mechanism at work in our mouse models.

Second is the determinant capture model. In Ea^d-transgenic BXSB mice, transgene-derived $E\alpha^d$ peptides bind to A^b molecules, possibly decreasing the use of A molecules for presentation of pathogenic self-peptides (18). Rudensky *et al.* (50) showed evidence for the binding of $E\alpha^d$ peptides to A^b molecules in a sequence analysis of peptides derived from the cell surface. This mechanism may be involved in the E-transgene-mediated protection of the collagen-induced arthritis model of $E\beta$-deficient B10.RQB3 mice (51, 52). However, such a mechanism may not be operative in our model, because the potential for chromatin presentation by $CD11C^+$ DCs was not affected by E molecules expressed in $A^{d/d}$ F_1 mice. This notion is in agreement with the finding by Nakano *et al.* (53) that antigen-presenting cells from both E^+ and E^- NOD mice can similarly stimulate diabetogenic T cells.

Third is the cytokine balance model. Hanson *et al.* (19) proposed that E molecule-mediated Th1/Th2 cytokine imbalance is one possible mechanism for the disease protection in $E\alpha^d$-transgenic NOD mice. Indeed, there is evidence that the differential MHC class II expression on antigen-presenting cells mediated by class II promoter polymorphism exerts a codominant effect on the Th1/Th2 cytokine balance (54, 55). In our preliminary studies, however, there was no clear difference in the potential of anti-CD3-stimulated T cells to produce IL-4 and IFN-γ between E^+ and E^- $A^{d/d}$ F_1 mice (data not shown).

An alternative is the signal transducer competition model, in which B cells may be the major cellular sites of E molecule-mediated autoimmune protection. Lang *et al.* (56) reported that antigen stimulation by means of the B cell antigen receptor on resting B cells induces association of MHC class II molecules with B cell antigen receptor-derived Ig-α/Ig-β heterodimers, which function as signal transducers on class II aggregation by the TCR. Because both A and E molecules physiologically associate with Ig-α/β heterodimers (56), when self-reactive B cells were stimulated by A-restricted T helper cells plus self-peptides, E molecules possibly associate competitively with Ig-α/β heterodimers. Hence, activation signals should be lower in E^+ than in E^- B cells. This idea well explains the observed dose dependency of E-mediated protection of SLE seen in the present studies.

Wild-type H-$2^{d/z}$ F_1 mice develop severe SLE, although they do express intact E molecules. This finding is in striking contrast to findings in H-$2^{d/d}$ F_1 mice that develop the disease only when lacking E molecules. Two mechanisms are thought to be involved. First, compared with H-$2^{d/d}$ F_1, the self-antigen-presenting capacity of DCs in H-$2^{d/z}$ F_1 is much higher, so that effects of E molecules may be insufficient for suppression. Alternatively, although not mutually exclusive, generation of H-$2^{d/z}$ F_1-unique self-reactive T cells restricted to haplotype-mismatched $A\alpha/\beta$ heterodimers in the thymus, as discussed above, may play a role in an E molecule-independent manner.

Taken collectively, our mouse models may provide means for further clarification of molecular mechanisms involved in the positive and negative regulation of autoimmune disease mediated by class II A and E molecules. Studies on the suppressive effect of E molecules are of particular importance, because one can apply this knowledge to future prophylactic and therapeutic approaches to autoimmune diseases.

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Suppression of T Cell Responses by Chondromodulin I, a Cartilage-Derived Angiogenesis Inhibitory Factor

Therapeutic Potential in Rheumatoid Arthritis

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Objective. **Chondromodulin I (ChM-I), a cartilage matrix protein, promotes the growth and proteoglycan synthesis of chondrocytes. However, it also inhibits angiogenesis. Since ChM-I is expressed not only in cartilage, but also in the thymus, we investigated the modulation of T cell function by ChM-I to assess its therapeutic potential in rheumatoid arthritis (RA).**

Methods. **The localization of ChM-I expression in mouse thymus tissue was examined by in situ hybridization. The proliferative response of peripheral blood T cells and synovial cells obtained from patients with RA was evaluated by ^{3}H-thymidine incorporation assay. The effects of ChM-I were examined using recombinant human ChM-I (rHuChM-I). Modulation of the antigen-specific immune response was evaluated by the recall response of splenic T cells and the delayed-type hypersensitivity response induced in the ear of mice primed with ovalbumin (OVA). Antigen-induced arthritis (AIA) was induced in mice by injecting methylated bovine serum albumin into the ankle joints 2 weeks after the priming.**

Results. **ChM-I was expressed in the cortex of the thymus. Recombinant human ChM-I suppressed the proliferative response of mouse splenic T cells and human peripheral blood T cells stimulated with anti-CD3/CD28 antibodies, in a dose-dependent manner. Production of interleukin-2 was decreased in rHuChM-I–treated mouse CD4 T cells. Ten micrograms of rHuChM-I injected intraperitoneally into OVA-primed mice suppressed the induction of the antigen-specific immune response. Finally, rHuChM-I suppressed the development of AIA, and also suppressed the proliferation of synovial cells prepared from the joints of patients with RA.**

Conclusion. **These results suggest that ChM-I suppresses T cell responses and synovial cell proliferation, implying that this cartilage matrix protein has a therapeutic potential in RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease in which massive synovial cell proliferation with leukocyte infiltration and abnormal capillary growth lead to the development of pannus and occasionally to disability due to the destruction of joints and bones. It has been suggested that T cells contribute to the pathogenesis of RA (1) on the basis of the massive infiltration of T cells into the synovial tissues (2), the oligoclonal expansion of T cells in the synovial fluid and synovial tissue (3–6), and the association between RA and particular HLA alleles (7,8). It has been proposed that these clonally expanded T cells play a role in disease pathogenesis by recognizing some

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arthritic antigens or by supporting synovial inflammation (3–6,9).

Since the formation of new blood vessels is one of the earliest histopathologic findings in RA and appears to be required for pannus development (10,11), it has been proposed that RA might be categorized as an "angiogenic disease." Since extension and flexion movements increase intraarticular pressure and collapse the capillaries, hypoxia and acidosis are induced in inflamed joints. The persistent growth of the synovial mass exceeds neovascularization, resulting in local ischemia (12). These metabolic demands and the decreased oxygen supply stimulate the production of angiogenic inducers, i.e., cytokines and growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, tumor necrosis factor α, interleukin-8 (IL-8), and vascular cell adhesion molecule 1 (13).

