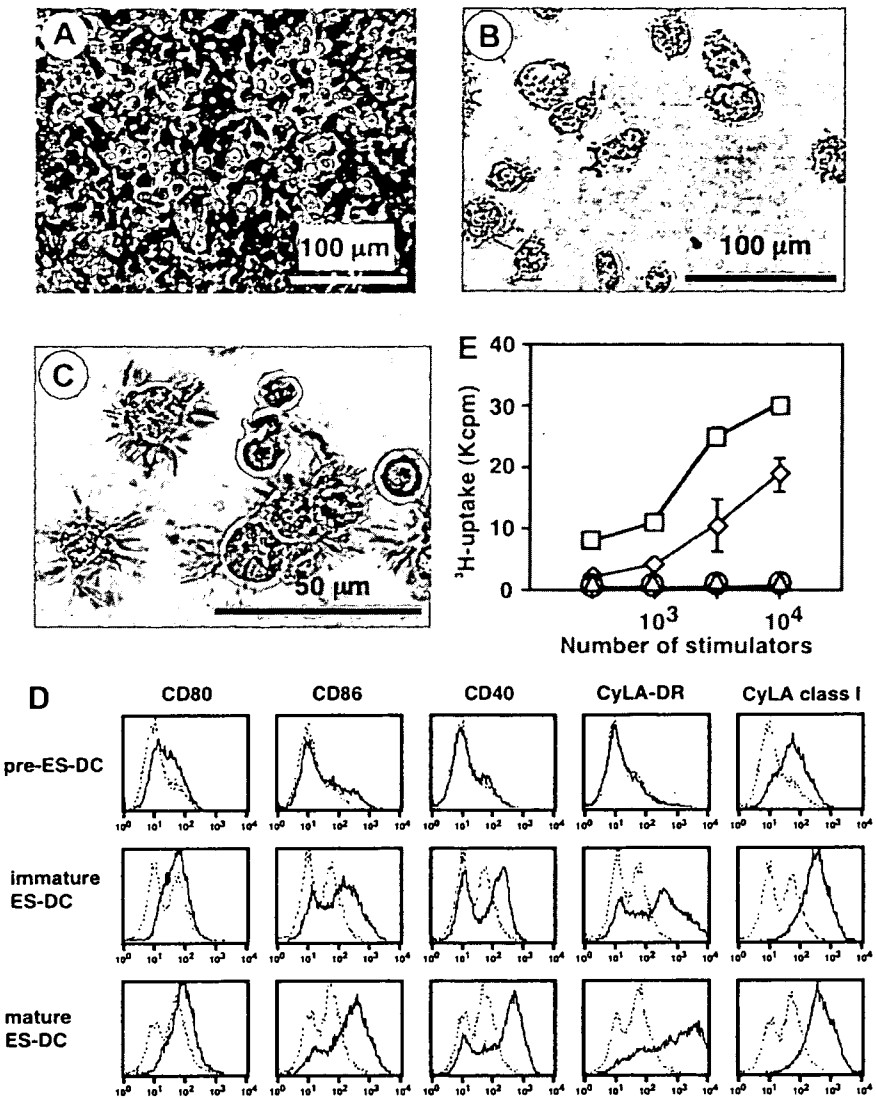

Figure 6. Generation of ES-DC from cynomolgus monkey embryonic stem (ES) cells. (**A–C**): The morphologies of cynomolgus monkey ES cell-derived differentiating cells (pre-ES-DC) at day 7 in the second step (**A**) and those in the third step before (**B**) and after (**C**) the addition of maturation stimuli are shown. (**D**): Cynomolgus monkey ES cell-derived cells harvested on day 8 in the second step (pre ES-DC) and from the third step before (immature ES-DC) and after (mature ES-DC) addition of maturation stimuli were analyzed for the cell surface expression of CD80, CD86, CD40, CyLA-DR, and CyLA class I. Staining patterns with specific monoclonal antibody (thick lines) and isotype-matched controls (thin, broken lines) are shown. (**E**): The indicated numbers of mature ES-DC (squares), immature ES-DC (diamonds), pre-ES-DC (circles), and undifferentiated cynomolgus ES cells (triangles) were x-ray-irradiated (40 Gy) and cocultured with allogeneic cynomolgus monkey peripheral blood T cells (4×10^4 cells per well) in a 96-well round-bottomed culture plate for 5 days. The proliferative responses of T cells in the last 16 hours of the culture were measured based on the [^{3}H]-thymidine uptake. Abbreviation: ES-DC, embryonic stem cell-derived dendritic cells.

with mitomycin C before use as feeder cells, and this was essential for efficient generation of hematopoietic cells. Treatment with mitomycin C may not only inactivate the mitosis of OP9 but also enhance the capacity of OP9 to support hematopoietic differentiation [26].

In the method of Slukvin et al., cells harvested from the first step of culture were directly transferred to 2-hydroxyethyl methacrylate-coated culture containers for the second step of culture [25]. In our method, cells harvested from the first step of culture were incubated in tissue culture-coated dishes for 2–5 hours to remove adherent cells. Removal of cells committed to nonmesodermal lineages by this procedure is essential. In addition, the second step of culture was also done with OP9 feeder in our method.

After the second step of culture, removal of dead cells and aggregated cells may be necessary in the method of Slukvin et al., as described in their report [25]. Indeed, we observed many dead cells, as well as DC-like cells, when we tried that method. In our method, most of recovered cells after the second step were viable, and removal of dead cells was not necessary.

The issues of safety and efficacy are critical for the establishment of ES-DC therapy. It is presumed that preclinical in vivo studies with the nonhuman primates will be required. Therefore, the ability to generate ES-DC from cynomolgus monkey ES cells is also considered to be important. It is probable that ES-DC can be generated from the ES cells of other nonhuman primates used in medical research, such as the rhesus monkey (*Macaca mulatta*) [27] and the common marmoset (*Callithrix jacchus*) [28]. For clinical application of the ES-DC technology, development of a feeder-free differentiation method may be required. Embryoid body-mediated differentiation methods may be one way to resolve this issue. In the mouse system, induction of mesodermal differentiation of ES cells using type IV collagen-coated culture plates has been reported [29, 30]. Several molecules have been reported to be involved in support of hematopoietic cell growth or differentiation by stromal cells [31–33]. Information on the molecular basis of the interaction between differentiating ES cells and feeder cells is valuable for the development of a feeder-free differentiation system.

Considering clinical applications, manipulation of function of ES-DC by genetic modification without use of viral vectors, demonstrated in the present study, has a significant advantage. However, random integration of multiple copies of transgenes into various genomic loci of ES cells is accompanied by risks such as activation of cellular oncogenes. Thus, a method to integrate transgenes into intended loci of the genome of human ES cells needs to be established.

Previously, we demonstrated a method for efficient targeted integration of expression vectors into specific genomic sites of mouse ES cells, using exchangeable gene-trap vector with Cre-

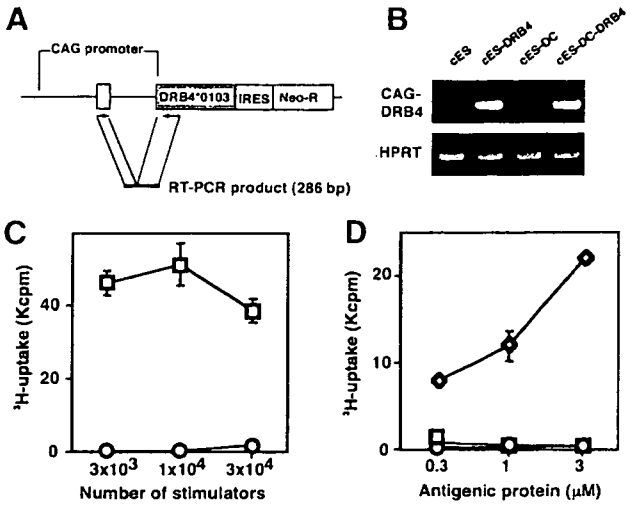

Figure 7. Antigen presentation to human T cells by genetically modified cES-DC. **(A):** The structure of HLA-DRB4*0103 expression vector is shown. The open box indicates the noncoding first exon of rabbit β-actin gene included in the CAG promoter. RT-PCR with PCR primers indicated by arrowheads generated PCR products of 286 base pairs from the transgene-derived mRNA. **(B):** Results of an RT-PCR analysis of parental cES and a transfectant embryonic stem cell clone (cES-DRB4) and derivative embryonic stem cell-derived dendritic cells (ES-DC) on the expression of transgene-derived mRNA (CAG-DRB4). The PCR products for HPRT transcript amplified from the same cDNA samples are also shown as control. **(C):** The indicated numbers of DRB4-transfectant ES-DC (squares) or nontransfectant ES-DC (circles) were preloaded with $GAD65_{111-131}$ peptide, x-ray-irradiated (40 Gy), and cocultured with SA32.5 T cells (3×10^4 cells per well) for 3 days. The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. **(D):** DRB4-transfectant ES-DC (diamonds) (1×10^4 cells per well) or nontransfectant ES-DC (squares) were cocultured with SA32.5 T cells (3×10^4 cells per well) in the presence of the indicated concentration of glutathione *S*-transferase (GST)-GAD recombinant protein for 3 days. DRB4-transfectant ES-DC and SA32.5 T cells were cocultured also in the presence of GST protein (circles). The proliferation of the T cells in the last 16 hours of the culture was measured by the [^{3}H]-thymidine uptake. Abbreviations: cES, cynomolgus embryonic stem cells; cES-DC, cynomolgus embryonic stem cell-derived dendritic cells; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRES, internal ribosomal entry site; RT-PCR, reverse transcriptase polymerase chain reaction.

Lox-mediated recombination system [1]. We are now trying to develop a system for targeted integration of transgenes into human ES cell genome. In this system, at first, gene-targeting vector conveying a drug resistance marker gene flanked by lox sequences is introduced, and then ES cell clones carrying the vector properly integrated by homologous recombination are selected. Subsequently, expression vectors with lox sequences are introduced with the aid of the Cre-Lox recombination system. Integration of a single copy of the transgene into the intended locus can be verified by Southern blot analysis. By this strategy, we can obtain ES cell clones with defined genetic modification, thus avoiding the risks accompanying the random integration of exogenous genes.

Allogenicity caused by differences in the genetic background between human ES cell lines and the recipients is considered to be a critical problem in medical application of ES-DC. We previously reported that mouse ES-DC administered into semiallogeneic recipients, sharing one MHC haplotype with the ES-DC, effectively primed antigen-specific cytotoxic T lymphocytes (CTL), suggesting that ES-DC can survive for a period long enough to stimulate antigen-specific CTL restricted by the shared MHC class I [4]. However, in the same semiallogeneic setting, we also observed five times that injection of no antigen-loaded ES-DC significantly reduced the efficiency of priming of antigen-specific CTL induced by the subsequent injection of antigen-loaded ES-DC (unpublished observations). Thus, repetitive stimulation with ES-DC expressing allogeneic MHC may result in activation and expansion of allogeneic MHC class I-reactive CTL, and in such recipients, subsequently transferred ES-DC may be rapidly eliminated. Repeated immunization may be required in clinical applications, for example, to induce antitumor immunity. Thus, we should resolve the problem of the histoincompatibilty between ES cell lines and recipients.

