immune modulation. Indeed, α-GalCer could prevent the onset and recurrence of Th1-skewed autoimmune disease models, such as type I diabetes in non-obese diabetic (NOD) mice [20, 21]. In this murine model, both IL-4 and IL-10 production from iNKT cells conferred protection against the development of diabetes [22]. In fact, defects of IL-4 production by Vα24 iNKT cells has been shown in diabetic patients, suggesting a correlation [23].

Although the mechanisms for controlling the multivalent functions of iNKT cells still remain unclear, some recent studies give us a clue to understanding *in vivo* iNKT cell function. Fujii *et al.* demonstrated that pretreatment of normal mice with intravenous injection of free α-GalCer inhibited the induction of IFN-γ-producing iNKT cells in α-GalCer-pulsed DCs injected mice [24]. And, Kojo *et al.* found that repeated intraperitoneal injections of α-GalCer generated splenic DCs of an immature phenotype that secreted IL-10, but not IL-12 [25]. Furthermore, Sonoda *et al.* showed in an experimental model of anterior chamber-associated immune deviation (ACAID) that iNKT cells, marginal zone B cells and their IL-10 production were required for the development of systemic immune tolerance [26, 27]. The results suggest that when the anti-tumor effects of iNKT cells, including IFN-γ production, are desired, α-GalCer should be delivered to DCs.

In contrast, for the treatment of allergic and autoimmune diseases, in which the reduction of iNKT cell inflammatory effects would be beneficial, α-GalCer should be targeted to cells other than DCs. In this review, we evaluate recent progress and the future perspective of the clinical application for iNKT cells and their ligands.

I. Treatment of Allergic Diseases with Liposomal α-GalCer

IFN-γ production by iNKT cells can attenuate the Th2 response seen in allergic diseases. Because of this, α-GalCer and its analogues have been studied as a potential adjuvant in the treatment of allergic conditions. A previous report showed that co-administration of the ragweed allergen Amb a 1 and immunostimulatory DNA sequences (ISS)-oligonucleotide (ODN) was much less effective in inducing a Th1 response, whereas the Amb a 1-ISS conjugated one induced de novo Th1 response and suppressed IgE antibody formation after challenge with Amb a 1 [28]. Then, attempts were made to generate α-GalCer-liposomes encapsulating OVA protein (liposomal-α-GalCer-OVA). Either liposomal α-GalCer or liposomal α-GalCer-OVA was intraperitoneally injected into groups of mice three days prior to immunization with alum-absorbed dinitrophenylated-ovalbumin (DNP-OVA). Results showed that primary antibody responses were completely suppressed by pretreatment with liposomal α-GalCer or liposomal α-GalCer-OVA, but not with α-GalCer or liposomes [29]. The results suggest that liposomal α-GalCer might use a novel mechanism different from the antigen-specific Th1-skewing effect of α-GalCer, because IgG2a antibody responses were completely repressed.

II. A Mechanism for Liposomal-α-GalCer-Mediated Primary Antibody Suppressive Responses

To investigate the mechanism of liposomal α-GalCer suppression, splenic $CD4^+$ T cells were obtained from mice after immunization with either liposomal α-GalCer with or without DNP-OVA or aqueous α-GalCer with or without DNP-OVA. The pretreatment with liposomal α-GalCer prevented T cell priming with DNP-OVA, however the pretreatment with aqueous α-GalCer enhanced the T cell priming with the antigen [29]. Splenic DCs taken from mice treated with liposomal α-GalCer showed expansion of splenic $CD11c^{low}CD45RB^{high}$ cells, which are consistent with either $CD180^+$ immature DCs or $CD49b^+$ cytotoxic cells [30]. The expansion of these cells by liposomal α-GalCer was not seen in mice deficient in IL-10 or iNKT cells, suggesting the importance of IL-10 secretion and iNKT cells in the generation of immature DCs.

To identify the source of IL-10 after treatment with liposomal α-GalCer, splenic DCs, B cells and macrophages were prepared and co-cultured with iNKT cells from normal mice. The concentrations of IFN-γ, IL-4 and IL-10 in culture supernatants were determined. DCs from the liposomal α-GalCer-treated mice induced the formations of more cytokines than those from the aqueous α-GalCer-treated mice [29]. On the other hand, when B cells from the liposomal α-GalCer-treated mice were co-cultured with NKT cell fractions, IL-10 became detectable in the culture supernatant [29]. IL-10 was undetectable in cultures in which anti-CD1d antibody was added, suggesting the importance of CD1d-associated α-GalCer presentation by B cells to iNKT cells for IL-10 production. In contrast, B cells from mice treated with aqueous α-GalCer barely induced the formation of IL-10 when they were co-cultured with NKT cell fractions [29]. The difference in IL-10 production between B cells from the liposomal α-GalCer-treated mice and those from the aqueous α-GalCer-treated mice is probably due to the enhanced uptake of α-GalCer by B cells.

In spite of the inhibitory effects of iNKT cells in allergic responses, it was shown that pulmonary iNKT cells produced IL-4 and IL-13, and played an essential role for the development of allergen-induced air way hyperresponsiveness (AHR) [31]. Indeed, direct activation of pulmonary iNKT cells in a model of α-GalCer-induced AHR in BALB/c mice has been observed [32], although the mechanism is not fully understood. However, in a recent study, Terashima *et al.* described that a novel subset of pulmonary iNKT cells expressing IL-17 receptor B (RB) that responds to IL-25 and is essential for the induction of AHR in BALB/c mice [33]. These results may suggest that systemic administration of α-GalCer would not activate pulmonary iNKT cell subsets responsible for allergic inflammation.

The immunosuppressive effects seen with liposomal α-GalCer, therefore, are triggered by the interaction of iNKT cells with α-GalCer-loaded splenic B cells including marginal zone B cells, which express high levels of CD1d. For an application of these findings into a treatment of allergic diseases such as pollinosis and asthma, specific antigens are encapsulated into liposomal α-GalCer because they are indispensable for the generation of antigen-specific Treg cells that are essential for the establishment of long term immune tolerance against the antigens (Fig. **1**).