It has therefore been proposed that inhibition of angiogenesis might be a therapeutic strategy in the treatment of RA (10). In fact, it has been demonstrated that treatment with several angiogenesis inhibitors such as AGM-1470, which is a cyclic peptide antagonist of integrin $\alpha v\beta 3$ and anti-Flt1, and gene delivery using angiostatin or endostatin ameliorated arthritis in experimental animal models including collagen-induced arthritis (CIA), adjuvant arthritis, and antigen-induced arthritis (AIA) (14–19).

Increasing attention has been paid to chondroprotective and chondroregenerative treatment of arthritis, since it is known that cartilage does not spontaneously regenerate. During the progression of arthritis, cartilage has been shown to be damaged by the invasion of pannus from the synovium–cartilage junction, by degradation of the cartilage matrix by IL-1, metalloproteinases, and other factors, and by apoptosis of chondrocytes (20). Moreover, bony erosion sometimes progresses without any obvious arthritic inflammation. Numerous factors have been reported to promote chondrogenesis, and a therapy that combines these factors with antiinflammatory or immunosuppressive agents has been proposed recently (21).

We previously identified chondromodulin I (ChM-I) as an angiogenesis inhibitor (22). ChM-I is a 25-kd glycoprotein originally purified from bovine epiphyseal cartilage on the basis of its promotion of chondrocyte growth (23). Both ChM-I protein and ChM-I messenger RNA are richly expressed in cartilage. ChM-I has been shown to stimulate the growth, proteoglycan synthesis, and colony formation of cultured chondrocytes (24). However, it has also been shown to inhibit DNA synthesis, the proliferation of vascular endothelial cells, tube morphogenesis, and chorioallantoic membrane angiogenesis, thereby demonstrating its angiostatic ability (22,25,26). As confirmation of this ability, ChM-I has been shown to suppress chondrosarcoma growth via angiogenesis inhibition in vivo (27). Therefore, ChM-I is thought to participate in the angiogenic switching of cartilage by deterring vascular invasion (22,25).

During the biologic characterization of ChM-I, our Northern blotting analysis revealed ChM-I expression not only in cartilage, but also in the thymus, suggesting a correlation of ChM-I with T cell function (26). In the present study, we found that recombinant human ChM-I (rHuChM-I) suppressed the T cell proliferative response. In addition, rHuChM-I was able to inhibit the proliferation of synovial cells. Finally, rHuChM-I was able to reduce the severity of AIA. ChM-I therefore appears to act beneficially in the treatment of arthritis in 4 ways: protection of chondrocytes, inhibition of angiogenesis, prevention of synovial cell proliferation, and suppression of the immune system.

MATERIALS AND METHODS

Mice. BALB/c mice and DBA/1 mice were obtained from SLC (Shizuoka, Japan) and Charles River (Tokyo, Japan), respectively. DO11.10 transgenic mice whose T cells express a receptor specific for ovalbumin (OVA) peptide 323–339 (28) were kindly provided by Dr. T. Watanabe (Medical Institute of Bioregulation, Kyushu University, Japan). The mice were maintained in a temperature- and light-controlled environment with free access to food and water under specific pathogen–free conditions. Female age-matched BALB/c and DO11.10 mice and male DBA/1 mice were used in the respective experiments, and all mice were 7–10 weeks old at the start of each experiment.

Cell lines. RAW264.7 cells were kindly provided by Dr. Takayanagi (Department of Immunology, University of Tokyo, Japan). J558L and WEHI-231 cells were kindly provided by Dr. Tsubata (Medical Research Institute, Tokyo Medical and Dental University, Japan), and Jurkat cells were purchased from Riken Bioresource Center (Tsukuba, Ibaraki, Japan).

Preparation of rHuChM-I. Recombinant human ChM-I was prepared as described previously (26). Briefly, we subcloned the coding region for the human ChM-I precursor protein into a pcDNA3 expression vector, repetitively transfected the resulting vector into CHO cells, and then selected the drug-resistant clone. Our preliminary experiment indicated that the recovered rHuChM-I molecules were eluted in the aggregated forms with an apparent molecular size of >200 kd, which requires reduction with β-mercaptoethanol in the presence of 6*M* urea for dissociation. Therefore, the culture supernatant was first loaded on a butyl-cellulofine column, which was then eluted by 6*M* urea. The eluted materials were reduced by β-mercaptoethanol at a final concentration of 1 m*M*. Contaminant proteins were eliminated by successive chromatography on QAE-toyopearl, butyl-toyopearl, and

sulfate-cellulofine columns. The purified rHuChM-1 was confirmed to have the same biologic activity as the native bovine ChM-1 on chondrocytes and endothelial cells (26).

RNA in situ hybridization. To synthesize the digoxigenin-labeled riboprobes, a 0.5-kb polymerase chain reaction fragment of ChM-I complementary DNA (627–1,163 bp) was inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA). Linearized DNA was transcribed using T7 and SP6 polymerases. Thymus tissue was dissected from a 4-week-old male BALB/c mouse. Tissue was embedded in paraffin, sectioned at 7 μm thickness, and collected on silane-coated glass slides (Matsunami, Osaka, Japan). After deparaffinization with xylene, rehydration, and rinsing with 0.1*M* phosphate buffer, sections were treated with proteinase K (10 μg/ml) in Tris–EDTA at room temperature for 10 minutes, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and then treated with 0.2*M* HCl for 10 minutes. Acetylation of the sections was performed by incubation for 10 minutes with 0.1*M* triethanolamine–HCl, pH 8.0, and 0.25% acetic anhydrate for 10 minutes.

A hybridization mixture (50% formamide, 10 m*M* Tris–HCl, pH 7.5, 200 μg/ml transfer RNA, 1× Denhardt's solution, 10% dextran sulfate, 600 m*M* NaCl, 0.25% sodium dodecyl sulfate, 1 m*M* EDTA, pH 8.0) was preheated for 10 minutes at 85°C. Ten micrograms of the sense or antisense RNA probe was added to the hybridization mixture and denatured by heating at 85°C for 3 minutes, and then applied to the sections. Hybridization was performed overnight at 50°C. After hybridization, sections were washed with 50% formamide in 2× saline–sodium citrate at 55°C for 30 minutes and treated with a solution of 10 m*M* Tris–HCl, pH 7.5, 0.5*M* NaCl, and 1 m*M* EDTA (TNE) at 37°C. Nonspecific bindings of the probes were reduced by RNase A treatment (10 μg/ml in TNE) at 37°C for 30 minutes. Hybridization signals were visualized by using nitroblue tetrazolium salt and BCIP. The sections were counterstained with methyl green.