Methods for targeted gene modification of human ES cells and for targeted chromosome elimination of mouse ES cells have been developed [34–36]. To overcome the problem of histoincompatibility, genetic modification to inhibit expression of endogenous HLA class I in ES-DC may be effective. Deletion of more than 1,000 kilobases of entire HLA class I region of human ES cell genome by gene targeting is infeasible by currently available technology. However, disruption of the genes of molecules necessary for the cell surface expression of HLA class I molecules, such as transporter associated with antigen processing (TAP) or β2-microglobulin (β2M), is presumably feasible. In our plan, we will introduce expression vector encoding the β2M-linked form of recipient-matched HLA class I heavy chain into TAP1- or β2M-deficient human ES cells. We are now testing this strategy by using a mouse system.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Expert Opinion

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4. Evaluation of antigen-specific T cells in autoimmune diseases
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Antigen-specific immunotherapy for autoimmune diseases

Kazuhiko Yamamoto†, Akiko Okamoto & Keishi Fujio
†*The University of Tokyo, Department of Allergy and Rheumatology, Graduate School of Medicine, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113, Japan*

The status of autoimmune disease therapies is not satisfactory. Antigen-specific immunotherapy has potential as a future therapy that could deliver maximal efficacy with minimal adverse effects. Several trials of antigen-specific immunotherapy have been performed, but so far no clear directions have been established. With regard to antigen-specificity in the immune system, T cells are essential components. However, at present, we do not have a sufficient range of strategies for manipulating antigen-specific T cells. In this review, the authors propose that T cell receptor gene transfer could be used for antigen-specific immunotherapy. In the proposed technique, important disease-related and, thus, antigen-specific T cells in patients would first be identified, and then a pair of cDNAs encoding α and β T cell receptors would be isolated from these single T cells. These genes would then be transferred into self lymphocytes. These engineered antigen-specific cells can also be manipulated to express appropriate functional genes that could then be applied to specific immunotherapy.

Keywords: autoimmune diseases, antigen-specific T cells, gene transfer, T cell receptor

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1. Introduction

Autoimmune diseases are fairly common disorders affecting ~ 5% of the population, predominantly women [1]. Existing treatment of autoimmune diseases is based mainly on the use of immunosuppressive drugs such as corticosteroids and cytotoxic reagents. These treatments reduce mortality and significantly lengthen the life expectations of patients in some diseases. However, because these drugs suppress overall immune reactions, they have several serious adverse effects. In this regard, selective immunotherapies are considered more promising. As cytokines are known to play a pivotal role in regulating immune reactions, the application of cytokines to control autoimmune diseases has been extensively studied. Systemic administration of suppressive cytokines, such as transforming growth factor-β, interleukin (IL)-4 and IL-10, has served as an effective therapy in models of autoimmune diseases. Some of these protocols seem to work by shifting the balance of cytokines; however, systemic cytokine therapy potentially leads to deleterious side effects, as in the case of recombinant human IL-4 [2]. Therapies neutralizing a certain cytokine have been successful and will serve as important strategies in many autoimmune diseases in the near future; however, because such strategies still have important drawbacks [3], it is also necessary to explore other specific immunotherapies.

In a variety of autoimmune diseases, trials to identify molecules recognized by T cells and autoantibodies have been extensively performed. These efforts are essential not only for understanding the pathogeneses of autoimmune diseases, but also for establishing antigen-specific immunotherapies. Although knowledge of target antigens in several autoimmune diseases has greatly increased, our ability to selectively silence particular pathogenic immune responses has not. One of the

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feasible approaches to treat autoimmunity is suppression of the activation and expansion of antigen-specific T cells before they differentiate into pathogenic T cells. This approach could be useful because once the pathogenic responses are established, intervention appears to be less effective on activated T cells subsets. However, the majority of patients who require clinical treatment have full-blown autoimmune disease and this approach would not be useful in such cases.

It is generally believed that in autoimmune diseases, an immune response to a single epitope on a self antigen at the start of the disorder can trigger immune responses to neighboring epitopes on the same molecule or to other epitopes on related molecules. This is termed 'epitope spreading'. Although it is not clear whether the epitope spreading occurs throughout the autoimmune process, some researchers argue against antigen-specific immunotherapy because of the difficulties of predicting such expanding autoimmune reactions. In this article, the authors discuss the behavior of antigen-specific T cells in autoimmune diseases. The authors propose that epitope spreading is not the sole mechanism of the T cell related pathogenesis of autoimmune diseases and that clonal restriction of T cells occurs in the late phase of autoimmunity. Therefore, antigen-specific immunotherapy would be feasible even for established autoimmune diseases. T cell receptor (TCR) gene transfer could be one of the possible strategies.

2. Immunotherapies and targeted immunotherapy in autoimmune diseases

Several autoimmune diseases, and particularly systemic types of autoimmune diseases, such as systemic lupus erythematosus and systemic vasculitis syndromes, are treated with corticosteroids and immunosuppressive drugs. The latter include cytotoxic drugs such as alkylating agents and purine analogues, and calcineurin inhibitors, such as cyclosporin and tacrolimus. At present, these drugs offer the best chance of suppressing – or sometimes inducing the remission of – these diseases. However, they have potentially life-threatening side effects due to their severe depression of immune function. Furthermore, a serious complication among patients undergoing immunosuppressive therapy would be the risk of developing cancer [4]. Similarly, corticosteroids are commonly used to treat several autoimmune diseases. Corticosteroids inhibit prostaglandin synthesis, block cytokine secretion and T cell activation, and are one of the most effective therapies against autoimmune diseases. However, they also have several effects on physiological systems, and if used over the long term, they can cause profound immunosuppression with increased risk of infection, cancer, osteoporosis, hypertension and endocrine abnormalities.

Due to improvements in our understanding of immune reactions and advances in molecular biology, new biological agents – especially monoclonal antibodies – are now available for specific blockade of effector molecules such as inflammatory mediators. Administration of monoclonal antibody against tumor necrosis factor (TNF) (infliximab) or a soluble recombinant TNF receptor–immunoglobulin fusion protein (etanercept) led to the suppression of inflammation and a remarkable improvement of function of patients with rheumatoid arthritis (RA) [5]. However, there are several limitations to the use of anti-TNF therapy. In fact, some patients do not respond to these TNF inhibitors. Furthermore, the blockade in pro-inflammatory cytokines can put some individuals at increased risk of tuberculosis or other infections [6,7].

Apart from anticytokine therapies, several molecules have been investigated as possible targets of selective immunotherapy. For example, activated lymphocytes bind to specific receptors along the vessel walls to initiate the process of penetrating the target organ. Therefore, clinical trails with antibodies against such homing receptors appear to be promising. In fact, a humanized, monoclonal antibody, an α_4 integrin antagonist, has shown some degree of success in a placebo-controlled trial; however, there was an increased rate of infection in the treated patients [8]. A depletion of 95% of circulating lymphocytes in patients with multiple sclerosis (MS) by a monoclonal antibody against CD52 suppressed the disease activity of MS, but a third of patients developed autoimmune thyroid disease [9]. Cytotoxic T lymphocyte antigen (CTLA)-4Ig and a high-affinity mutant form, LEA29Y, are now in clinical trials in patients with autoimmunity [10]. These agents appear to work by blocking CD28 costimulation, leading to an inhibition of pathogenic T cell activation. However, it should be emphasized that a subset of regulatory T cells also depend on CD28/B7 interaction for their development and function. Furthermore, in the majority of autoimmune situations, effector T cells have already been established and are less dependent on costimulation for their activity effectiveness [11,12]. Thus, the situation is more complex. Recently, an approach for depleting B cells with antibodies against CD20 (rituximab) proved successful in several autoimmune disorders [13]. Further candidates for immunotherapy would include complement inhibitors [14] and Toll-like receptor modulations [15].

3. Trials of antigen-specific suppression in autoimmune diseases

Immunologists have tried to develop methods to treat autoimmune diseases by identifying and applying self-antigens, which are the target of autoimmune processes. In this regard, stimulation of T cells with the target antigen is an attractive direction. The spectrum of possible approaches involving T cell stimulation includes ablation of antigen-specific T cells, achieving specific T cell anergy, induction of regulatory T cells, and induction of a shift in the predominant phenotype of the antiself response from T helper (Th)1 to Th2 type. In fact, specific antigen

vaccination by administration of a target antigen in aqueous solution has been shown to significantly decrease the disease severity in several animal models. However, there is a high degree of anaphylactic sensitization in this method, making it difficult to directly apply it to human disorders [16,17]. Vaccination of antigen-coding DNA plasmids could bypass the immunological sensitization in protein administration. DNA-based immunotherapy, composed of unmethylated CpG repeats, is capable of inducing a shift in the cytokine profile and immune response that favours the Th1 arm. This observation makes DNA-based immunotherapy a promising candidate for the treatment of allergic diseases, which are known to be mediated by Th2-based responses [18]. DNA vaccination combined with a Th2 inducing costimulation is also effective in the treatment of several animal models of autoimmune diseases [19,20]. Although DNA vaccination appeared to have great potential as a safe and efficacious type of antigen-specific immunotherapy, the route of DNA administration and the combination of costimulation should be carefully examined before clinical application.

Oral antigen administration suppresses animal models of autoimmune diseases, including experimental autoimmune encephalitis, uveitis, arthritis and diabetes in the non-obese diabetic mouse [21]. Oral antigen induces antigen-specific Th2 and $CD4^+CD25^+$ regulatory cells. Based on the success in these animal experiments, oral tolerance has been examined in human autoimmune diseases, including MS, arthritis, uveitis and diabetes. Although positive results have been observed in Phase II trials, no effects were observed in Phase III trials of CII in RA or oral myelin and glatiramer acetate in MS [21,22]. Recently, oral insulin has prevented progression of immune-mediated (Type 1) diabetes [23]; however, further analysis of the immunological basis of oral tolerance is required for the effective therapy in human autoimmune diseases.

Altered peptide ligand (APL) is an antigenic peptide with amino acid modifications and is expected to block antigen-specific T cell responses by acting as a partial agonist or TCR antagonist, or by inducing regulatory T cell populations. In MS, myelin basic protein (MBP)-specific T cells are considered to be essential in the pathogenesis and MBP_{83-99} are estimated to be one candidate epitope. One APL has already been designed and submitted to a Phase II clinical trial; however, the treatment was poorly tolerated at the dose tested and the trial was halted. Some patients developed exacerbations of the disease due to the expansion of T cells specific for MBP_{83-99} by this APL [24]. A more sophisticated approach of antigen-specific T cells redirected against autoreactive T lymphocytes was reported [25]. However, it is not clear whether such methods could be used in the clinic.