III. The Use of α-GalCer-Pulsed DCs to Generate Anti-Tumor Immunity

α-GalCer-pulsed DCs have been shown to be more effective in generating a cytotoxic immune response than α-GalCer alone in pre-clinical cancer studies [34]. A single

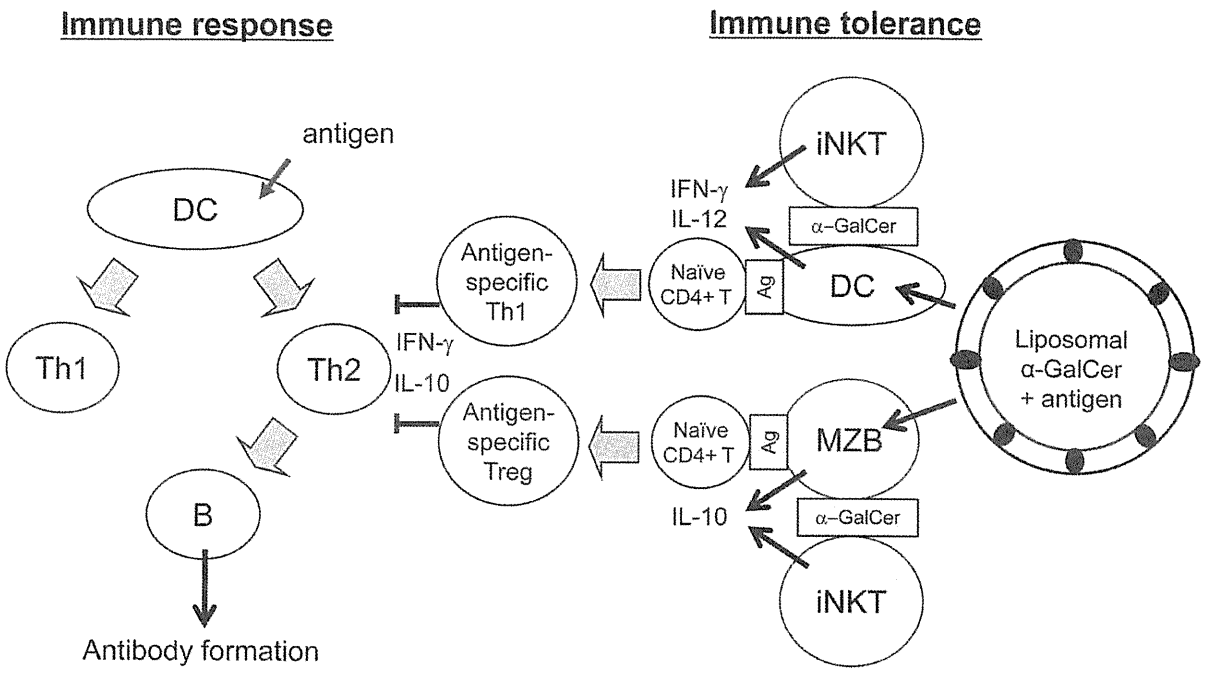

Fig. (1). Generation of antigen-specific Treg cells by liposomal α-GalCer encapsulating antigens. α-GalCer in liposomal formulation, but not aqueous formulation, preferentially enters into marginal zone B (MZB) cells. After uptake of liposomal α-GalCer encapsulating antigens, MZB cells present both α-GalCer on CD1d and antigenic peptide (Ag) on MHC class II, and simultaneously interact with both iNKT cells and Ag-specific CD4+T cells. Then, antigen-specific Treg cells are differentiated from naïve T cells in the presence of IL-10 produced by the interaction of MZB with iNKT cells and suppress helper T (Th) cell activation in an antigen-specific manner.

injection of α-GalCer-pulsed DCs (DC/Gal) induced a prolonged burst of IFN-γ secretion by iNKT cells, peaking at 4 days in murine models. IL-4 secretion was also seen in mice given DC/Gal, but the number of IL-4-producing cells was significantly less than those producing IFN-γ [24]. Furthermore, in a murine model of lung cancer, DC/Gal induced expansion of iNKT cells in the lung and inhibited *in vivo* tumor growth [35].

IV. Clinical Immunotherapy with α-GalCer-Pulsed Antigen Presenting Cells (APCs)

Because of the early promise of iNKT cell immunotherapy in murine lung cancer models, a phase I dose escalation study of α-GalCer-pulsed APCs was initiated in patients with primary lung cancer at Chiba University Hospital [36]. Whole PBMCs from patients were cultured with GM-CSF and IL-2 for 1 or 2 weeks to generate antigen presenting cells including DCs (α-GalCer-pulsed APCs). These cells were then pulsed with α-GalCer for use in studies [37].

In the first clinical study, 11 patients with advanced or recurrent non-small cell lung cancer (NSCLC) refractory to the standard treatment were enrolled. Patients were treated with APCs without α-GalCer on day 0. On day 7, 14, 56 and 63, patients were administered with α-GalCer-pulsed APCs (level 1, $5x10^7/m^2$; level 2, $2.5x10^8/m^2$; and level 3, $1x10^9/m^2$) intravenously. No severe adverse events were observed in any patients. After the first and second injection of α-GalCer-pulsed APCs, a dramatic increase in the peripheral blood Vα24 iNKT cells was observed in one case and modest increase in two other cases receiving the level 3 dose of cells. Increased IFN-γ mRNA from circulating Vα24 iNKT cells was confirmed after the first and second injections of α-GalCer-pulsed APCs. Although no objective tumor regression was found, one patient with a striking increase of Vα24 iNKT cells in peripheral blood demonstrated an intra-thoracic lesion that remained stable for more than two and half years with a good quality of life (Table **1**). This study verified that therapy using α-GalCer-pulsed APCs was well tolerated and could be used safely even in patients with advanced disease.