Lymphocyte proliferation assay. Naive T and B cells were purified with a magnetic cell sorting system (Miltenyi Biotech, Bergisch Gladbach, Germany), as previously described (29,30). Naive T cells were stimulated with 1 μg/ml of anti-CD3 antibody and 1 μg/ml of anti-CD28 antibody in the presence of various concentrations of rHuChM-I (from 0 to 1 μ*M*) in RPMI 1640 medium supplemented with 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum for 24, 48, or 72 hours. Naive B cells were stimulated with 10 μg/ml of lipopolysaccharide (LPS) in the presence of various concentrations of rHuChM-I (from 0 to 1 μ*M*) for 24 hours. Naive OVA T cell receptor transgenic mouse DO11.10 T cells were cultured at 1×10^5 cells/well with irradiated antigen-presenting cells, various concentrations of OVA peptide (0.01, 0.1, and 1 μ*M*), and various concentrations of rHuChM-I (0, 1, 10, and 100 n*M*) for 24, 48, or 72 hours. This procedure was followed by a final 4 hours of culture in the presence of 1 μCi of ^{3}H-thymidine per well.

In some experiments, media without 2-mercaptoethanol contained either E-64 protease inhibitor (100 n*M*; Calbiochem, La Jolla, CA), iodoacetamide (50 n*M*; Sigma, St. Louis, MO), or *N*-ethylmaleimide (50 n*M*; Sigma) (31,32). The incorporated radioactivity was counted with a β-scintillation counter. The proliferative response was expressed as the mean ± SD counts per minute of test cultures.

Human peripheral blood T cell and synovial cell proliferation assays. Human peripheral blood T cells obtained from healthy volunteers were selected by lymphoprep (Axis Shield, Oslo, Norway) and stimulated with human anti-CD3 antibodies (0.001, 0.01, and 0.1 μg/ml) in the presence of rHuChM-I (0, 1, 10, and 100 n*M*) for 24 hours. Synovial cells were obtained from the joints of RA patients, who gave their informed consent, before undergoing total knee arthroplasty or total hip replacement. Synovial cells (1×10^4 cells per well), within 4 passages of culture (33), were seeded in culture plates with various concentrations of rHuChM-I (0, 10, 30, 100, and 300 n*M*) and cultured for 5 days. This procedure was followed by a final 16 hours of culture in the presence of 1 μCi of ^{3}H-thymidine per well. The cells were detached with 50 μl of 0.25% trypsin–0.2% EDTA, and harvested onto glass-fiber filters. The incorporation of ^{3}H-thymidine was measured by scintillation counting.

Naive T cells viability assay. Mouse naive T cells (1×10^6 cells per well) were cultured with various concentrations of rHuChM-I (0–1 μ*M*) for 24 hours. Viable cells were counted by trypan blue exclusion.

Evaluation of IL-2 production. The concentration of IL-2 was determined in the supernatant from mouse CD4+ T cells or CD8+ T cells. These T cells were activated with immobilized anti-CD3 (1 μg/ml) + anti-CD28 (1 μg/ml) for 24 hours, and the IL-2 concentration was determined by sandwich enzyme-linked immunosorbent assay (Genzyme, Cambridge, MA).

Assessment of delayed-type hypersensitivity (DTH). The evaluation of the DTH response was based on the degree of ear swelling. BALB/c mice were immunized with 100 μg of OVA in Freund's complete adjuvant (CFA) with or without a concomitant intraperitoneal injection of rHuChM-I. DTH was induced by an injection of 200 μg of OVA into the left ear pinnae of the mice 14 days after the priming. The right ear served as an untreated control. Both ear pinnae were measured immediately before the injection and 24 hours later with a dial-gauge caliper (Mitsutoyo, Kawasaki, Japan). The measurements were performed in triplicate.

Synovial cells viability assay. Synovial cells (1×10^5 cells per well) were cultured with various concentrations of rHuChM-I (0–1 μ*M*) for 5 days. Viable cells were counted by trypan blue exclusion. Each experiment was performed in triplicate.

MTT assay of synovial cells. MTT is a substrate that is cleaved by living cells. Since this process requires active mitochondria and even freshly dead cells do not cleave significant amounts of MTT, this colorimetric assay is able to determine the amount of live cells (34,35). Therefore, to evaluate the proliferation of synovial cells, we conducted an MTT assay according to the manufacturer's protocol (Chemicon, Temecula, CA). Briefly, synovial cells were seeded in a 96-well microtiter plate (1×10^4 cells/well) and were incubated in the growth medium in the presence or absence of rHuChM-I for 5 days. Four hours before the termination of culture, MTT (5 mg/ml) was added to each well. At the end of the incubation, 100 μl of isoproanol was added to each culture to dissolve the formazan complex. The optical density at 590 nm was measured using a 96-well multiscanner. Each experiment was performed in triplicate.

Induction of AIA. BALB/c mice were injected intradermally with 100 μg of methylated bovine serum albumin (mBSA) in CFA at the base of the tail on day 0. Mice received 10 μg of rHuChM-I in PBS on day 0 (for the single-injection protocol) or day 0 to day 3 (for the 3-consecutive-days delivery protocol), and control mice received PBS alone on day 0 or day 0 to day 3. Fourteen days later, 20 μg mBSA dissolved in 20 μl of PBS was injected intraarticularly into the left ankle joint. The right ankle joint was injected with 20 μl of PBS alone as a negative control. The joint thickness was measured with a dial gauge caliper, and the net increase in thickness was calculated (30,36).

Induction of CIA and treatment with rHuChM-I. Male DBA/1 mice were injected intradermally with 100 μg of bovine type II collagen (BII; Chondrex, Redmond, WA) in CFA (Difco, Detroit, MI) at the base of the tail on day 0. A booster was administered on day 21. The mice were injected intraperitoneally with 10 μg of rHuChM-I dissolved in PBS on day 0 (for the single-injection protocol) or from day 0 to day 3 (for the 3-consecutive-days delivery protocol). Control mice were injected with PBS alone on day 0 or from day 0 to day 3, and signs of arthritis appeared at around days 25–28, which is consistent with the findings in previous reports (37–40).

Assessment of CIA. Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or other parts of the paws. Arthritis was scored using the following scale: 0 = no change; 1 = redness or mild inflammation; 2 = swelling or inflammation; 3 = severe swelling or severe inflammation; 4 = ankylosis (41). The scoring was done by 2 independent observers.