Dendritic cells (DCs) are professional antigen-presenting cells with the potential to either stimulate or inhibit immune responses. DCs loaded with antigen can be used as a DC vaccine. Although most DC vaccines have been used to stimulate immune responses in patients with cancer [26], an increasing number of preclinical studies are focusing on the capacity of immature DCs to induce antigen-specific non-responsiveness [27-29]. DCs in the steady-state are immature and can induce tolerance in an antigen-specific manner. Immature DCs incubated with an agent, such as dexamethasone [30], vitamin D [31] or the Rel-B inhibitor [32], can induce tolerance. However, as discussed in cancer therapy, a careful study design incorporating standardized and quality-controlled clinical and immunological criteria is needed [33]. At present, we do not know exactly how robust the immunostimulatory DCs and tolerogenic DCs are.

Regulatory T cells are now recognized as one of the most central mechanisms of immune regulation. $CD4^+CD25^+$ T cells, in particular, have been shown to develop in the thymus or in the periphery to maintain the homeostatic equilibrium of immunity and tolerance [34]. Thus, researchers are now trying to expand these regulatory T cells and use them for the treatment of autoimmune diseases. However, this is not effective in some cases. For example, in the case of non-obese diabetic mice, which spontaneously develop diabetes, the suppression was relatively inefficient when heterogeneous regulatory T cells were simply expanded and adoptively transferred [35-37]. It appears that effective, regulatory T cell activity depends on both an appropriate phenotype and a high frequency of autoantigen specificity. Therefore, management to expand regulatory T cells in an antigen-specific manner would be required [34].

As discussed, regulatory cytokines and cytokine antagonists have been considered for the treatment of autoimmune diseases. However, systemic administration could potentially lead to deleterious side effects. Thus, local delivery of such molecules would be more efficient and eliminate the possible systemic side effects. In this regard, antigen-specific T cells or T cell hybridomas are believed to be suitable vehicles for targeted immunotherapies. In fact, the authors, as well as others, have reported T cell-mediated gene therapy for autoimmune animal models [38-41]. T cell-mediated, adoptive, cellular gene therapy is based on site-specific homing and retention of the vehicle, and local effects of the delivered effector molecules.

4. Evaluation of antigen-specific T cells in autoimmune diseases

With respect to the modes of autoimmune reactions, the idea of epitope spreading or determinant spreading has been widely accepted [42,43]. At the T cell level, this is a diversification of specificity from the initial, limited epitope-specific immune response to a hierarchical cascade of autoreactive T cell specificities. This mechanism may explain, for example, the pathway of infection induced autoimmunity. According to this idea, the initial phase of the autoimmune reaction might be carried out by a few activated T cells against limited numbers of epitopes. These T cells may be crossreactive T cells that recognize both microbial epitopes and self epitopes. However,

in the late phase of the disorders, the reactive epitopes might spread, and T cells recognizing a variety of different epitopes on several different self-molecules would be activated. However, if epitope spreading is the only mechanism involved in the T cell immune responses in autoimmune disorders, development of effective antigen-specific immunotherapies will be difficult, as target epitopes and molecules will always have the potential to spread and it would be difficult to define the pattern of spreading in a chronic human autoimmune disorder. The hierarchy of immune response to multiple tissue antigens would depend partially on individual HLA genotypes, but also on unknown factors and probably stochastic events.

In order to determine whether the phenomenon of epitope spreading is operative throughout the entire autoimmune process, it is important to detect how specific T cells behave within the lymphocyte population in the pathological lesions. Antigen-specific T cells should proliferate and form accumulated T cell clones in the heterogeneous lymphocyte population to exert their function. Therefore, evaluation of accumulated T cell clones in the pathological lesions would be informative. Several years ago, the authors' group established a system to analyze accumulated T cell clones in the lymphocyte population using reverse transcriptase polymerase chain reaction (RT-PCR) and single-strand conformation polymorphism (SSCP) on TCR messages [44]. In this system, a heterogeneous T cell population exhibits a smear pattern of amplified TCR messages. If there is an accumulation of certain T cell clones in the heterogeneous lymphocyte population, bands corresponding to each clone are observed in the background smear. Identification of accumulated T cell clones in different samples can be easily compared because the same clone exhibits the same electrophoretic mobility. Separation using cell surface phenotypes can be used to further identify disease- related important clones.

Using this system, the authors' group analyzed several synovial tissue samples of an RA patient. As a result, the same clones were found to exist in different joints [44,45]. These data clearly suggested that immune responses in RA were uniform throughout all of the arthritic lesions of the patient. In order to compare this finding in human samples with murine models, the authors' group analyzed several cases of spontaneous autoimmune models. For example, human T cell leukemia virus-1 env-pX transgenic mice exhibit spontaneous symmetrical arthritis similar to human RA [46]. In one study, the T cell clonality among different arthritic lesions in each stage was compared [47]. In the early stage, there were vigorous accumulations of T cells in the joints, but they were different among the different lesions. In the middle stage, several identical clones were found to be accumulated in the different lesions. In the late stage, the majority of the accumulated clones in one lesion were found to exist in the other lesions, suggesting that the autoimmune responses in the pathological lesions were rather uniform in the mouse. The number of dominant clones did not necessarily increase. The finding in the late stage was similar to what was observed in human RA synovial samples.

From the analyses of several spontaneous autoimmune animal models [48,49], it is now speculated that epitope spreading does not necessarily work in the late phase of the whole disease progression and it is possible that some form of clonal restriction of T cells occurs in autoimmune disorders. During the progression of autoimmune disease, immune response might become rather restricted to certain targets. Some restricted T cell clones directed towards such target self-antigens might be sustained. A similar, limited T cell oligoclonality as a 'driver clone' in autoimmunity was described in experimental autoimmune encephalomyelitis [50,51]. Oligoclonally expanded insulin-reactive T cells were also identified in the pancreatic draining lymph nodes of Type 1 diabetes patients with prolonged disease durations, [52]. It was reported that avidity maturation of a pathogenic T cell population may be the key event in the progression of benign inflammation to overt disease in autoimmunity [53] Therefore, an ideal way to control the disorder would be to suppress such sustained pathologic responses without globally interfering with the immunity of the host.

With respect to general immune responses to foreign antigens, T cell responses are reported to be dominated by few clonotypes that express a restricted set of TCRs [54]. This clonal selection and dominance may be due to the competitive advantages of the higher-affinity receptor, the duration of TCR–pMHC interaction or the affinity threshold [55]. In addition, in response to viruses, clonal T cell 'immunodomination' appears to occur in $CD8^+$ T cells, probably due to proliferation advantages, differences of TCR affinity or cosignal requirements [56]. Therefore, the clonal restriction of T cells found in autoimmune disorders is not behaviour specific to these diseases, but can be considered as a usual T cell response.

5. TCR gene transfer for controlling autoimmune diseases

From the results of the evaluation of antigen-specific T cell clonality, continuous expansion of immune response by acquisition of self-reactive epitopes does not appear to occur in the advanced stages of autoimmune disorders. Thus, the authors now believe that antigen-specific, immunotherapy targeting T cells would be feasible in autoimmune diseases. In this regard, extensive attempts have been made to try and establish antigen-specific T cell clones or lines by *in vitro* culture; however, there are several difficulties with this process. Usually, the culture should be performed without the information of appropriate autoantigens. A candidate autoantigen in cloning culture of autoantigen-specific T cells has to be selected based on the limited information. Moreover, there is no guarantee that *in vitro* established T cell clones represent real disease-associated T cells, mainly because *in vivo*-activated T cells are more easily rendered in

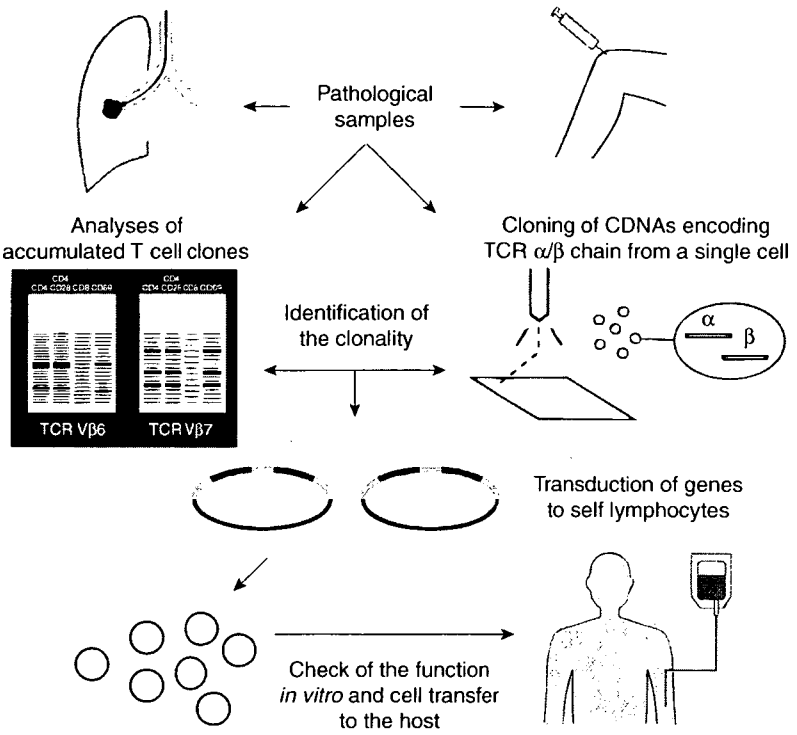

Figure 1. Proposed antigen-specific immunotherapy. Clonal analyses of TCRs in the pathological lesions could be performed as indicated on the upper left hand of the panel. This information could be combined with the *in vitro* reconstitution of the TCR function by cloning TCR cDNAs from a single cell and transferring them into self lymphocytes as in the right-hand panel. These engineered cells could be applied for antigen-specific immunotherapy.
TCR: T cell receptor.

activation-induced cell death. Therefore, the authors' group is now trying to produce autoimmune-associated T cells by gene transfer of TCR obtained from *in vivo*, as discussed below.

TCRs of the accumulated T cell clones in the established autoimmune lesions can be visualized by RT-PCR/SSCP analysis. If we obtain a pair of full-length cDNAs encoding α and β chains of TCR expressed in a single cell in the lesion, and if we express them efficiently by gene transfer to self lymphocytes, TCR function can be reconstructed using the information obtained *in vivo*. A schematic of the authors' system is given in **Figure 1**. For the gene transfer to lymphocytes, the authors' group have established a highly efficient retrovirus vector system with PLAT-E and pMX. PLAT-E is a packaging cell transfected with *gag–pol* and *env* segments separately. Two independent, monocistronic retrovirus vectors harbouring α and β TCR cDNAs were generated. For the first study, the class II MHC-restricted *α* and *β TCR* genes specific for chicken ovalbumin (OVA) were used. These *TCR* genes were cloned from TCR transgenic mice designated DO11.10. These TCRs were transduced to splenocytes from BALB/c mice. The results indicated that *α* and *β TCR* gene transfer into peripheral T cells reconstituted the antigen-specific immunity [57]. The amount of TCR expression and both the *in vitro* and *in vivo* antigen-specific functions were comparable with those obtained with splenocytes from DO11.10 transgenic mice.