Based on these results, a phase I-II clinical trial of 17 patients with recurrent and advanced NSCLC was initiated using α-GalCer-pulsed APCs ($1x10^9/m^2$, at the maximal dose of previous study) [38]. No severe adverse event related to the treatment was observed. Six out of 17 patients showed a remarkable increase in the number of circulating Vα24 iNKT cells after the administration of α-GalCer-pulsed APC. The number of IFN-γ producing cells in PBMCs after restimulation with α-GalCer *in vitro* was assessed by an enzyme-linked immunospot (ELISPOT) assay. In 10 patients, IFN-γ-producing cells were obviously increased and these patients showed prolonged overall survival compared to the patient group with no increase in IFN-γ producing cells. In contrast, neither baseline Vα24 iNKT cell number nor the extent of Vα24 iNKT cell expansion appeared to correlate with the outcome of survival time (Table **1**).

Table 1. Immunological Responses and Anti-Tumor Effects

	Disease	Treatment	Number of Patients	Immunological Responses	Anti-Tumor Effects	Ref.
Ishikawa *et al.*	NSCLC	α-GalCer-APC	11	iNKT cell expansion (3) augmentation of IFN-γ mRNA level (1)	SD (3)	[36]
Motohashi *et al.*	NSCLC	α-GalCer-APC	17	iNKT cell expansion (5) augmentation of IFN-γ spot forming cells (10)	SD (5), prolonged overall survival	[38]
Uchida *et al.*	HNSCC	α-GalCer-APC	9	iNKT cell expansion (4) augmentation of IFN-γ spot forming cells (8)	PR (1), SD (7)	[39]
Kunii *et al.*	HNSCC	α-GalCer-APC and iNKT cell	8	iNKT cell expansion (7) augmentation of IFN-γ spot forming cells (7)	PR (3), SD (4)	[40]

Abbreviations: NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; SD, stable disease; PR, partial response. The number of positive cases was depicted in the parentheses.

To expand on these findings, we also performed a study with nasal submucosal injections of α-GalCer-pulsed APCs in patients with head and neck squamous cell carcinoma [39]. After α-GalCer-loaded APCs were injected into the nasal submucosa, they quickly migrated to the ipsilateral regional neck lymph nodes and remained, which should be sufficient for the induction of regional anti-tumor immune responses. α-GalCer-pulsed APCs were administered into the inferior turbinate submucosa, which were ipsilateral to the tumor, in patients with unresectable or recurrent head and neck squamous cell carcinoma. Nine patients received 2 times of α-GalCer-pulsed APCs. Relatively smaller numbers of APCs were able to increase the number of Vα24 iNKT cells and IFN-γ-producing cells in peripheral blood as measured by ELISPOT (Table **1**). In addition to the immune responses, positive clinical effects were observed for the first time (Table **1**).

V. Combination Immunotherapy with α-GalCer-Pulsed APCs and *In Vitro* Activated Vα24 iNKT Cells

Based on these promising results, we tested whether adoptive immunotherapy with *in vitro* expanded Vα24 iNKT cells would further augment the anti-tumor effects seen with active immunotherapy [40]. A phase I clinical study was carried out in eight patients with locally recurrent head and neck squamous cell carcinoma (HNSCC) refractory to standard treatments. The primary endpoint of this study was to test the safety and efficacy of intra-arterial infusion of *in vitro* expanded Vα24 iNKT cells into tumor-feeding arteries combined with nasal submucosal injections of α-GalCer-pulsed APCs into the ipsilateral nasal submucosa. A grade 3 pharyngocutaneous fistula related to local tumor reduction was observed in one patient, otherwise no severe adverse events were observed. The number of Vα24 iNKT cells and interferon-γ-producing cells in peripheral blood increased in seven out of eight patients (Table **1**). Furthermore, three cases exhibited objective tumor reduction, four were classified as stable disease, and one patient was evaluated as having progressive disease (Table **1**). Then, the use of the intra-arterial infusion of activated Vα24 iNKT cells and the submucosal injection of α-GalCer-pulsed APC has been shown to induce the beneficial clinical effects in the management of advanced HNSCC.

VI. Adjuvant Effect of iNKT Cell Activation Toward Adaptive Immunity

Systemic application of adjuvants such as α-GalCer [41], CD40-specific antibody [42] and Toll-like receptor (TLR) ligands [43] enhance $CD8^+$ T cell responses to co-administered antigens. Recent studies show that innate lymphocytes, such as NK cells, iNKT cells, and γδ T cells, activate and mature DCs after detecting infected or transformed cells [44].

We have studied ways of directing specific tumor antigens linked with maturation stimuli to *in vivo* DCs in murine models. α-GalCer is capable of activating iNKT cells *in vivo*, thus enabling them to mature DCs *via* cytokine secretion and cell-cell interactions. Even transient activation of iNKT cells is sufficient for the development of adaptive T cell immunity. Innate lymphocyte activation as well as TLR stimuli has been evaluated for use as adjuvants in DC-based vaccines.

We have shown that adoptive transfer of tumor cells loaded with α-GalCer led to both innate and adaptive immunity after tumor antigen cross presentation by host DCs [45]. Specifically, administration of B16 melanoma cells loaded with α-GalCer (B16/Gal) or B16 melanoma cells transduced to express high levels of CD1d and loaded with α-GalCer ($CD1d^{hi}$B16/Gal) protected mice from the development of B16 lung metastases after tumor challenge. This resistance to tumor challenge continued for more than 6 months after vaccination and has been replicated using a number of different tumor cell lines. In all cases, this resistance was dependent on NK and iNKT cells. Thus, mice given i.v. tumor cells loaded with α-GalCer (tumor/Gal) developed innate iNKT cell-dependent immunity against subsequent i.v. tumor challenge, as expected. But, these mice also became resistant to subcutaneous challenge with tumor cells in tumor challenge models [17, 46]. After tumor regression occurred, T cells responding to a variety of specific tumor antigens remained as memory T cells. $CD4^+$ and $CD8^+$ T cell-mediated resistance to tumor seen in the previous mouse model was due to antigen uptake by host DCs and their subsequent maturation by activated iNKT cells. DC maturation leads to an upregulation of co-stimulatory molecules, enhancement of antigen presenting