Histologic examination. Ankles and knees were fixed in 10% phosphate-buffered formalin and decalcified. Tissues were then dehydrated in a gradient of alcohol, and then paraffin-embedded, sectioned, mounted on glass slides, and stained with hematoxylin and eosin (30,36). The histopathologic arthritis score of AIA was quantified according to the method of Brackertz et al (42), based on the degree of synovial hypertrophy, mononuclear cell infiltration, and pannus formation. Each section was studied by 3 blinded examiners in the AIA experiment.

The histopathologic arthritis score of CIA was assessed according to the method of Hietala et al (43), based on the degree of synovial hypertrophy, cartilage destruction, and pannus formation. Each section was studied by 2 blinded examiners in the CIA experiment. The average scores of each parameter from 4 joints (rear ankles and knees) in each mouse were calculated.

Statistical analysis. Statistical significance was determined by the Student's unpaired t-test. P values of less than 0.05 were considered to indicate a statistically significant difference. Results are reported as the mean ± SD.

RESULTS

ChM-I expression in the cortex of the thymus. When we previously examined the tissue distribution of ChM-I in DDY mice by Northern blot analysis, we found that ChM-I is expressed not only in cartilage, but also in the thymus and the eye (26). In order to further verify the ChM-I expression in the thymus, we performed in situ hybridization using BALB/c mice in addition to DDY mice. We found that ChM-I is expressed in the cortex, but not in the medulla (Figure 1). The ChM-I–expressing cells seemed to be thymic stromal cells.

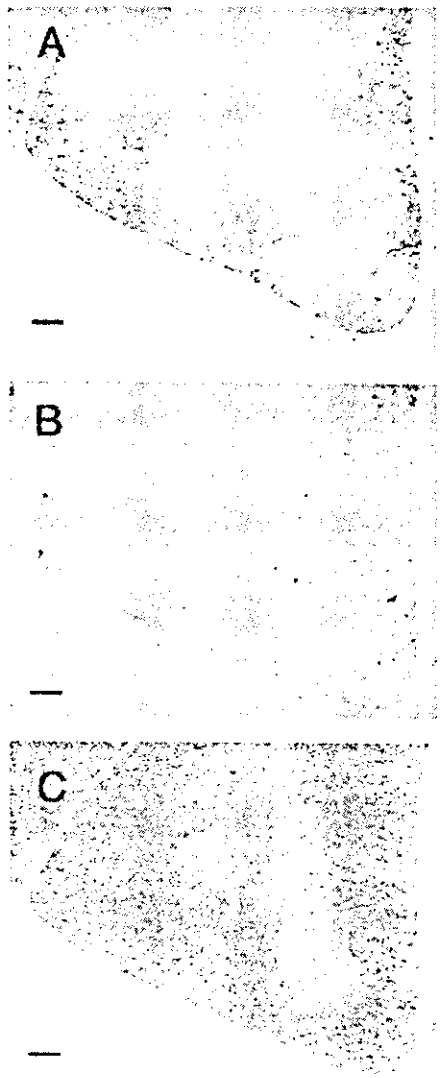

Figure 1. Expression of chondromodulin I (ChM-I) mRNA in the cortex of the thymus of 4-week-old mice. **A**, In this thymus section, which was hybridized with the antisense ChM-I cRNA probe, there are obvious hybridization signals in the cortex. **B**, In this semiserial section, which was hybridized with the sense probe as a control, no signal was detected. The sections were counterstained with methyl green. **C**, The cortex of mouse thymus tissue is purple stained with hematoxylin and eosin. Bar = 100 μm.

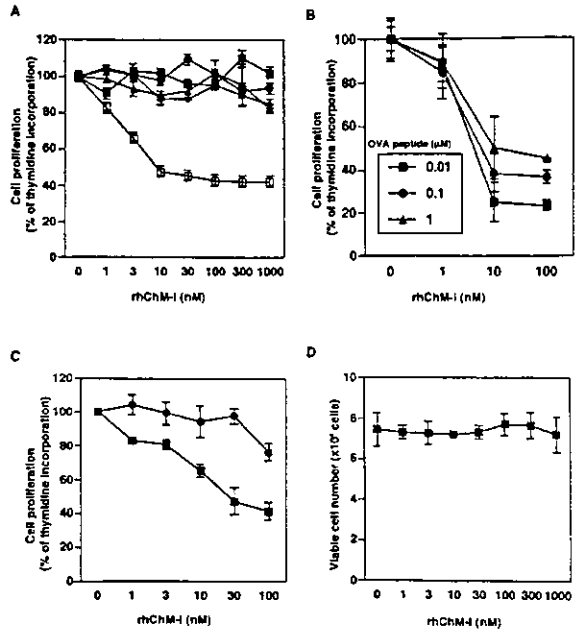

Figure 2. Suppression of the T cell proliferative response in vitro by recombinant human chondromodulin I (rhChM-I). A, Mouse splenic T cells (1×10^5/well) (□) were stimulated with immobilized anti-CD3 (1 μg/ml) + anti-CD28 (10 μg/ml) in the presence of varying concentrations (0.1–1,000 n*M*) of rhChM-I, and cultured for 24 hours. As a reference, the growth, in the presence of various concentrations of rhChM-I, of various cell sources derived from mouse blood cells is shown: 5×10^4 cells/well of the RAW264.7 mouse macrophage-derived cell line (■), J558L mouse myeloma cell line (▲), and WEHI-231 mouse lymphoma cell line (◆), as well as 10^5 cells/well of mouse splenic B cells stimulated with lipopolysaccharide (●). B, Splenic DO11.10 T cells were stimulated with ovalbumin (OVA) peptide and irradiated antigen-presenting cells in the presence of various concentrations of rhChM-I. C, Human peripheral blood T cells (■) were purified by lymphoprep and stimulated with human anti-CD3 antibodies (0.1 μg/ml) in the presence of rhChM-I (1, 3, 10, 30, and 100 n*M*) for 24 hours. As a reference, the growth, in the presence of rhChM-I, of the Jurkat human T lymphocyte cell line (●) is shown. Bars in A–C show the mean ± SD ^{3}H-thymidine incorporation as a proportion of that in the absence of rhChM-I. D, To demonstrate lack of toxicity of rhChM-I, mouse T cells were cultured in the absence or presence of various concentrations of rhChM-I. Bars show the mean ± SD number of live cells.