The authors' group next attempted to use this TCR gene transfer to control autoimmune disorders [58]. The target was lupus nephritis. NZB/W F1 mice spontaneously develop a lupus-like syndrome and nephritis. Anti-DNA antibodies are believed to be one of the major pathogenic autoantibodies for nephritis. Datta and co-workers [59,60] have pointed out that nucleosome is a major immunogen in systemic lupus erythematosus. As DNA and the nucleosome are physically linked, it is speculated that nucleosome reactive T cells help the activation of anti-DNA specific B cells as the hapten-carrier model. Therefore, the authors tried to generate nucleosome-specific T cells with an immunosuppressive function. The authors selected CTLA-4Ig as a suppressive molecule. TCR cDNAs were engineered based on the published sequence of nucleosome-specific TCR by fusing a TCR V region sequence with a synthesized CDR3 sequence and a TCR J-C region sequence [59]. The V regions used were V α 13 and V β 4. This TCR recognizes the immunodominant I-A^d-restricted nucleosomal epitope.

In the authors' usual experimental protocol, the proportion of clonotypic TCR expression cells with two transferred TCR genes was estimated to be ~ 25% in $CD4^+$ T cells. The

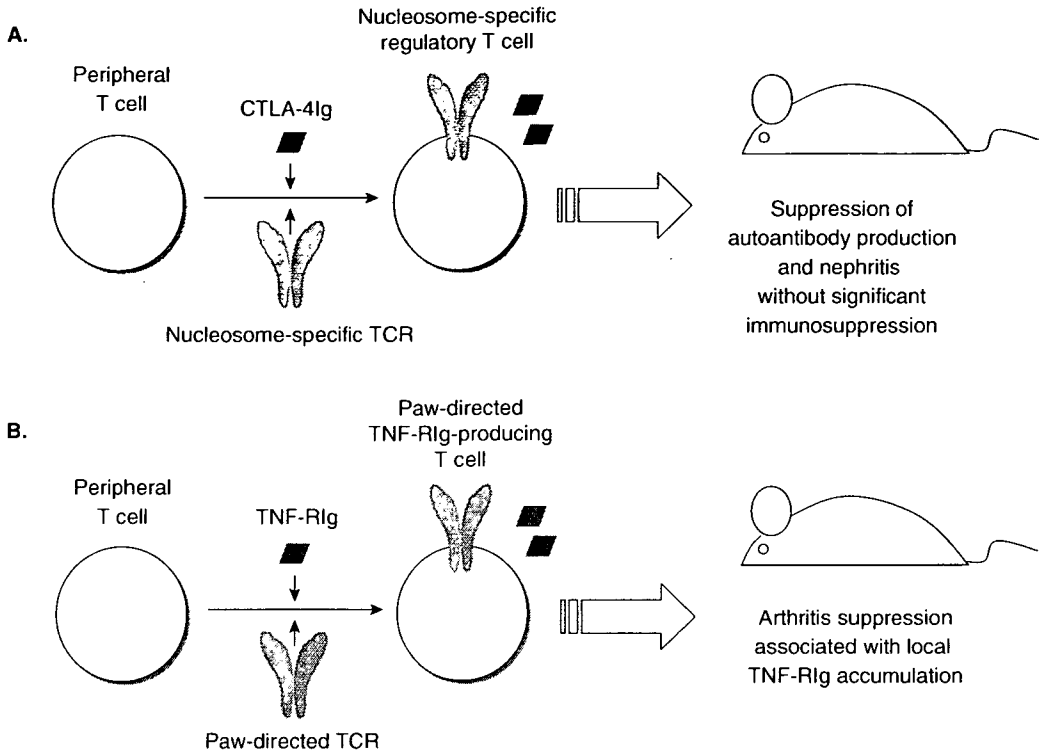

Figure 2. Experimental outlines of TCR gene transfer for controlling autoimmune diseases. A and **B** illustrate images of triple gene transfer to generate nucleosome-specific regulatory T cells and paw-directed TNF-R Ig-producing T cells.
CTLA: Cytotoxic T lymphocyte antigen; TCR: T cell receptor; TNF-R: Tumor necrosis factor receptor.

introduction of TCR was found to reconstitute the specificity for the nucleosome. Triple gene transfer was then performed together with CTLA-4Ig to generate regulatory T cells (**Figure 2A**). Calculations showed that ~ 10% of the total $CD4^+$ cells expressed all three genes. The CTLA-4Ig secreted from transduced T cells blocked the proliferation of the polyclonal T cell population. The TCR and CTLA-4Ig transduced cells showed the increase of CTLA-4Ig secretion on T cell activation in the presence of DCs. A million of the nucleosome-specific regulatory T cells engineered by the triple genes were then transferred into 10-week-old NZB/W F1 mice. The mice were monitored for proteinuria. By week 22, all of the control mice that received phosphate-buffered saline, cells transferred with mock vectors, TCR alone and CTLA-4Ig started to develop severe nephritis diagnosed by persistent proteinuria of > 300 mg/dl. By 30 weeks of age, the majority of these control mice showed severe proteinuria; however, none of the mice treated with cells transferred by the TCR and CTLA-4Ig showed excess proteinuria. The kidneys of the control mice showed severe glomerulonephritis with membrano-proliferation, glomerular sclerosis and tubular casts. The treated mice had mild glomerular disease with less deposition of IgG and complement, especially in the capillary loop. The autoantibodies usually found in NZB/W F1 mice were measured in the sera from different groups. The elevations of anti-dsDNA and antihistone antibodies were suppressed at the age of 22 weeks in the TCR and CTLA-4Ig-treated mice. The T cell-dependent humoral response to active immunization of OVA was also analyzed. The level of anti-OVA IgG antibody titre was not significantly different from those of the control mice, indicating there was not an overt systemic immunosuppression of the triple gene-treated mice.

In order to obtain the whole TCR information from pathological lesions, the authors' group next tried to clone a pair of full-length TCR cDNAs from a single cell accumulated in the inflamed joints of DBA/1 mice with collagen-induced arthritis [61]. Cloning of full-length cDNA encoding TCR was already established [62]. Single-cell sorting with $CD4^+$ and Vβ 8.1/8.2-positive cells was performed and TCR messages were amplified with three-step nested PCR using a fixed Vβ primer and multiple Vα primers. The authors then compared the clones obtained from the single cells with accumulated clones observed in the arthritis joints using the RT-PCR/SSCP method. Some TCRs from sorted single cells were actually identical to major clones accumulated in the joints. These TCR cDNAs were converted into full-length cDNAs and transferred to DBA/1 splenocytes. Interestingly, some of the pairs of TCR were found to be nonspecific to immunized type II collagen, but specific to self-antigen because

TCR-transferred cells proliferated in the culture with DCs from normal and arthritic mice. The carboxyfluorescein diiacetate succinimidyl ester (CSFE) labelling experiments showed that such TCR-transduced cells accumulated and proliferated in the arthritic joints. The authors' group next performed experimental therapy using the triple-gene engineered T cells. In this experiment, a soluble fusion protein consisting of TNF receptor p75 and Fc domain of IgG2a (TNF-RIg), was used as a regulatory molecule. Control cells were transduced with either TCR alone or TNF-RIg alone. With regard to the arthritis score and the percentage of severe arthritis, only TCR plus TNF-RIg-transduced cells significantly suppressed the arthritis (**Figure 2B**). Interestingly, the serum concentration of TNF-RIg was not the main determinant of arthritis suppression in the TCR plus TNF-RIg group, as the serum concentrations of TNF-RIg protein in the TCR plus TNF-RIg group were equivalent to those in the TNF-RIg group. In contrast, the amount of TNF-RIg in the paws of the TCR plus TNF-RIg group was significantly higher than that in the paws of the TNF-RIg group. Therefore, local accumulation of the TNF-RIg transcript suppressed arthritis in the TCR plus TNF-RIg group. Therefore, biological agents producing T cells may have an advantage over the conventional biological agents that depends on serum concentration. A reduced serum concentration may be associated with less systemic immunosuppression. Taking these results together, the system illustrated in the **Figure 2** was shown to be feasible for use in experimental animals.

Recently, the clinical appreciation of retroviral TCR gene transfer was reported in the treatment of melanoma patients. T cells transduced with melanoma antigen-specific TCRs suppressed disease progression in patients with advanced melanoma [63]. This result showed the essential efficacy and safety of TCR gene transfer in human. Therefore, autoimmune disease can be a suitable target for TCR gene transfer.

6. Expert opinion and conclusion

T cells are one of the most decisive components in immune responses, especially in terms of antigen specificity. TCR determines the specificity. However, the *TCR* genes are rearranged in each cell to obtain a variety of antigenic specificities, and so T cells are enormously heterogeneous. A small number of T cells in the total lymphocyte population participate in an antigen-specific immune response. Therefore, this limited population should be the main target in antigen-specific immunotherapy, without affecting systemic immunity. However, it is rather difficult to evaluate and further manipulate such specific T cells. The authors believe that much effort should be required for the analysis and manipulation of antigen-specific T cells in the future research. The authors propose that TCR gene cloning using the information of TCR clonal analysis, and reconstitution of the TCR function by gene transfer would be a promising strategy for antigen-specific immunotherapy in autoimmune disorders.

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Affiliation

Kazuhiko Yamamoto[†1], Akiko Okamoto & Keishi Fujio

[†]Author for correspondence

[1]The University of Tokyo, Department of Allergy and Rheumatology, Graduate School of Medicine, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113, Japan

E-mail: yamamoto-tky@umin.ac.jp

Hepatocyte Growth Factor Significantly Suppresses Collagen-Induced Arthritis in Mice

Katsuhide Okunishi,* Makoto Dohi,[1]* Keishi Fujio,* Kazuyuki Nakagome,* Yasuhiko Tabata,[†] Takahiro Okasora,[†] Makoto Seki,[‡] Mihoko Shibuya,* Mitsuru Imamura,* Hiroaki Harada,* Ryoichi Tanaka,* and Kazuhiko Yamamoto*

Hepatocyte growth factor (HGF) plays an important role in angiogenesis, cell proliferation, antifibrosis, and antiapoptosis. Moreover, recent studies have highlighted the immunosuppressive effect of HGF in animal models of allogenic heart transplantation and autoimmune myocarditis and in studies in vitro as well. We also reported that HGF significantly suppresses dendritic cell function, thus down-regulating Ag-induced Th1-type and Th2-type immune responses in allergic airway inflammation. However, the immunosuppressive effect of HGF in many other situations has not been fully clarified. In the present study, using a mouse model of collagen-induced arthritis (CIA) and experiments in vitro, we examined the effect of HGF on autoimmune arthritis and then elucidated the mechanisms of action of HGF. To achieve sufficient delivery of HGF, we used biodegradable gelatin hydrogels as a carrier. HGF suppressed Ag-induced T cell priming by regulating the functions of dendritic cells in the Ag-sensitization phase with down-regulation of IL-10. In contrast, under continuous Ag stimulation HGF induced IL-10-producing immunocytes both in vivo and in vitro. Moreover, HGF potently inhibited the development of CIA with enhancing the Th2-type immune response. We also confirmed that HGF significantly suppressed the production of IL-17 by immunocytes. These results indicate that HGF suppresses the development of CIA through different ways at different phases. They also suggest that HGF could be an attractive tool for treating patients with rheumatoid arthritis. *The Journal of Immunology,* 2007, 179: 5504–5513.