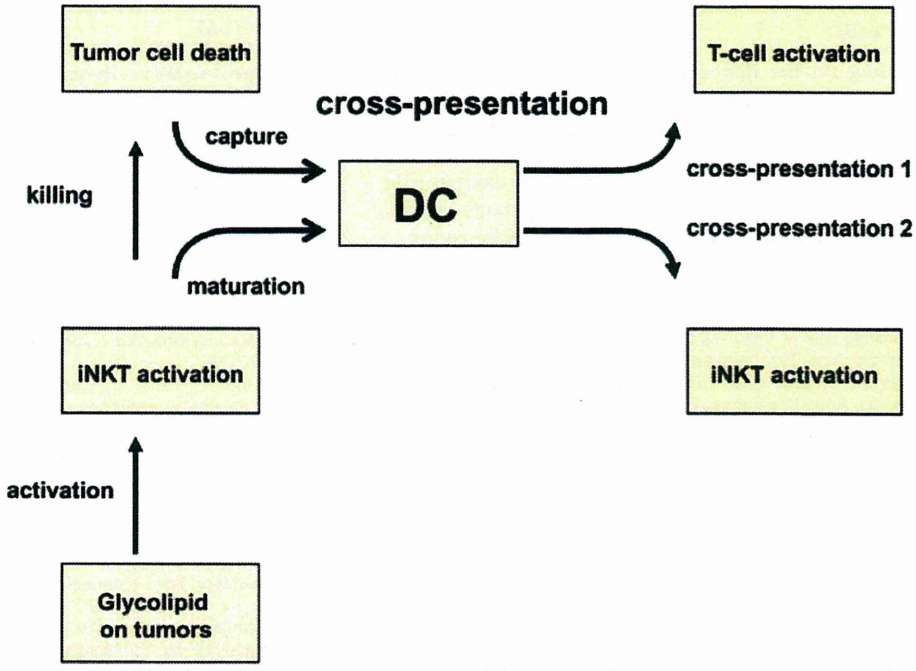

Fig. (2). Activation of iNKT cells to adaptive immunity as an adjuvant effect through DC cross presentation. After intravenous injection of a low dose of α-GalCer-loaded tumor cells, NK and NKT cells in the spleen become activated and kill tumor cells. Immature DCs then capture released tumor antigens and undergo maturation *via* cell-cell interactions and pro-inflammatory cytokines. DCs process tumor antigens and present them in the context of MHC molecules to $CD8^+$ and $CD4^+$ T cells (cross presentation 1). DCs also present the ligand α-GalCer to iNKT cells (cross presentation 2). The two types of cross presentation augment the antitumor immune response.

capacity and expression of chemokine receptors that promote migration to nodal T cell-areas. The injection of tumor/Gal in this study elicited two forms of cross-presentation: the presentation of antigen peptide to $CD8^+$ T cells and the presentation of glycolipids to iNKT cells (Fig. **2**). Both *ex vivo* and *in vivo* DC immunotherapies have been well tolerated in clinical trials and offer new strategies for the treatment of cancer.

In addition, other groups have also focused on the adjuvant effects of iNKT cell activation. A combination therapy with α-GalCer, anti-DR5 Ab and anti-4-1-BB Ab successfully resulted in eradication of tumor cells [47]. This therapy combined an agonistic Ab reactive to tumor necrosis factor related, apoptosis-inducing ligand receptor (DR5) expressed by tumor cells, α-GalCer to mature DCs, and an agonist anti-4-1-BB Ab to costimulate $CD8^+$T cells. Another combination therapy used α-GalCer and the TLR4 ligand, monophosphoryl lipid A, and demonstrated an increase in antigen specific $CD8^+$T cells [48]. Stirneman *et al.* showed that α-GalCer-loaded, soluble CD1d (sCD1d/Gal)-fused anti-HER2-scFv fusion protein demonstrated antigen specific killing of HER-2-expressing tumor cells in an antigen specific manner [49]. Such findings are encouraging for the development of future immunotherapeutic strategies.

CONCLUSION

Because iNKT cells bridge innate and adaptive immunity, they are important potential targets for therapies that modulate the immune response. Ishii *et al.* demonstrated that vaccines with liposomal α-GalCer induced iNKT cells to produce IL-10 and subsequently diminish the magnitude of allergic responses [29]. Using this strategy, a liposomal α-GalCer vaccine encapsulating a recombinant cedar antigen is currently under investigation for the treatment of seasonal allergies.

Furthermore, a number of clinical trials have investigated the anti-tumor properties of iNKT cell immunotherapy. Studies of iNKT cell-based immunotherapy using α-GalCer-loaded, GM-CSF and IL-2-stimulated PBMCs in patients with NSCLC demonstrated clinical safety and efficacy. Cancer patients receiving this therapy had prolonged stable disease with extended time to tumor progression [36, 38, 50, 51]. Treatment strategies which utilize tumor antigen and α-GalCer recapitulate the natural course of an immune response in which immature DCs take up antigen and are subsequently matured by activated iNKT cells *via* CD40-CD40L signaling and pro-inflammatory cytokines. Several recent studies have highlighted the promise of this approach.

The immune dysregulation that allows tumor development is complex and includes local tumor effects that inhibit immune surveillance by the innate immune system. For example, $CD4^+$ type II NKT cells have shown to produce IL-13 in tumor-bearing mice [52]. It is believed that IL-13 secreted from NKT cells activates $CD11b^+Gr\text{-}1^+$ myeloid suppressor cells to produce TGF-β, which directly suppresses CTL-mediated tumor rejection [53]. Moreover, in a ConA-hepatitis model, sulfatide-generated type II NKT cells also trigger tolerance through plasmacytoid DC-induced anergic type I NKT cells in a CD1d-dependent manner [54], although type II NKT cells have not been well characterized in humans. Therefore, in the future, we need to

make a combination strategy to inhibit such kinds of suppressor cells.

Depending on the delivery vehicle used for α-GalCer, iNKT cells can be manipulated to either inhibit the immune response as in the treatment of autoimmune and allergic diseases or augment it as in the treatment of cancer and infectious diseases. This flexibility and the unique role linking the innate and adaptive immune systems makes iNKT cells an important target for future immunotherapies.