Suppression of the T cell proliferative response by rHuChM-I. The thymic expression of ChM-I suggested that ChM-I might be associated with the development or function of T cells. Therefore, we examined the possibility that rHuChM-I modifies the T cell immune response. As shown in Figure 2A, rHuChM-I suppressed the proliferative response of mouse T cells stimulated with anti-CD3 + anti-CD28 antibodies. Similarly, rHuChM-I suppressed the antigen-specific proliferation of OVA-stimulated T cells (Figure 2B). These inhibitions of T cell proliferative response occurred in a dose-dependent manner, and the maximum inhibition of 76.5% was obtained at an rHuChM-I concentration of 100 n*M*. This suppressive effect was not due to the toxicity of rHuChM-I, since incubation with variable amounts of rHuChM-I did not alter the number of live T cells (Figure 2D).

The proliferation of human peripheral blood T cells was also inhibited by rHuChM-I in a dose-dependent manner (Figure 2C). The dose–response curves revealed that the dose required for 50% inhibition (ID_{50}) of the T cell proliferative response was ~3 n*M* for mouse T cells and ~10 n*M* for human T cells (see Figures 2A and C). These ID_{50} values for mouse and human T cells are fairly consistent with our previous observation that the ID_{50} of endothelial cell proliferation was almost 8 n*M* (22). Since mouse and human ChM-I are 87% similar in their amino acid sequences, it is possible that human ChM-I can bind the receptor for mouse ChM-I with almost the same affinity.

Since ChM-I inhibits the spontaneous growth of endothelial cells, we examined the possibility that ChM-I is a general inhibitor of growth. As shown in Figure 2A, rHuChM-I did not inhibit the spontaneous growth of the RAW264.7 mouse macrophage-derived cell line, J558L mouse myeloma cell line, or WEHI-231 mouse lymphoma cell line, and it did not inhibit the proliferation of mouse splenic B cells stimulated with LPS. Moreover, rHuChM-I did not inhibit the spontaneous proliferation of the Jurkat human T lymphocyte line, although at higher doses, partial inhibition did occur (Figure 2C). Considering that the ID_{50} is ~3 n*M* for mouse T cells, ~10 n*M* for human T cells, and almost 8 n*M* for endothelial cells, the dose needed to inhibit Jurkat proliferation was extremely high, implying that the suppressive mechanism in Jurkat cells might be different. These results indicate that rHuChM-I is not a general growth inhibitor and that T cell proliferation is one of the selective targets of rHuChM-I.

Furthermore, IL-2 production in the supernatant was significantly decreased by rHuChM-I in CD4+ T cells, but not in CD8+ T cells (Figure 3). This result indicates that the inhibitory mechanism of T cell proliferation involves, at least in part, the suppression of IL-2 production in CD4 T cells, and again supports the idea that the biologic effect of rHuChM-I is specific to certain cell types.

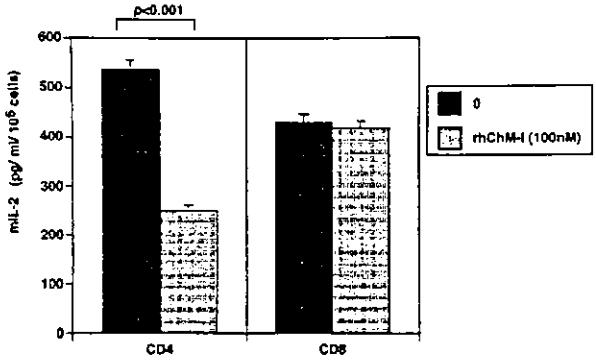

Figure 3. Reduction by rhChM-I of interleukin-2 (mIL-2) production from mouse CD4+ T cells. Levels of IL-2 in the supernatant of either CD4+ or CD8+ mouse splenic T cells stimulated with anti-CD3 + anti-CD28 antibodies in the presence of 100 n*M* of rhChM-I were evaluated by enzyme-linked immunosorbent assay. The IL-2 concentrations were normalized to the number of live T cells. Bars show the mean and SD IL-2 production from 10^6 T cells. See Figure 2 for other definitions.

Suppression of the antigen-specific immune response in vivo by rHuChM-I. To confirm that rHuChM-I is able to suppress an antigen-specific immune response in vivo, we immunized mice with a nominal antigen, OVA. Splenic T cells from mice primed with OVA exhibited a decreased recall response to OVA in vitro when they were injected with rHuChM-I at the time of their priming (Figure 4A). Ear swelling, which was induced by OVA injection into the ear of mice primed with OVA, was diminished in a dose-dependent manner (Figure 4B) in the mice treated with rHuChM-I in comparison with untreated control mice. Since the background level of ^{3}H-thymidine incorporation was not significantly altered between the rHuChM-I–injected mice and the control mice, rHuChM-I seems to suppress the immune response to the primed antigen preferentially. These results indicate that ChM-I suppressed the immune response to the antigen in vivo.

Duration of effect of rHuChM-I on the T cell proliferative response. The suppressive activity of rHuChM-I on T cells began to diminish by 48 hours, and it was completely abrogated by 72 hours in the in vitro culture experiments (Figure 5). When we repeatedly added rHuChM-I every 24 hours, the suppression lasted for at least 4 days. Recently, it was reported that plasma contains a reductase that can reduce disulfide bonds in proteins and reduce the average size of von Willebrand factor secreted by endothelial cells (31,32). Since ChM-I contains 4 intramolecular disulfide bonds, a feature that is assumed to be critical for its activity (22,25), we assumed that the short duration of rHuChM-I activity might be due to a reduction of disulfide bonds by some molecules contained in the culture. To verify this hypothesis, we examined the kinetics of the suppressive activity of rHuChM-I on T cells in the presence of reductase inhibitors. Although the reductase inhibitors were not toxic on T cells, rHuChM-I was able to retain its suppressive activity for 72 hours in the presence of reductase inhibitors (Figure 5). These results suggest that the short duration of the rHuChM-I suppressive activity in vitro might have been due to reductase in the culture.