Hepatocyte growth factor (HGF),[2] originally identified and cloned as a potent mitogen for hepatocytes (1–3) and a scatter factor (4), targets various cell types (5). HGF has many functions such as induction of angiogenesis, promotion of cell proliferation and migration (5), and inhibition of apoptosis (6, 7). HGF exhibits these functions through its receptor c-Met (5). It is well established that HGF promotes tumor progression (8–12) and suppresses the development of fibrosis after injury (13–15).

The role of HGF in immune-mediated disorders has not been fully studied. HGF promotes adhesion and migration of B (16, 17) and T cells (18) and enhances dendritic cell (DC) migration (19, 20). HGF frequently counteracts TGF-β, a potent immunosuppressive cytokine (13, 14, 21). These results indicate that HGF might accelerate immune responses. In contrast, recent studies clarified an immunosuppressive effect of HGF. In a mouse model of allogenic heart transplantation, HGF reduced acute and chronic rejection of the allograft with increased expression of TGF-β and IL-10, indicating that HGF might induce allograft tolerance (22). HGF ameliorates the progression of experimental autoimmune myocarditis, a Th 1-type dominant immune response, inducing production of Th2 cytokines (23). In addition, other articles reported that HGF suppresses the development of Th2-type responses as well (24–26). HGF attenuates allergic airway inflammation (24, 25), and one article recently reported that HGF prevents lupus nephritis in a murine lupus model of chronic graft-vs-host disease through suppression of Th2-type immune responses (26). These results indicate that HGF could suppress both Th1-type and Th2-type immune responses. As to the mechanisms of immune suppression by HGF, two major possibilities have been reported. One is the down-regulation of functions of DCs, a mechanism elucidated in the case of allergic airway inflammation that was reported by us previously (24). Another mechanism is to induce the regulatory phenotype of $CD4^+$ T cells that produce IL-10 or TGF-β, which was studied in an experimental system of allogenic heart transplantation (22) and in vitro (23).

Rheumatoid arthritis (RA) is an autoimmune disorder and a systemic chronic inflammatory disease characterized by persistent synovial cell proliferation with inflammatory cell infiltration and destruction of joints (27). The mechanism and pathogenesis of RA have not been fully clarified. RA has traditionally been assumed to be a Th1-type disease (28, 29). However, recent studies revealed a new lineage of effector $CD4^+$ T cells characterized by the production of IL-17, and this Th17 lineage plays an essential role in both the development of autoimmune arthritis (30, 31) and bone destruction (32). In addition to the T cell-mediated immune responses, angiogenesis plays a very important role in maintaining and promoting RA (33).

The role of HGF in RA has been reported in a few cases. HGF and its receptor c-Met were found in the synovial tissue of patients with RA (34). HGF levels in synovial fluids were significantly higher in patients with RA than in those with arthritis of other

*Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [†]Institute of Frontier Medical Sciences, Kyoto University, Kyoto, Japan; and [‡]Research Laboratory III, Pharmaceutical Research Division, Mitsubishi Pharma Corporation, Yokohama, Japan

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[1] Address correspondence and reprint requests to Dr. Makoto Dohi, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan. E-mail address: mdohi-tky@umin.ac.jp

[2] Abbreviations used in this paper: HGF, hepatocyte growth factor; CIA, collagen-induced arthritis; CII, type II collagen; DC, dendritic cell; EU, ELISA unit; LN, lymph node; RA, rheumatoid arthritis; rhHGF, recombinant human HGF; Treg, regulatory T.

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Table I. *Time course of HGF concentration in the sera (pg/ml)*[a]

Hours or Days after Injection	4 h	Day 1	Day 2	Day 4
HGF protein				
HGF (10 μg)	ND[b]	ND	ND	ND
HGF (100 μg)	1658 ± 447	216 ± 71	ND	ND
Gelatin/rhHGF complex				
HGF (0 μg)	ND	ND	ND	ND
HGF (100 μg)	801 ± 117	188 ± 34	174 ± 174	ND

[a] Data are the mean ± SEM from three to four animals per group.
[b] Not detected.

from CII/CFA-sensitized mice on day 10 were restimulated with CII (10 μg/ml) in the presence or absence of rhHGF at several concentrations. After 3 to 4 days of incubation, cytokine production was measured.

Flow cytometry

Expression of surface molecule on DCs obtained from each group of mice on day 10 was examined as reported previously (43) by flow cytometry (EPICS XL System II; Beckman Coulter). We also examined the expression of CD25 and Foxp3 in $CD4^+$ T cells on days 10, 20, and 40. Staining of spleen or LN cells with anti-mouse CD4, CD25, and Foxp3 Abs was conducted following the manufacturer's protocol. In brief, first the cells were stained with allophycocyanin-conjugated anti-mouse CD4 Ab and FITC anti-mouse CD25 Ab (BD PharMingen). Then, intracellular Foxp3 staining was conducted using anti-mouse Foxp3 Ab and fixation/permeabilization solution and permeabilization buffer contained in a mouse regulatory T cell staining kit (eBioscience). Then stained cells were analyzed by flow cytometry (EPICS Elite; Beckman Coulter).

RT-PCR

mRNA was extracted from $CD4^+$ T cells by the acid-guanidium phenol chloroform method using Isogen (Nippon Gene). Then, RT-PCR was conducted as reported previously (39). PCR for GATA-3 consisted of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C for 26 cycles. PCR for β-actin consisted of 1 min of denaturation at 94°C, 1 min of annealing at 61°C, and 1 min of extension at 72°C for 18 cycles. The sense primer for the transcription factor GATA-3 was 5′-TCT GGAGGAGGAAACGCTAATGG-3′ and the antisense primer was 5′-GAACTCTTCGCACACTTGGAGACTC-3′. The sense primer for β-actin was 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and the antisense primer was 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. PCR products were electrophoresed in a 3% agarose gel, and the results were visualized by ethidium bromide staining.

Statistical analysis

Values are expressed as the mean ± SEM. The Mann-Whitney *U* test was used to analyze the clinical scores and histologic findings. The unpaired *t* test was used to analyze the other results. Values of $p < 0.05$ were considered to be significant.

Results

HGF significantly suppresses T cell priming induced by OVA/alum

Generally, exogenously administered HGF protein delivered by i.v. injection vanishes from organs within several hours (44). So, to achieve efficient delivery of HGF we adopted biodegradable gelatin hydrogels as a carrier for the CIA model and delivered the HGF/gelatin complex by s.c. injection (37). First, we examined the time course of HGF concentration in sera after s.c. injection of HGF protein, gelatin, or gelatin/rhHGF complex. We confirmed that the more sustained release of HGF was achieved by s.c. injection of gelatin/rhHGF complex compared with the injection of HGF protein alone (Table I). Then, we examined the effect of this gelatin/rhHGF complex (designated HGF in figures) on OVA-induced immune responses. Spleen cells obtained from the mice

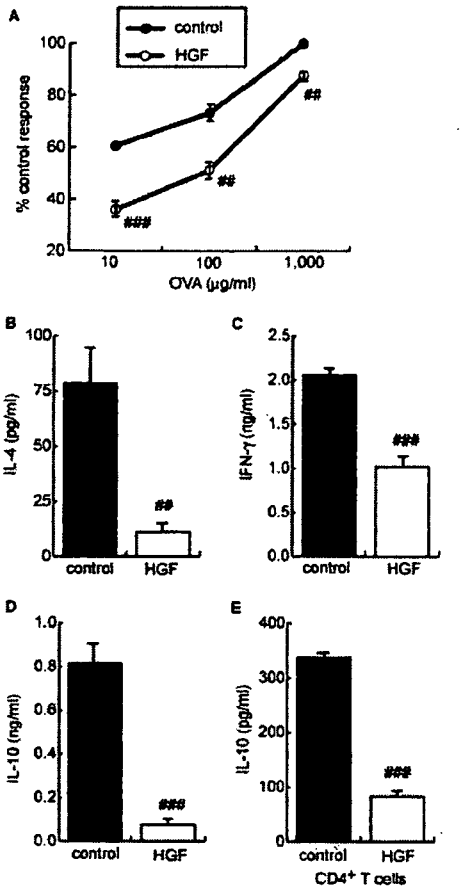

FIGURE 1. Controlled release of HGF in vivo potently suppresses T cell priming with OVA/alum. BALB/c male mice were sensitized with OVA/alum and a few hours later received a s.c. injection of gelatin (2 mg) (control mice) or gelatin/rhHGF (100 μg) complex (HGF) on day 0. On day 10, spleen cells were obtained from each group of mice. *A–E*, Spleen cell responses (2.5×10^6 cells/ml) to OVA restimulation in vitro were examined. *A*, Cell proliferation was measured after 3 days of incubation with the indicated concentrations of OVA. Data are expressed as a percentage of the response compared with that of spleen cells from control mice at OVA (1000 μg/ml). *B–D*, Production of IL-4 (*B*) and IFN-γ (*C*) as well as IL-10 (*D*) was measured by ELISA after 4 days of incubation with OVA (100 μg/ml). *E*, IL-10 production by $CD4^+$ T cells after nonspecific stimulation. $CD4^+$ T cells were negatively selected and then stimulated in vitro with PMA (1 ng/ml) and ionomycin (0.1 μg/ml) for 2 days. IL-10 concentrations in the supernatants were measured. Data were obtained from four wells per group of mice. ##, $p < 0.01$; and ###, $p < 0.001$ (vs control mice).

treated with HGF demonstrated significantly reduced cell proliferation (Fig. 1*A*) and the production of IL-4 (Fig. 1*B*), IFN-γ (Fig. 1*C*), and IL-10 (Fig. 1*D*) upon stimulation with OVA-Ag. Then, we also confirmed that treatment with HGF in vivo significantly suppressed IL-10 production by $CD4^+$ T cells in response to nonspecific stimulation with PMA and ionomycin (Fig. 1*E*). These results indicated that HGF potently suppressed Ag-induced T cell priming with a down-regulation of IL-10 production.