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Increase of regulatory T cells and the ratio of specific IgE to total IgE are candidates for response monitoring or prognostic biomarkers in 2-year sublingual immunotherapy (SLIT) for Japanese cedar pollinosis

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Abstract The aims of this study were to examine the therapeutic effects of sublingual immunotherapy (SLIT) and to identify potential biomarkers that would predict the therapeutic response in a randomized, double-blind, placebo-controlled clinical trial. The trial was carried out over two pollinosis seasons in 2007 and 2008. Carry-over therapeutic effects were analyzed in 2009. SLIT significantly ameliorated the symptoms of pollinosis during the 2008 and 2009 pollen seasons. Cry j 1-specific cytokine production in a subgroup of patients with mild disease in the SLIT group was significantly attenuated. The ratio of specific IgE to total IgE before treatment correlated with the symptom-medication score in the SLIT group in 2008. Patients with increased

Abbreviations: DBPC, double-blind, placebo-controlled; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; iTreg, induced regulatory T cells; ITT analysis, intention-to-treat analysis; JAU, Japanese allergy unit; N.S., not significant; OT analysis, on-treatment analysis; PBMCs, peripheral blood mononuclear cells; RAST, radioallergosorbent test; SLIT, sublingual immunotherapy; SMS, symptom-medication score; Treg, regulatory T cells; QOL, quality-of-life.

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Cry j 1-iTreg in the SLIT group had significantly improved QOL and QOL-symptom scores. In summary, the specific IgE to total IgE ratio and upregulation of Cry j 1-iTreg are candidates for biomarker of the clinical response to SLIT.

1. Introduction

Japanese cedar (*Cryptomeria japonica*) pollinosis is a common allergy in Japan, with a prevalence estimated to be 26.5% in a nationwide survey conducted in 2008 [1].

A 2000 Japanese allergy unit (JAU) sample of standardized extract from Japanese cedar pollen is the only available allergen for subcutaneous and sublingual immunotherapy (SLIT) against pollinosis in Japan. The 2000 JAU extract contains 1.5 to 4.2 μg of the major allergen, Cry j 1 [2]. The common monthly cumulative dose for SLIT is 8000 JAU, which contains approximately 10 μg of Cry j 1. This maintenance dose is 200-fold higher than that used in traditional subcutaneous immunotherapy using 0.2 ml of a 200 JAU/ml extract, which contains approximately 50 ng of Cry j 1. Despite using a low dose of the major allergen compared with that in European trials, positive effects on pollinosis have been shown in randomized double-blind, placebo-controlled (DBPC) studies, in which SLIT significantly ameliorated the symptom score, symptom-medication score (SMS), and quality-of-life (QOL) score [3,4].

SLIT induces Cry j 1-specific IgG4 production and attenuates the seasonal increase in the number of Th2 cells specific to epitopes from Cry j 1 and Cry j 2 [3]. Involvement of antigen-specific Tr1 cells or regulatory T cells (Treg) in the therapeutic mechanism has also been suggested [5,6]. We previously found that SLIT increased the levels of Cry j 1-specific induced Treg cells (Cry j 1-iTreg; $IL10^+Foxp3^+$ cells in $CD25^+CD4^+$ leukocytes) and that the increase in Cry j 1-iTreg after the pollen season may serve as a response monitoring biomarker that correlates with a positive therapeutic effect based on the QOL-symptom score and distinguishes responders from non-responders after SLIT [6].

In this report, we examined the reproducibility of the positive therapeutic effects and safety of SLIT and upregulation of iTregs as a response monitoring biomarker, with the goal of confirming our previous results in a larger randomized DBPC study. Therefore, the safety and clinical effect of SLIT for Japanese cedar pollinosis were used as the primary endpoint, and carry-over effects, immunological changes, and biomarkers for a positive clinical effect induced by SLIT were secondary endpoints.

2. Materials and methods

2.1. Study population

The study was conducted as a randomized, DBPC, parallel-group, single center trial in subjects with Japanese cedar pollinosis. This study was performed for two pollen seasons between September 2006 and May 2008, with follow-up in the pollen season in 2009. We recruited 130 participants in September 2006. Diagnosis of Japanese cedar pollinosis was based on clinical history and the presence of IgE specific to Japanese cedar pollen of at least class 2 (CAP-RAST method, Phadia, Tokyo, Japan). Participants with a history of immunotherapy or a diagnosis of asthma, or those who were pregnant, were excluded from the study. Patients who suffered seasonal or chronic rhinitis that required medical treatment were also excluded.

A total of 103 patients were eligible for the study, and all had moderate or severe symptoms in the previous pollen season [7]. We anticipated that some participants in the SLIT group would drop out from the study due to side effects and we planned to evaluate the risk of mild or severe side effects due to the vaccination. Therefore, we randomly divided the patients into treatment (SLIT) and placebo groups with a ratio of 6:4 according to the table of random numbers prepared by the Department of Pharmacy at Chiba University Hospital (Fig. 1). The sample size was determined based on a previous study [3]. Briefly, we planned to have 50 patients in each group with anticipation of dropout. We set 1.0 as a magnitude for the difference of average SMS between that from the SLIT and placebo groups and 1.5 as a standard deviation according to the result of previous study. Therefore, when the power was set to 0.8 and the α-error to 0.05, the number of required cases was 35 in each group. A person who was not directly involved in the study was responsible for group allocation. To prevent leakage of information, the allocation table was kept by this person and a member of the ethics committee who was also not directly involved in the study, until accessed with the key after completion of the study. The protocol was approved by the Ethics Committee of Chiba University, and written informed consent was obtained from each patient prior to participation in the study.

2.2. Clinical protocols

The SLIT group included 58 patients who received standardized Japanese cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) [8], and the placebo group included 45 patients who received an inactive placebo. The protocol consisted of treatment with graded courses of the extract in 50% glycerol, followed by maintenance therapy [6]. Briefly, the extract was graded in three strengths: 20, 200, and 2000 JAU/ml. Patients received increasing doses with each vial, beginning with 0.2 ml from the 20 JAU/ml vial and increasing by 0.2 ml a day for 5 days per week. The vaccine was taken sublingually, kept in place for 2 min without a retention reagent, and then spit out. The procedure was repeated until the maximum dose (1.0 ml of 2000 JAU/ml) was reached. The maintenance dose was 1.0 ml of 2000 JAU/ml given once a week until the end of May 2008. The patients in the placebo group received inactive 50% glycerol in saline. All participants were allowed to take symptom-reducing drugs as needed.