Suppression of the proliferation of synovial cells by rHuChM-I. Since our studies of ChM-I have consistently revealed its potential to ameliorate arthritis, we decided to further examine the effect of ChM-I on synovial cell proliferation, which must be controlled to treat RA. As expected, the incorporation of ^{3}H-thymidine into synovial cells prepared from RA joints decreased in the presence of rHuChM-I. The maximal

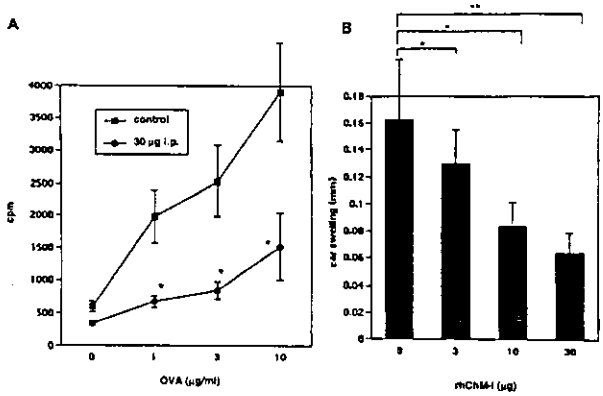

Figure 4. Suppression of the T cell response in vivo by rhChM-I. **A,** In splenocytes primed with OVA, rhChM-I reduced the recall response against OVA. BALB/c mice were immunized with OVA and intraperitoneally (i.p.) injected with rhChM-I at the time of OVA immunization. The secondary proliferative response of the splenocytes was examined 14 days later, by culturing for 72 hours with various concentrations of OVA (1, 3, and 10 μg/ml). **B,** The delayed-type hypersensitivity response, evaluated by ear swelling, was suppressed by rhChM-I. Fourteen days after the immunization, 200 μg of OVA was injected into the left ear pinnae of the mice. The right ear served as an untreated control. Both ear pinnae were measured immediately before and 24 hours after the injection. Bars show the mean ± SD of 5 mice per group. * = $P < 0.05$; ** = $P < 0.01$. See Figure 2 for other definitions.

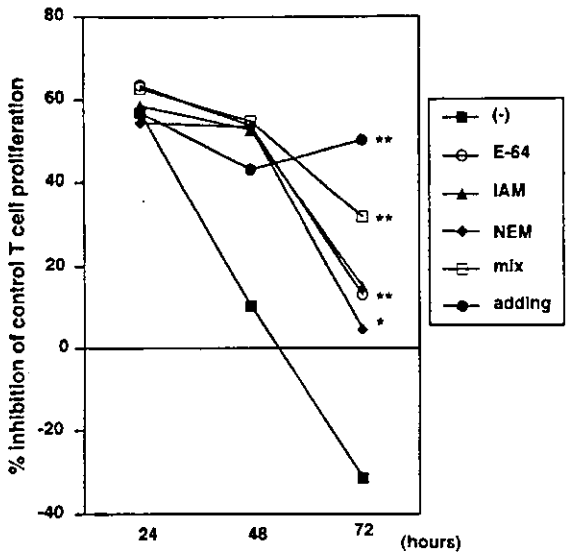

Figure 5. Preservation of the suppressive effect of recombinant human chondromodulin I (rHuChM-I) on the T cell response up to 72 hours by sequential addition of rHuChM-I or by the presence of reductase inhibitors. Splenic T cells (1×10^5/well) were plated in 96-well plates with 1 μg/ml of anti-CD3 + 10 μg/ml of anti-CD28. The rHuChM-I was added every 24 hours (adding). Either E-64, iodoacetamide (IAM), or *N*-ethylmaleimide (NEM) was added separately or mixed (E-64 + IAM + NEM) (mix) at the beginning of the culture. * = $P < 0.05$; ** = $P < 0.01$.

inhibition was 48% at 100 n*M* of rHuChM-I (Figure 6A). In order to discern whether the suppression of ^{3}H-thymidine incorporation was simply due to cytotoxicity of rHuChM-I, we conducted additional studies involving direct counting of the live cells and an MTT assay that is able to determine the amount of live cells. Both studies confirmed that rHuChM-I suppressed the proliferation of synovial cells (Figures 6B and C) and that the decreased ^{3}H-thymidine incorporation did not simply reflect the decreased cell number due to rHuChM-I cytotoxicity, because the number of cells after the culture increased compared with that at the start of culture, even at the 1 μ*M* concentration of rHuChM-I.

Suppression of the development of AIA by rHuChM-I. We next examined whether rHuChM-I is able to suppress the induction of experimental arthritis. We primed BALB/c mice with mBSA so that they would develop AIA after intraarticular injection of mBSA, and evaluated the severity of arthritis using the maximum hind-paw thickness. We injected rHuChM-I intraperitoneally at the time of the priming. The rHuChM-I significantly suppressed the development of AIA. In addition, when rHuChM-I was delivered to the mice for 3 consecutive days, the development of the arthritis was markedly suppressed (Figure 7A). Histologic examination of the ankle joints revealed that the number of inflammatory cells invaded into periarticular soft tissues and bone marrow in the tarsus was reduced in the rHuChM-I–treated mice in comparison with the control mice (Figures 7C and D). The evaluation of histopathologic severity revealed a significant amelioration by rHuChM-I treatment ($P < 0.001$) (Figure 7B). These

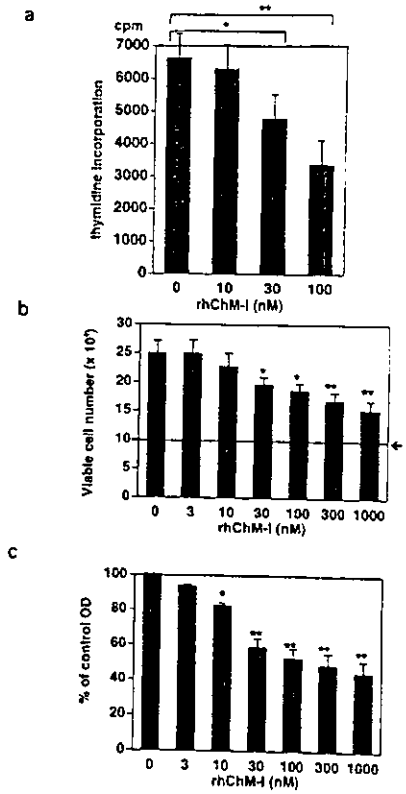

Figure 6. Reduction of rheumatoid arthritis (RA) synovial cell proliferation by recombinant human chondromodulin I (rhChM-I). a, Synovial cells (1×10^4) from RA patients were plated in 96-well plates and incubated with rhChM-I (10, 30, and 100 n*M*) for 5 days, and the proliferative response was measured by ^{3}H-thymidine incorporation. b, Synovial cells (1×10^5) from RA patients were plated in 24-well plates and incubated with rhChM-I (3, 10, 30, 100, 300, and 1,000 n*M*) for 5 days, and viable cells were counted by trypan blue exclusion. The arrow denoting the horizontal line indicates the initial number of cells at culture start (1×10^5 cells). c, Synovial cell proliferation was determined using MTT assay. Results are expressed as the percentage of the values detected in cells in the absence of rhChM-I. OD = optical density. Bars show the mean and SD. * = $P < 0.05$; ** = $P < 0.01$.