HGF significantly suppresses T cell priming induced by CII/CFA

Then, we examined the immunosuppressive effect of HGF in the CIA model. DBA/1 mice were sensitized with CII/CFA and received a s.c. injection of gelatin or gelatin/rhHGF complex once on day 0. On day 10, spleen cells were obtained and then restimulated

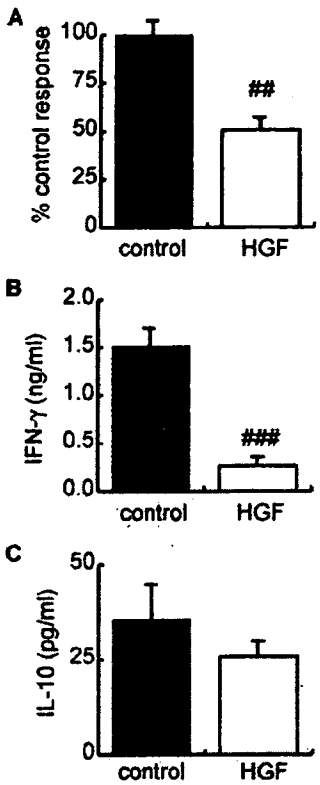

FIGURE 2. Controlled release of HGF in vivo potently suppresses T cell priming by CII/CFA. DBA/1 male mice were sensitized with CII/CFA and a few hours later, received a s.c. injection of gelatin (control) or gelatin/rhHGF (HGF) complex on day 0. On day 10, spleen cells were obtained from each group of mice and spleen cells (5×10^6 cells/ml) were restimulated with CII (10 μg/ml) in vitro. *A*, Cell proliferation after 3 days of incubation was measured by BrdU incorporation. Data are expressed as a percentage of the response compared with that of spleen cells from control mice. *B* and *C*, Production of IFN-γ after 3 days of incubation (*B*) and IL-10 after 4 days of incubation (*C*) was measured by ELISA. Data were obtained from four wells per group of mice. ##, $p < 0.01$ (vs control mice).

in vitro with CII. Spleen cells obtained from the mice treated with HGF demonstrated significantly reduced cell proliferation (Fig. 2*A*) and IFN-γ production (Fig. 2*B*). The production of IL-10 by spleen cells from mice treated with HGF also tended to decrease compared with that by cells from control mice (Fig. 2*C*). At this time point, IL-4 production was very low. We obtained almost the same results using femoral LN cells instead of spleen cells (data not shown). In preliminary experiments, we confirmed that the s.c. injection of HGF protein (10 μg/mouse/day) once daily on days 0–9 had no effect on CII/CFA-induced T cell priming (data not shown). These results indicated that the controlled release of HGF using the gelatin/rhHGF complex could suppress Ag-induced T cell priming independently of the kind of Ag and mouse strain and that this immunosuppressive effect might be exhibited without up-regulation of IL-10 production.

HGF significantly suppresses Ag-induced DC activation

We previously reported that HGF significantly suppressed DC functions such as Ag presentation and cytokine production, thus inhibiting OVA-induced not only Th2-type immune responses but also Th1-type immune responses (24). In the present study, we examined the mechanism of immunosuppression by HGF in CII/CFA-induced sensitization. DBA/1 mice were sensitized and treated as described above, and on day 10 DCs were purified from each group of mice. Then cytokine production by DCs after in vitro LPS stimulation was examined. Treatment with the HGF complex in vivo significantly suppressed the production of IL-10 (Fig. 3*A*), IL-12p70 (Fig. 3*B*), and IL-23 (Fig. 3*C*) by DCs after LPS stimulation. Moreover, compared with DCs from control mice, DCs from HGF-treated mice demonstrated a significantly decreased capacity to induce the proliferation of $CD4^+$ T cells (Fig. 3*D*) and the production of IL-10 (Fig. 3*E*) and IFN-γ (Fig. 3*F*) from $CD4^+$ T cells obtained from the CII/CFA-sensitized mice in the presence of CII in the medium. Moreover, we also confirmed that CD40 expression was reduced in DCs obtained from HGF-treated mice compared with that in DCs from control mice (Fig. 3*G*). These results suggested that HGF significantly suppressed DC function in the early stages of the Ag-induced immune response, thus suppressing Ag-induced $CD4^+$ T cell activation.

HGF up-regulates IL-10 production by immunocytes under continuous Ag stimulation

Next, we examined the effect of HGF on Ag-primed T cells using ex vivo and in vitro experiments. In ex vivo experiments, mice were sensitized with CII/CFA on day 0, received gelatin or gelatin/rhHGF complex on days 0 and 10, and spleen cells were collected on day 20 from each group of mice. Then the spleen cells were restimulated in vitro with CII. Spleen cells obtained from the mice treated with HGF demonstrated significantly increased IL-10 production (Fig. 4*A*). The production of IFN-γ by spleen cells from mice treated with HGF tended to decrease compared with that of cells from control mice (Fig. 4*B*). IL-4 production by spleen cells from each group of mice was very low and did not differ between each group at this time point (data not shown). We also confirmed that $CD4^+$ T cells obtained on day 20 from the mice treated with HGF demonstrated significantly increased IL-10 production after nonspecific PMA and ionomycin stimulation (Fig. 4*C*). Moreover, we examined the cytokine profile of splenic DCs purified on day 20 and found that IL-10 production by DCs from mice treated with HGF tended to increase compared with that of DCs from control mice (Fig. 4*D*), while IL-12p70 production by DCs was as significantly suppressed by HGF as it was on day 10 (Fig. 4*E*). These results indicated that, under continuous Ag-stimulation, HGF could induce IL-10-producing immunocytes including T cells and DCs. To confirm this possibility, we then conducted in vitro studies. Spleen cells obtained on day 10 from CII/CFA-sensitized mice were restimulated in vitro with CII in the presence or absence of HGF in the medium. Like the treatment with HGF in vivo, HGF in vitro significantly up-regulated IL-10 (Fig. 4*F*) production by splenocytes without affecting IFN-γ and IL-4 production (Fig. 4*G*).

HGF significantly reduces IL-17 production by T cells

We also examined the effect of HGF on the production of IL-17 by T cells. The femoral LN cells from HGF-treated mice produced significantly less IL-17 than those from control mice on days 10 (Fig. 5*A*) and 20 (Fig. 5*B*), although no significant difference was detected in spleens (data not shown).

Controlled release of HGF significantly suppresses development of CIA in mice

Then, we examined the effect of HGF on the development of experimental arthritis. DBA/1 mice were sensitized with CII/CFA on day 0 and received a booster injection of CII/IFA on day 21. Mice received s.c. injections of gelatin or gelatin/rhHGF complex on day 0 and every 10 days. The severity of the arthritis in the mice was scored on a scale of 0–4 for each limb. Progression of the

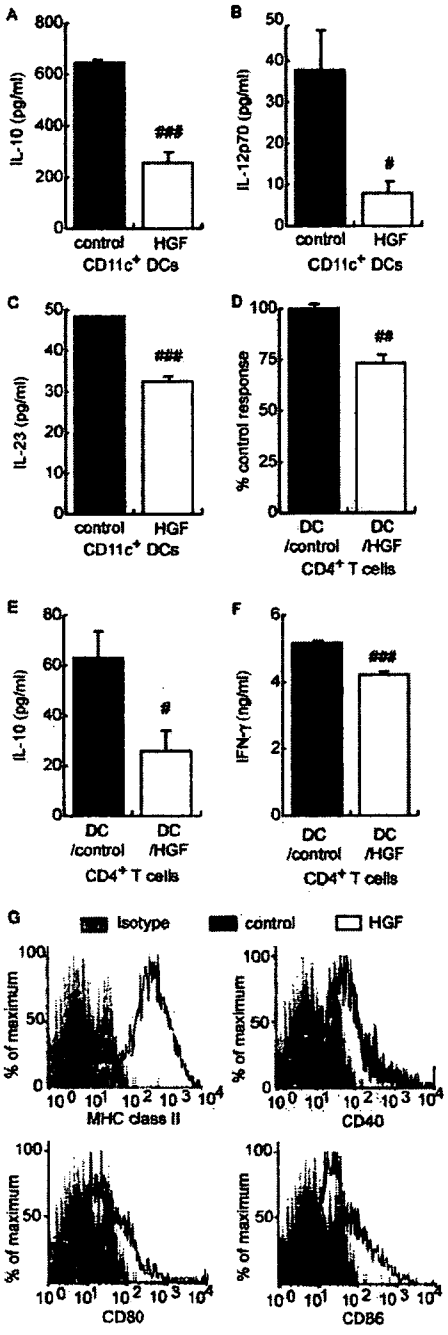

FIGURE 3. Controlled release of HGF in vivo potently suppresses DC functions, thus down-regulating Ag-induced $CD4^+$ T cell activation. Mice were treated as described in Fig. 2. On day 10, $CD11c^+$ DCs and $CD4^+$ T cells were purified from spleen cells as described in *Materials and Methods*. Then, the functions of DCs from each group of mice were examined. *A–C*, Cytokine production by DCs after LPS stimulation in vitro. DCs (1×10^6 cells/ml) from each group of mice were stimulated with LPS (1 μg/ml) in vitro. After 2 days, IL-10 (*A*), IL-12p70 (*B*), and IL-23 (*C*) in the supernatants were measured. *D–F*, Effects of DCs from each group of mice on the cell proliferation of and cytokine production by primed $CD4^+$ T cells. $CD4^+$ T cells (1×10^6 cells/ml) were obtained from control mice and cocultured with DCs (1×10^5 cells/ml) from each group of mice in the presence of CII (3 μg/ml for *D* and 10 μg/ml for *E* and *F*) in the medium. After 3 days (*D*), the cell proliferation of $CD4^+$ T cells was measured. After 4 days of incubation, the production by $CD4^+$ T cells of IL-10 (*E*) and IFN-γ (*F*) was measured. Data were obtained from three to four wells per group of mice. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ (vs DCs from control mice). *G*, Effect of HGF on surface molecule expression on