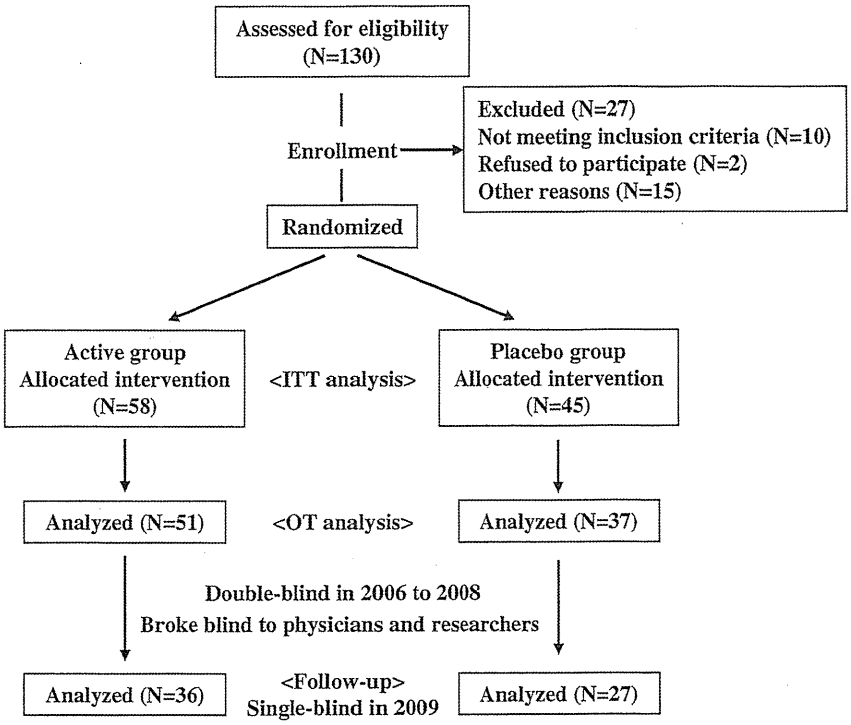

Figure 1 Flow diagram for groups and individuals in the phases of the randomized trial. Fifteen participants from the SLIT ($N=7$) and placebo ($N=8$) groups were lost to follow-up due to reasons such as moving house and transfer. The double-blind status was maintained until completion of analysis of all clinical and immunological parameters (December 2008). Follow-up analysis in 2009 was undertaken in a single-blind manner.

2.3. Clinical symptoms and safety measurements

The patients completed a pollinosis diary to record their nasal symptoms and use of symptom-reducing drugs in the 2007, 2008, and 2009 pollen seasons. The total amounts of pollen scattered from Japanese cedar and Japanese cypress (*Chamaecyparis obtusa*) in Chiba prefecture were 2777, 6596, and 5486 grains/cm^2 during the 2007, 2008, and 2009 pollen seasons, respectively, based on measurements with a Durham pollen sampler. The duration and amount of scattered Japanese cedar pollen differed greatly among these years, but the daily amount of scattered pollen typically followed a wide-based bell-shaped curve over the whole pollen season from the middle of January or early February to the middle or end of May. The duration of the peak pollen season was relatively constant in the 3 years, and therefore, we analyzed the SMS during the peak period. The peak pollen season was defined as the period from the first day that the pollen count was $\geq$20 grains/cm^2/day for 3 consecutive days until the last day that the pollen count was $\geq$20 grains/cm^2/day before a period in which the pollen count was <20 grains/cm^2/day for 7 consecutive days.

The daily SMS was calculated as described previously [3]. Briefly, daily episodes of sneezing and nose blowing were rated as 0–4: none, 0; 1–5 episodes, 1; 6–10 episodes, 2; 11–20 episodes, 3; >20 episodes, 4. Daily medication was recorded based on drug types and duration of usage using the following guidelines: antihistamines, mast cell stabilizers, and vasoconstrictors, 1; topical ocular or nasal steroids, 2. Patients with an average daily SMS in the peak pollen season of $\leq$4 were judged to have mild symptoms based on guidelines for allergic rhinitis [7].

In the middle of the 2007 and 2008 pollen seasons, the participants completed the Japanese Allergic Rhinitis QOL Standard Questionnaire No.1 (JRQLQ No.1) for assessment of QOL-symptom and total QOL scores [9]. These scores were calculated as previously described [4,6]. The total QOL-symptom score was calculated as the sum of each component score: none, 0; mild, 1; moderate, 2; severe, 3; and very severe, 4. Nasal and ocular symptoms covered by the questionnaire included runny nose, sneezing, nasal congestion, itchy nose, itchy eyes, and watery eyes. Adverse events were graded using Common Terminology Criteria for Adverse Events (CTCAE) v.3.0 [10]. Briefly, adverse events were graded as mild, grade 1; moderate, grade 2; severe, grade 3; life threatening, grade 4; death, grade 5 according to a category for allergy/immunology in the CTCAE v.3.0 scoring system.

2.4. Blood samples

Peripheral blood was obtained from each patient before treatment (September to October 2006) and before and after the pollen seasons in 2007 (December 2006 to January 2007, and May to June 2007, respectively) and 2008 (November to December 2007, and May 2008, respectively). Peripheral blood mononuclear cells (PBMCs) were isolated, frozen, and stored in liquid nitrogen [6]. However, the PBMCs isolated before treatment, and before and after the 2007 pollen season were damaged during storage and we were unable to

analyze their immunological responses. Therefore, immunological data were obtained only from PBMCs collected before and after the 2008 pollen season.

2.5. Total and antigen-specific immunoglobulin titer

The Cry j 1-specific IgE and IgG4 titers in plasma were measured by ELISA [3,11]. Total IgE and specific IgE titers for Japanese cedar, orchard grass, mugwort, and house dust mites were evaluated by the CAP-RAST method (Phadia).