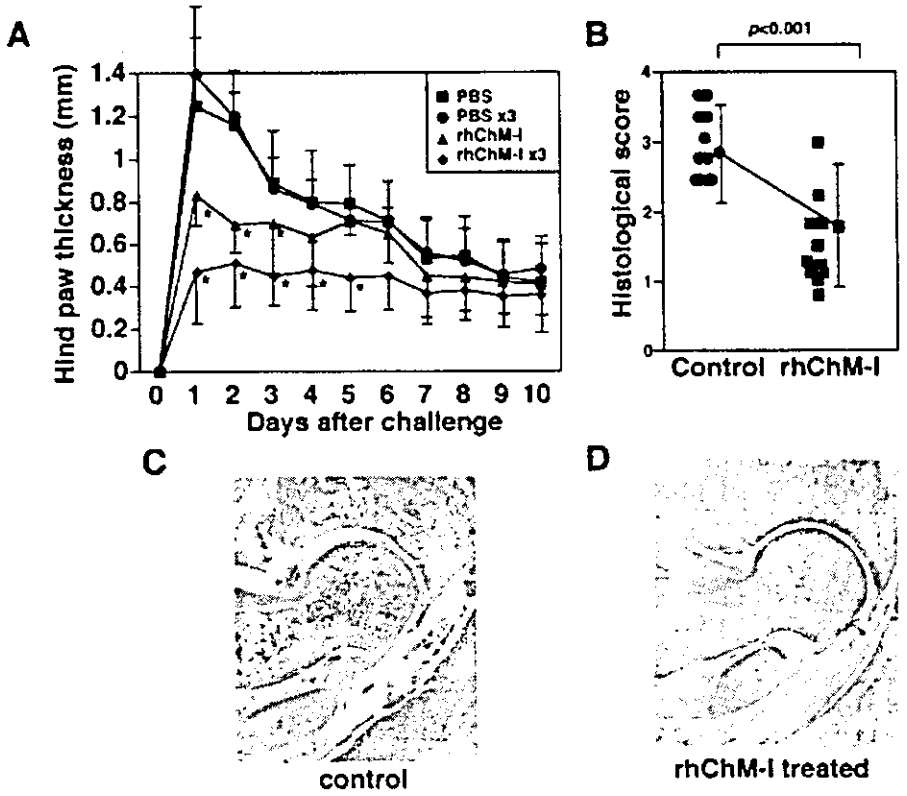

Figure 7. Suppression of the development of antigen-induced arthritis (AIA) by rhChM-I. A, For induction of AIA, BALB/c mice were immunized with 100 μg of methylated bovine serum albumin in Freund's complete adjuvant at the base of the tail. Ten micrograms of rhChM-I was intraperitoneally injected once on the same day or on 3 consecutive days (×3). The control mice received phosphate buffered saline (PBS) alone. The primed mice were challenged intraarticularly with the antigen on day 0. Bars show the mean ± SD increase in hind-paw thickness during the course of the disease (n = 10 per group). B, Histologic examination of the ankle joints. The histopathologic arthritis score for AIA was assessed by 3 blinded examiners as the extent of synovial hypertrophy, mononuclear cell infiltration, and pannus formation. C and D, Massive cell infiltration in the control AIA mice was ameliorated in the rhChM-I–treated AIA mice, respectively. See Figure 6 for other definitions.

results confirmed that rHuChM-I is able to modulate AIA.

Reduction of incidence of arthritis in CIA by rHuChM-I. In order to further evaluate the effect of ChM-I on arthritis, we also investigated its ability to suppress CIA. We injected 10 μg of rHuChM-I (or PBS for the control) intraperitoneally when we immunized the mice with BII. While all the control mice treated with PBS fully developed CIA, only 60% of the mice receiving a single injection of 10 μg rHuChM-I developed the disease (Figure 8A); however, this reduction was not statistically significant. The incidence of CIA significantly decreased to 50% in the mice receiving rHuChM-I injection for 3 consecutive days ($P < 0.05$). The mean arthritis score in the group of rHuChM-I–treated mice also decreased significantly (Figure 8B). Since the arthritis score in the mice that developed the disease in spite of rHuChM-I delivery eventually increased to the full value, similar to that in the control mice, this reduction might simply reflect a decrease in arthritis development.

The histopathologic examination revealed massive mononuclear cell infiltration and edema in the control mice (Figure 8C), whereas both of these features were suppressed in the rHuChM-I–treated mice (Figure 8D). The grading of histopathologic severity revealed