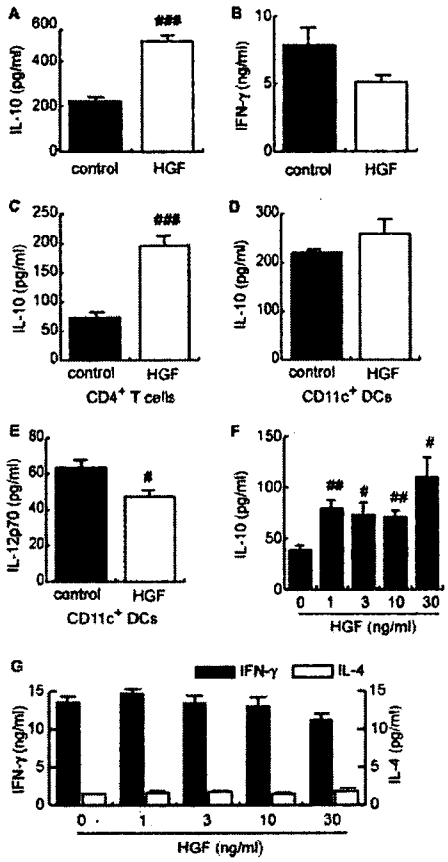

FIGURE 4. HGF significantly increased IL-10 production by Ag-primed immunocytes. *A–E*, Effect of treatment with HGF in vivo after Ag priming on cytokine production by spleen cells, $CD4^+$ T cells, or DCs. Mice were sensitized with CII/CFA on day 0. Mice also received gelatin (control) or gelatin/HGF complex (HGF) on days 0 and 10. On day 20, whole spleen cells, splenic $CD4^+$ T cells, or DCs were obtained from each group of mice. Then, spleen cells (5×10^6 cells/ml) were restimulated with CII (10 μg/ml) in vitro. Production of IL-10 (*A*) and IFN-γ (*B*) after 4 days of incubation was measured. $CD4^+$ T (1×10^6 cells/ml) cells were stimulated in vitro with PMA (1 ng/ml) and ionomycin (0.1 μg/ml) for 2 days, and IL-10 concentrations in the supernatants were measured (*C*). DCs (1×10^6 cells/ml) were stimulated with LPS (1 μg/ml) for 2 days, and IL-10 (*D*) and IL-12p70 (*E*) concentrations in the supernatants were measured. Data were obtained from four wells per group of mice. *F* and *G*, Effect of in vitro treatment with HGF on cytokine production by spleen cells induced by Ag restimulation. Mice were sensitized with CII/CFA on day 0, and spleen cells were obtained on day 10. Spleen cells (5×10^6 cells/ml) were restimulated with CII (10 μg/ml) in vitro in the presence or absence of rhHGF at several concentrations for 4 days. Concentrations of IL-10 (*F*), IFN-γ (■), and IL-4 (□) (*G*) in the supernatant were measured. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ (vs spleen cells, $CD4^+$ T cells, or DCs from control mice, respectively).

arthritis was evaluated until day 39 after immunization. On day 40, the most severely swollen hind paw was obtained from each mouse, and a histologic examination was conducted. HGF treatment significantly suppressed the severity (Fig. 6*A*) and incidence (Fig. 6*B*) of CII-induced arthritis. Histologic examination demonstrated that HGF potently reduced articular destruction such as cartilage destruction, synovial hypertrophy, pannus formation, and

$CD11c^+$ DCs. The expression of MHC class II, CD40, CD80, and CD86 was examined by flow cytometry. Representative data from three independent experiments are shown.

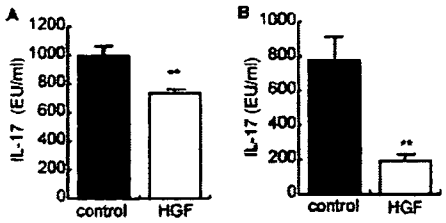

FIGURE 5. Treatment with gelatin/HGF complex in vivo potently suppresses IL-17 production. Mice were sensitized with CII/CFA and a few hours later received a s.c. injection of gelatin (control) or gelatin/rhHGF complex (HGF) on day 0. On day 10, femoral LN cells were obtained from each group of mice. Some mice also received additional treatment with gelatin (control) or gelatin/rhHGF complex on day 10 and femoral LN cells were obtained on day 20. Then the cells obtained on the indicated days were restimulated with CII (10 μg/ml) in vitro for 4 days and IL-17 concentrations in the supernatants were measured. IL-17 production by LN cells obtained from control mice on day 10 was defined as 1000 EU. *A*, IL-17 production by LN cells obtained on day 10. *B*, IL-17 production by LN cells obtained on day 20. ##, $p < 0.01$ (vs control mice).

bone erosion (Fig. 6, *C–F* and Table II). HGF significantly reduced CII-specific total IgG (Fig. 6*G*) and IgG2a (Fig. 6*H*) production. In a preliminary experiment, we confirmed that the s.c. injection of

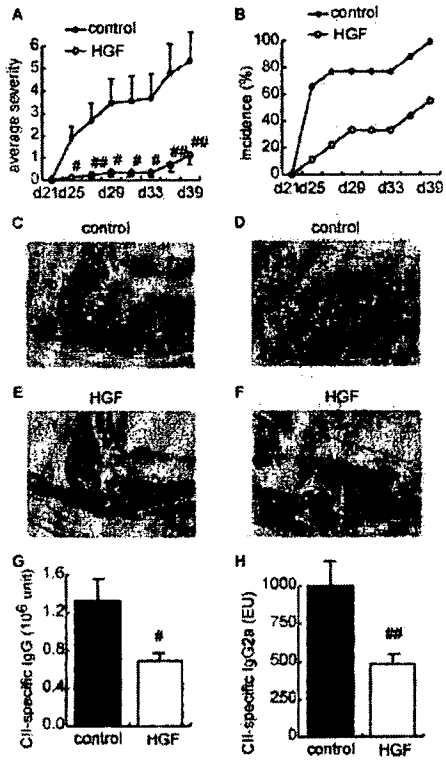

FIGURE 6. Treatment with gelatin/HGF complex in vivo significantly suppresses development of CIA. Arthritis was induced in DBA/1 mice by immunization with CII in Freund's complete adjuvant on day 0. On day 21, mice were injected s.c. with CII in Freund's incomplete adjuvant. Mice also received gelatin (control; $n = 9$) or gelatin/HGF complex (HGF: $n = 9$) on day 0 and every 10 days. *A*, Arthritis scores in the two groups. Clinical scores were determined as described in *Materials and Methods*. *B*, Incidence of arthritis in the two groups. *C–F*, H&E staining of representative hind paws from control mice (*C* and *D*) and mice treated with gelatin/HGF complex (*E* and *F*). Original magnification: ×16 for *C* and *D* and × 32 for *E* and *F*. *G* and *H*, CII-specific total IgG (*G*) and IgG2a (*H*) concentration in the sera obtained from each group of mice on day 40. Data were obtained from nine mice per group. #, $p < 0.05$; ##, $p < 0.01$ (vs control mice).

Table II. *Impact of treatment with HGF in the murine CIA model*[a]

Pathologic Category	Control	HGF
Cartilage	1.33 ± 0.441	0.111 ± 0.111[b]
Cellularity	1.22 ± 0.521	0.222 ± 0.222
Pannus	1.11 ± 0.455	0.111 ± 0.111
Bone erosion	1.11 ± 0.484	0.111 ± 0.111

[a] Data are the mean ± SEM pathologic score from nine animals per group (0, normal; 1, minimal; 2, mild; 3, moderate; and 4, marked).
[b] $p < 0.05$ vs control mice (Mann-Whitney *U* test).

HGF protein (10 μg/mouse/day) once daily on days 0–40 had no suppressive effect on the development of CII-induced arthritis (data not shown). These results indicated that controlled release of HGF could suppress Ag-induced arthritis.

Continuous treatment with HGF during Ag-induced chronic inflammation enhances Th2-type immune responses

Finally, we elucidated the mechanism of suppression by HGF in the chronic phase of arthritis. Mice were sensitized and then treated as described above. On day 40, spleen cells were obtained from each group of mice and restimulated in vitro with CII. Spleen cells obtained on day 40 from the mice treated with HGF demonstrated significantly reduced cell proliferation (Fig. 7*A*) and enhanced IL-10 production (Fig. 7*B*) in response to in vitro CII restimulation. Interestingly, in this chronic phase of Ag-induced

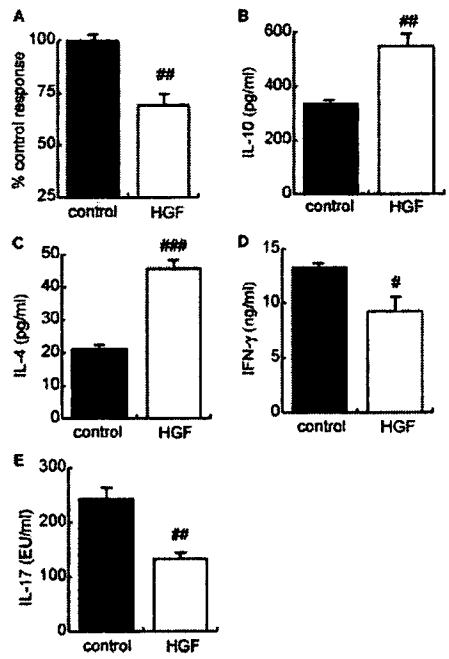

FIGURE 7. In vivo treatment with gelatin/HGF complex (HGF) in the presence of persistent Ag stimulation enhances Ag-specific Th2-type immune responses. Mice were treated as described in Fig. 6. On day 40, spleen cells were collected from each group of mice. *A–E*, Spleen cell responses to in vitro CII (10 μg/ml) stimulation were examined. *A*, Cell proliferation after 3 days of incubation. Data are expressed as a percentage of the response compared with that of spleen cells from control mice. *B–E*, Concentrations of IL-10 (*B*) and IL-4 (*C*) after 5 days of incubation, IFN-γ after 4 days of incubation (*D*), and IL-17 (*E*) after 3 days of incubation in the supernatants were measured. Data were obtained from four wells per group of mice. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ (vs spleen cells from control mice).

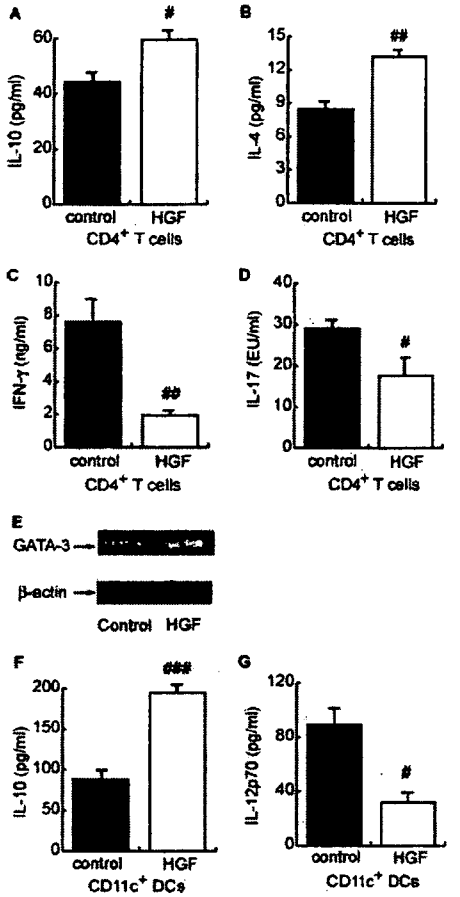

FIGURE 8. Effect of repeated treatment with gelatin/HGF complex (HGF) in vivo on cytokine production by $CD4^+$ T cells and DCs. Mice were treated as described in Fig. 6. On day 40, splenic $CD4^+$ T cells and DCs were purified from each group of mice. Then, $CD4^+$ T cells (1×10^6 cells/ml) were stimulated with PMA (1 ng/ml) and ionomycin (0.1 μg/ml) and IL-10 production after 1 day of incubation (*A*), IL-4 production after 20 h of incubation (*B*), and IFN-γ (*C*) and IL-17 (*D*) production after 2 days of incubation were measured. *E*, GATA-3 mRNA expression in $CD4^+$ T cells. RNA was extracted from splenic $CD4^+$ T cells and then RT-PCRs for GATA-3 and β-actin were conducted. *F* and *G*, DCs were stimulated with LPS (1 μg/ml) for 2 days, and IL-10 (*F*) and IL-12p70 (*G*) concentrations in the supernatants were measured. Data were obtained from three to four wells per group of mice. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ (vs $CD4^+$ T cells or DCs from control mice, respectively).

immune response, spleen cells obtained from control mice produced a significant amount of IL-4 in response to Ag restimulation, and spleen cells from HGF-treated mice demonstrated significantly enhanced production of IL-4 after Ag restimulation (Fig. 7*C*) with down-regulation of cytokine production for IFN-γ (Fig. 7*D*) and IL-17 (Fig. 7*E*). Further, the cytokine profiles of $CD4^+$ T cells from each group of mice after PMA and ionomycin stimulation (Fig. 8, *A–D*) were the same as those of spleen cells after CII restimulation (Fig. 7, *B–E*). We also confirmed that treatment with HGF enhanced mRNA expression of the transcription factor GATA-3, which is known as a master gene for Th2 cell development (45), in splenic $CD4^+$ T cells obtained on day 40 (Fig. 8*E*). Moreover, we found that continuous treatment with HGF in vivo significantly increased IL-10 production (Fig. 8*F*) and decreased IL-12p70 production (Fig. 8*G*) by DCs after LPS stimulation. These results indicated that repeated treatment with HGF in chronic inflammation could induce Th2-type immune responses with up-regulation of IL-10 production by DCs.