2.6. Flow cytometric analysis

The levels of Cry j 1-iTreg were analyzed by flow cytometry [6]. Briefly, PBMCs were cultured with or without Cry j 1 for 3 days, followed by a culture with 10 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin, and 2 μM monensin for 6 h. The PBMCs were stained with PE-Cy7-anti-CD4 antibody, APC-anti-IL10 antibody (BD Biosciences, San Diego, CA, USA), PE-anti-CD25, and FITC-anti-Foxp3 (clone: PCH101) using a Foxp3 staining buffer set (eBioscience, San Diego, CA, USA).

2.7. Analysis of the number of IL4-producing cells and the concentration of cytokines

The number of IL4-producing cells stimulated with Cry j 1 was determined by enzyme-linked immunospot (ELISPOT) assay, and the concentrations of IL2, IL5, and IL13 in the culture supernatant were measured using a BD™ Cytometric bead assay (CBA) Flex system (BD Biosciences) [6]. Briefly, a 96-well sterile filter plate (Millipore, Billerica, MA, USA) was coated with monoclonal antibody to human IL4 (Mabtech AB, Nacka Strand, Sweden). The plate was pre-incubated with AIM-V medium at 37 °C for 1 h. The medium was discarded, and then PBMCs (3×10^5 cells/well) were cultured with fresh medium alone or with 10 μg/ml Cry j 1 for 17 h at 37 °C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich, St. Louis, MO, USA). The plates were then incubated with a biotinylated monoclonal antibody to human IL4 for 2 h, and then with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. After washing with PBS, the plates were incubated with BCIP/NBT^{PLUS} (Mabtech) for 5 min at 37 °C. For the CBA, isolated PBMCs were cultured at 2.5×10^6 cells/ml with or without 5 μg/ml Cry j 1 for 3 days at 37 °C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich). After centrifugation at 300×g for 10 min, the supernatant was divided into aliquots and stored at −20 °C until the cytokine assay was performed.

2.8. Data representation

The full analysis set ($N=103$) was used for the intention-to-treat (ITT) analysis and per protocol populations ($N=88$) were used for on-treatment (OT) analysis (Fig. 1). Cry j 1-specific cytokine production is shown as the difference between cells stimulated with Cry j 1 and controls stimulated with medium only. Changes after the 2008 pollen season are shown as differences between pre- and post-pollen season values.

2.9. Statistical analysis

Two-group comparisons were performed using a Wilcoxon *t*-test or Mann–Whitney *U*-test to determine the significance of differences, or using an unpaired *t*-test as indicated. *P*-values <0.05 were considered to be significant.

3. Results

3.1. Clinical effects and adverse events

A total of 103 patients were included in the overall analysis of efficacy for the 2007 and 2008 pollen seasons. These patients were randomly divided into the SLIT ($N=58$) and placebo ($N=45$) groups at a ratio of 6:4. Diaries and QOL questionnaires for 88 patients were available at the end of the DBPC study. The overall randomized population was considered to be the ITT population. The SMS in the SLIT group did not differ significantly from that in the placebo group in ITT analysis after 2-year SLIT (*P*=N.S.; Student *t*-test, data not shown).

The final sample size included 88 subjects for OT analysis (SLIT; $N=51$, placebo; $N=37$, ratio 4:3). The demographic characteristics of the OT population before treatment are shown in Table 1. The SMS in the SLIT group did not differ significantly from that in the placebo group in the 2007 peak pollen season (February 19 to March 31, *P*=N.S.; Student *t*-test). However, the average SMS in the 2008 peak pollen season (February 29 to April 1) was significantly ameliorated in the SLIT group compared with the placebo group (4.2 vs. 5.3, $P=0.02$; Student *t*-test). The percentages of subjects with mild symptoms (SMS ≤ 4) were 55% and 28% in the SLIT and placebo groups, respectively, in the peak pollen

Table 1 Clinical data of participants at the start of the study.

Group	SLIT	Placebo	*P*-value
Number	51	37	
Sex (M/F)	17/34	8/29	N.S.[a]
Mean age	44.4	42.3	N.S.[b]
Range	16–73	19–70	
Total IgE [IU/ml]	198	258	N.S.[b]
Range	6.8–1480	8.6–2090	
Specific IgE[c]	27	29	N.S.[b]
Range	0.8–100	1.5–100	
Class [mean]	3.5	3.8	N.S.[b]
Range	2–6	2–6	
Other allergies[d] (%)			
Orchard grass	16 (31%)	11 (30%)	N.S.[e]
Mugwort	5 (10%)	3 (8%)	<0.05[f]
House dust mite	24 (47%)	13 (35%)	N.S.[e]

[a] Yates2×2 Chi-squared test.
[b] Student *t*-test.
[c] Specific IgE to Japanese cedar pollen; CAP-RAST raw value [kAU/L], mean.
[d] Number of subjects with specific IgE of at least CAP-RAST class 2.
[e] 2×2 Chi-squared test.
[f] Fisher exact probability.

season (Fig. 2A). QOL-symptom and total QOL scores were also significantly ameliorated in the SLIT group compared to those in the placebo group in the middle of the 2008 pollen season (Fig. 2B).

There were no severe adverse events that required a patient to withdraw from the study; however, some subjects reported adverse events of mild discomfort: six of grade 2 (oral pruritus: 2; gingivostomatitis: 2; asthma: 1; rash in nasal cavity: 1) in the SLIT group (6/51; 11.8%); and one of grade 1 (bitter taste) in the placebo group (1/37; 2.7%).

3.2. Immunoglobulin production

There were no significant differences in Cry j 1-specific IgE and IgG4 production between patients in the SLIT and placebo groups before treatment, or before and after the pollen seasons. The SLIT group was divided into subgroups based on the SMS in the 2008 peak pollen season: a mild subgroup with SMS ≤ 4 (classified as responders; $N=28$) and a severe subgroup with SMS >4 (non-responders; $N=23$). IgE and IgG4 production in patients in the mild subgroup were both similar to those in patients in the severe subgroup and in the placebo group at various time points (data not shown).