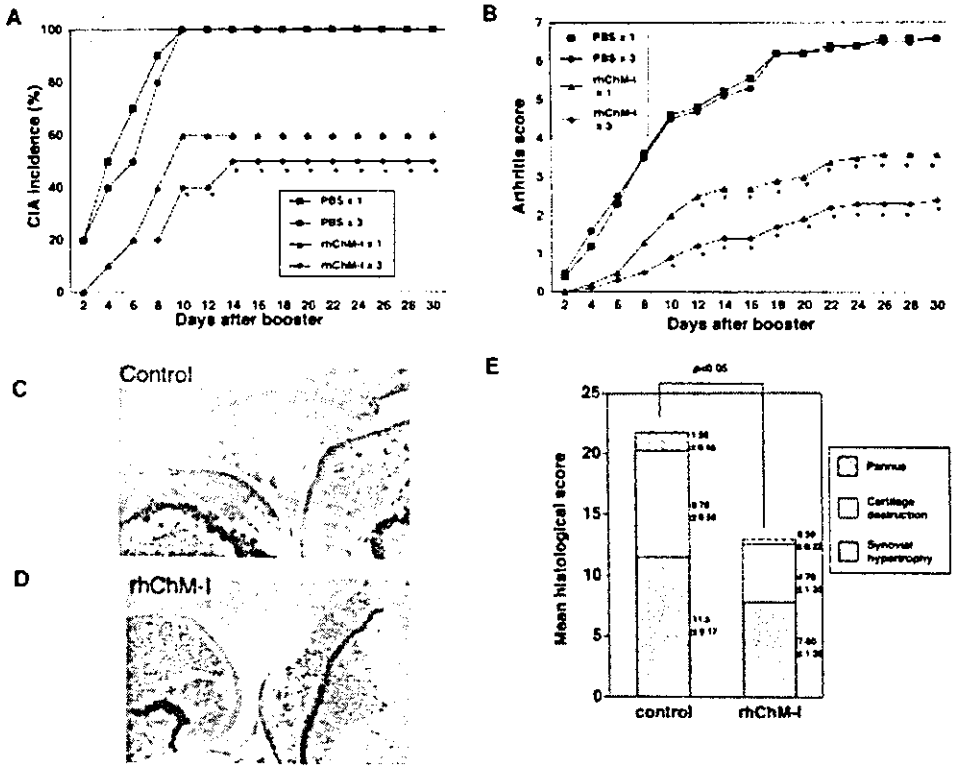

Figure 8. Decreased incidence of collagen-induced arthritis (CIA) by treatment with rhChM-I. A, For induction of CIA, mice were injected intradermally with 100 μg of bovine type II collagen in Freund's complete adjuvant at the base of the tail on day 0. A booster was administered on day 21. Mice received 10 μg of rhChM-I in phosphate buffered saline (PBS) on day 0 or from day 0 to day 3 (×3). Control mice received PBS instead of rhChM-I. Each group consists of 10 mice. B, For the arthritis score of CIA with or without rhChM-I treatment, 2 independent observers scored the ankle joints on a scale from 0 to 4. C and D, For histopathologic analysis, CIA control mice and CIA mice treated with rhChM-I were killed on day 50 and their knee joints were sectioned and stained by hematoxylin and eosin. C, The joints of control mice showed severe inflammation in the synovium and joint space with synovial hyperplasia. D, The joints of rhChM-I–treated mice showed mild inflammation in the synovium. E, To determine the histopathologic severity of arthritis, the histologic arthritis score of CIA was assessed by 2 blinded examiners as the extent of synovial hypertrophy, cartilage destruction, and pannus formation. The scores of each parameter from 4 joints of each mouse were summed. Values are the mean ± SD. * = $P < 0.05$. See Figure 6 for other definitions.

that rHuChM-I treatment significantly prevented the development of CIA ($P < 0.05$) (Figure 8E), but that once the mice developed arthritis in spite of rHuChM-I delivery, the pathology of the arthritic joints was almost the same as in the CIA control mice.

Taken together, these results show that rHuChM-I suppressed the proliferation of both T cells and synovial cells. In addition, rHuChM-I suppressed the development of AIA as well as CIA, although its effect on the latter was partial.

DISCUSSION

This study revealed 2 novel features of ChM-I, namely, that ChM-I suppressed both T cell activation and synovial cell proliferation. These findings combined with our previous findings (that ChM-I promotes chondrocyte growth and inhibits angiogenesis) would suggest a therapeutic potential for ChM-I in arthritis.

The therapeutic effect in CIA was partial, and we were unable to confirm that T cell suppression occurred in our CIA model. We did not observe a significant

decrease in the T cell proliferative response against BII, although the antibody titer against BII in the mice treated for 3 consecutive days was slightly decreased (data not shown). Therefore, we cannot conclude that ChM-I exerted its therapeutic effect on CIA via the suppression of the T cell response.

In contrast to the CIA experiments, rHuChM-I exhibited a distinct suppressive effect on the development of AIA. The prevention of T cell priming in vivo was also confirmed, as shown in Figures 4A and B, indicating that the therapeutic effect of AIA depends on T cell suppression. CIA requires a rather longer time course (almost 40 days) to develop in comparison with AIA. Therefore, we suspect that the short duration of the suppressive activity of rhChM-I on T cell proliferation, as demonstrated in Figure 5, might have been related to this discrepancy of the outcome between AIA and CIA, since the arthritis severity in AIA as well as the arthritis incidence in CIA decreased more significantly in the mice receiving rHuChM-I for 3 consecutive days than in the mice receiving a single injection.

It is possible that some factors expressed or secreted by activated T cells might have been involved in decreasing the rHuChM-I activity, since the suppression of T cells did not last as long as in our previous study using endothelial cells. In addition, this short duration of activity might have prevented us from observing chondrocyte protective and synovial cell growth retardation effects, which require a longer period to examine clearly. In order to dissect those effects, we would have to deliver rHuChM-I more frequently throughout the entire disease course, although at this time we cannot prepare a sufficient amount of rHuChM-I to conduct such a study.

In any case, the current form or protocol of ChM-I delivery might limit its practical use in arthritis in which activated T cells are involved. The development of methods to compensate for the short activity of ChM-I, e.g., the development of a form that is made less susceptible to reduction or joint expression by using adenoviral vector, would provide a new innovative therapy not only for RA, but also for other rheumatic diseases, including osteoarthritis and seronegative spondylarthropathy. In addition, identification of the mechanism of ChM-I activity would help in the development of a more refined therapy.

To date, no molecule derived from bone or joint tissues has been shown to modulate the immune response. Although the primary role of ChM-I must be related to its antiangiogenic activity in cartilage, one other physiologic role of ChM-I might be the control of T cell positive selection. It is interesting to note that the effective dosage of ChM-I is almost the same irrespective of its various biologic outcomes; that is, the dose required for a 50% effect in chondrocyte growth promotion is between 4–8 n*M*, while the suppressive ID_{50} values are almost 8 n*M* for endothelial cells (22) and ~3–10 n*M* for T cells. It seems that the opposite functions in the different cells share a single type of receptor. This interesting phenomenon should stimulate further studies to elucidate its mechanism.

During the inflammatory process or the drastic pressure change caused by joint movement, the molecules released from damaged joint tissues could be presented as antigens by synovial cells or dendritic cells. Once these molecules are recognized by the immune system, the resulting immune response might contribute to the exacerbation or initiation of arthritis. In fact, a number of joint-derived matrix molecules, including type II collagen, BjP, YKL-39, YKL-40, matrilin-1, proteoglycan aggrecan, and p205, have been demonstrated to be the target of autoreactive T cells and to be involved in the pathogenesis of not only RA, but also osteoarthritis and polychondroarthritis (44–50). In addition, since it is known that the autoreactive immune response becomes aggressive as the immunologic determinant spreads (51–53), it would be important to prevent the immune system from recognizing new antigens or additional epitopes. In this context, it is interesting to note that fetal bone is rich in ChM-I and the expression level decreases with age (54), whereas aged cartilage contains very little ChM-I and aging increases the susceptibility to arthritis. It would be interesting to examine whether ChM-I is able to prevent priming of these arthritic antigens under physiologic conditions.

The biologic features of ChM-I not only provide us with a therapeutic strategy, but also contribute new insights into the relationship between the cartilage matrix and the immune system. Future studies will be undertaken to clarify the mechanism or factors that promote the degradation or reduction of ChM-I or the loss of its activity, thereby contributing to the treatment of arthritis.

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