Discussion

The results of the present study clearly demonstrated that HGF strongly suppresses collagen-induced immune responses, thus attenuating experimental arthritis. In the early phase, systemic delivery of HGF suppressed the activation of DCs in the spleen that was provoked by sensitization with CII, thus down-regulating CII-induced $CD4^+$ T cell activation. During continuous Ag stimulation, HGF up-regulated IL-10 production by immunocytes. Further, the delivery of HGF attenuated the severity and incidence of arthritis in the CIA model with down-regulation of IL-17 production. To our knowledge, this is the first report that clearly demonstrates the effect of HGF on immune-mediated arthritis.

The presentation of Ag by APCs to T cells initiates the differentiation of naive Th cells into the effector T cells. During the differentiation into each phenotype such as Th1, Th2, or regulatory T (Treg) cells, the expression of costimulatory molecules on APCs and the cytokine profile produced by APCs play a critical role (46). Among various APCs, DCs are most efficient and crucial (47).

Recent articles reported the effect of HGF on DC functions (24, 48). Rutella et al. (48) reported that, in in vitro experiments, HGF suppresses alloantigen-presenting capacity, modulates the costimulatory molecule expression and cytokine production of DCs, and generates DCs that induce Treg cells ("tolerogenic DCs"). In contrast, we reported that HGF potently suppresses Ag-presenting capacity and IL-12p70 production of DCs, thus inhibiting the development of both Th1- and Th2-type immune responses induced by OVA (24).

In the present study, we confirmed that treatment with HGF in vivo suppressed the production of both IL-10 and IL-12p70 by CII/CFA-induced DCs (Fig. 3, *A* and *B*). When the DCs and $CD4^+$ T cells were cocultured in the presence of CII, DCs from HGF-treated mice showed a reduced capacity to present Ag to $CD4^+$ T cells (Fig. 3*D*) and to induce IFN-γ and IL-10 production by CII/CFA-primed $CD4^+$ T cells compared with DCs obtained from CII/CFA-sensitized control mice (Fig. 3, *E* and *F*). Moreover, we also found that HGF decreased CD40 expression on DCs (Fig. 3*G*), which was consistent with our previous study (24). We also confirmed that HGF potently inhibited CII/CFA-induced T cell priming (Fig. 2). Based on these results, in a situation such as Ag-induced T cell priming in which DCs play an essential role, HGF would suppress immune responses through down-regulation of DC function.

Then, with continuous Ag stimulation, HGF up-regulated IL-10 production by immunocytes including T cells (Fig. 4, *A*, *C*, and *F*). IL-10 is an immunosuppressive and regulatory cytokine (49–51). This is consistent with a recent report that HGF reduced acute and chronic rejection of allografts with the increased expression of IL-10 in a mouse model of allogenic heart transplantation (22). The exact mechanism of induction of IL-10-producing T cells remains unclear. Generally, exogenous IL-10 itself plays an important role in the induction of IL-10-producing T cells (50, 51). In our study, HGF did not directly increase IL-10 production when added to cocultures of DCs and $CD4^+$ T cells obtained from CII/CFA-sensitized control mice on day 10 in the presence of CII (data not shown). HGF did not increase PMA and ionomycin-induced production of IL-10 by $CD4^+$ T cells obtained from CII/CFA-sensitized mice (data not shown). Moreover, to clarify whether IL-10 was produced by $Foxp3^+$ Treg cells, we also examined the percentage and the absolute number of $CD4^+$ ($CD25^+$) $Foxp3^+$ cells in the spleens or draining LNs of each group of mice on days 10, 20, and 40. We found that treatment with HGF in vivo did not

increase $CD4^+Foxp3^+$ Treg cells in spleens and LNs in the present study (data not shown). Treatment of splenocytes with HGF in vitro during CII restimulation did not increase $Foxp3^+$ Treg cells either. In contrast, repeated treatment with HGF in vivo gradually increased IL-10 production by DCs (Figs. 4*D* and 8*F*). These results indicated that the augmented IL-10 production by $CD4^+$ T cells was not mediated by $Foxp3^+$ Treg cells but, at least in vivo, by up-regulation of IL-10 production by DCs after repeated HGF treatment. The precise mechanism of induction of IL-10-producing $CD4^+$ T cells by HGF is not clear at present and should be further investigated.

IL-10 also enhances the formation of Th2 cells by down-regulating IL-12 production by DCs (52). Moreover, some reports also emphasize the importance of IL-10 in the induction of Th2 cells (53, 54). As described above, after T cells were primed with Ag, HGF in the presence of continuous Ag stimulation increased IL-10 production by immunocytes, including DCs, along with suppression of IL-12 production by DCs (Figs. 4, 7, and 8), indicating that under continuous Ag stimulation HGF could induce Th2-type immune responses in the chronic phase. In fact, in the chronic phase of CII-induced immune responses, repeated treatment with HGF up-regulated both IL-4 and IL-10 production in T cells (Figs. 7 and 8). These results were consistent with a recent report that HGF ameliorates the progression of experimental autoimmune myocarditis with the induction of Th2 cytokines (23). We also confirmed that HGF enhanced mRNA expression of GATA-3, which specifies Th2 cell development, in $CD4^+$ T cells in the chronic inflammatory phase (Fig. 8*E*). Th2-type immune responses suppress Th1-type immune responses (55), and a recent study reported that IL-4 significantly suppresses the development of Th17 cells, a new subset of effector $CD4^+$ T cells distinct from Th1 or Th2 cells (56). However, in the current study we found that neutralization of IL-4 in vitro did not increase IL-17 production by splenocytes after CII restimulation (data not shown). Collectively, HGF would enhance Th2-type immune responses in chronic inflammation, thus inhibiting both Th1- and Th17-type responses at least in vivo.

Recent studies clarified that IL-17 produced by Th17 cells has a crucial role in the induction of autoimmune tissue injury (30–32, 57, 58). Accumulating evidence indicates that IL-17 plays an essential role not only in the induction of autoimmune arthritis (30, 31) but also in the subsequent bone destruction (32). In the current study, HGF potently suppressed IL-17 production by draining LN cells after in vitro CII restimulation in the early stage of Ag-induced immune responses (Fig. 5). Further, in addition to the sensitization phase, even in the chronic inflammation phase with joint destruction HGF significantly suppressed IL-17 production by spleen cells (Fig. 7*E*). Moreover, HGF significantly suppressed DC production of IL-23 (Fig. 3*C*), which is now recognized as a very important cytokine for IL-17 secretion from activated $CD4^+$ T cells (57, 59). These results indicated that HGF would be beneficial in treating autoimmune arthritis.

TGF-β is an immunosuppressive growth factor. Some phenotypes of T cells function as Treg cells by producing TGF-β. In contrast, the role of TGF-β in the induction of the Th17 cell lineage to promote an autoimmune response has been recently highlighted (52, 60, 61). Generally, HGF counteracts the biological functions of TGF-β such as promoting fibrosis (13, 14). In the immune response, however, the relation between HGF and TGF-β differs among experimental systems. HGF suppresses acute and chronic rejection in a mouse model of cardiac allograft transplantation with unexpectedly enhanced expression of TGF-β mRNA (22). In contrast, in allergic airway inflammation HGF did not up-regulate TGF-β production in the lung (24). In the present study on arthritis, HGF reduced mRNA expression of TGF-β in $CD4^+$ T cells at both early and chronic phases (data not shown).

Generally, exogenously administered HGF proteins vanish from organs within several hours (44). In a preliminary study, we confirmed that s.c. injection of HGF protein (10 μg per mouse) once daily failed to suppress the Ag-induced T cell priming and development of CII-induced arthritis (data not shown). Previously, we used a hydrodynamic-based transfer system to deliver HGF effectively and confirmed that a slight but continuous up-regulation of HGF protein in the sera potently suppressed OVA/alum-induced T cell priming and allergic airway inflammation (24). However, this delivery system could not be applied to an experimental model of arthritis due to an anatomical narrowing of the tail vein provoked by injection of CII/CFA into the subcutis of the tail. Thus, to achieve a controlled release of HGF, we adopted biodegradable gelatin hydrogels as carriers of HGF. We previously confirmed that when this gelatin/HGF complex was s.c. injected into mice, HGF was delivered under a controlled release based on hydrogel degradation and that the degradation occurred over 10 days (37). We reconfirmed that controlled release of HGF was achieved using a gelatin/rhHGF complex by examining the time course of concentration of HGF in the sera (Table I). In this study, the controlled release of HGF potently suppressed Ag-induced T cell priming and development of CII-induced arthritis. Thus, gelatin hydrogels would be an ideal carrier for HGF to exhibit its biological effects, and further application in various models can be expected.

Pulmonary fibrosis is often associated with RA and is one of the major causes of death in RA patients (62). To date, several articles, including our own, reported that HGF inhibits the progression of experimental pulmonary fibrosis (15, 63, 64). Considering the simultaneous effect on pulmonary fibrosis and arthritis, HGF could be an attractive tool in treating RA with pulmonary involvement in a clinical situation. In contrast, in the clinical use of HGF the possibility of promoting tumor progression should be considered. Therefore, for practical usage of HGF in clinical situations further studies should be performed.

In summary, our results in the present study indicated that HGF could exhibit its immunosuppressive effects in different manners at different stages of immune response. In the early phase of Ag-induced immune responses HGF potently suppressed DC function, thus inhibiting T cell priming by Ag. In contrast, during chronic inflammation HGF gradually increased IL-10 production by DCs, which subsequently induced IL-10 producing T cells and Th2-type immune responses. The precise mechanism should be further investigated in detail.

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Disclosures

The authors have no financial conflict of interest.

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