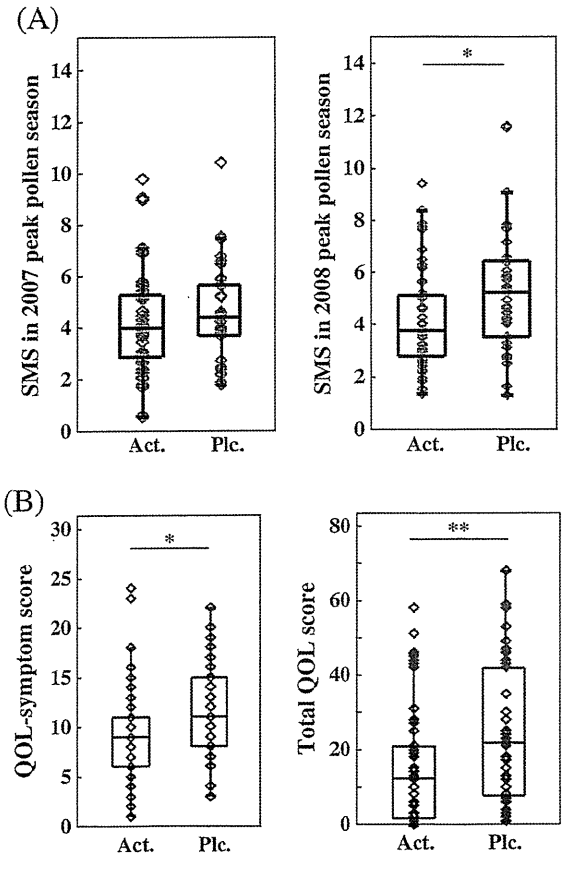

Figure 2 Clinical scores after 2-year SLIT. (A) Average daily symptom-medication scores (SMS) in the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups in the 2007 and 2008 peak pollen seasons. (B) QOL-symptom and total QOL scores from the QOL questionnaire were plotted for the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups in the middle of the 2008 pollen season. Each diamond shows a value for an individual. Two-group comparisons were performed using an unpaired Student t-test. $^{*}P<0.05$, $^{**}P<0.01$.

3.3. Cry j 1-specific cytokine production

IL2, IL5, and IL13 levels were analyzed in the culture supernatant. The number of IL4-producing cells was measured by ELISPOT because IL4 was undetectable in the supernatant. There were no significant differences between the SLIT and placebo groups in the production of each cytokine following stimulation with Cry j 1 (Fig. 3A). IL5 was significantly increased after the pollen season in all groups ($P<0.05$; Wilcoxon t-test), and the IL2 and IL13 levels and the number of IL4-producing cells were significantly increased after the pollen season in the SLIT and placebo groups and in the severe subgroup ($P<0.05$; Wilcoxon t-test). Patients in the mild subgroup (responder to SLIT) did not show significant increase of IL2 and IL13 or of IL4-producing cells after the pollen season (P=N.S.; Wilcoxon t-test). The increases in the number of IL4-producing cells and IL5 level after the pollen season in the mild subgroup were significantly less than those in the severe subgroup (non-responders) and the placebo group. The increase of IL13 in the mild subgroup was significantly less than that in the severe subgroup and showed a tendency to be attenuated compared with the placebo group (P=N.S.; Mann–Whitney U-test). The increase of IL2 in the mild subgroup was significantly less than that in the placebo group ($P<0.05$) and showed a tendency to be attenuated compared with the severe subgroup ($P=0.053$; Mann–Whitney U-test, Fig. 3B).

3.4. Prognostic biomarkers for clinical effects

The average ratio of Japanese cedar pollen-specific IgE to total IgE (sIgE/tIgE ratio) in all patients in the study was 0.193 before treatment. The SLIT group was divided into subgroups with a sIgE/tIgE ratio ≤ 0.19 (low, $N=28$) and >0.19 (high, $N=23$) before treatment. Similar subgroups were established in the placebo group. The SMS in the 2008 peak pollen season for the low subgroup was significantly improved compared to that in the high subgroup in the SLIT group ($P=0.02$; Mann–Whitney U-test); however, in the placebo group, the low and high subgroups had comparable SMSs (P=N.S.; Mann–Whitney U-test, Fig. 4A). Furthermore, the SMS was correlated with the sIgE/tIgE ratio in the SLIT group ($Rs=0.39$, $P<0.01$; Spearman correlation analysis), but not in the placebo group ($Rs=0.08$, P=N.S.; Spearman correlation analysis, Fig. 4B).

3.5. Upregulation of Cry j 1-iTreg levels as a response monitoring biomarker

A population of $IL10^{+}Foxp3^{+}$ cells in $CD25^{+}CD4^{+}$ leukocytes was evaluated as a potential marker for iTreg after stimulation with Cry j 1 or medium only before and after the pollen season in 2008. Neither the changes in Cry j 1-iTreg levels after stimulation with and without Cry j 1 nor the upregulation of Cry j 1-iTreg from pre- to post-pollen season differed significantly different between the groups (data not shown).

We previously reported that upregulation of Cry j 1-iTreg is a candidate biomarker that may distinguish SLIT responders from non-responders based on QOL-symptom scores [6]. Therefore, we divided the SLIT group into subgroups based on an increase (N=24) or decrease (N=27) in Cry j 1-iTreg levels from before to after the pollen season in 2008. QOL-symptom and total QOL scores in the increased iTreg subgroup significantly improved compared with those in the placebo group. In contrast, the scores in the decreased iTreg subgroup were similar to those in the placebo group (Fig. 4C).

3.6. Carry-over effects in the year after treatment

A total of 63 patients completed a pollinosis-symptom diary during the 2009 pollen season; 1 year after the 2-year SLIT treatment (Fig. 1). All participants remained blinded to their treatment with SLIT or a placebo. The SMS in the peak pollen season in 2009 (February 15 to March 6) in the SLIT group (N=36) was significantly attenuated compared to the placebo group (N=27, P=0.03). The average SMSs for the SLIT and placebo groups were 3.5 and 4.5, respectively, in the peak pollen season (Fig. 5).

4. Discussion

The primary endpoint of this randomized DBPC trial was the therapeutic effect evaluated in ITT analysis. No significant positive effect was observed between the SLIT and placebo groups after exchanging the perceived improvement of patients who dropped out with each median score from the counter group. In OT analysis, the SMS in the SLIT group was

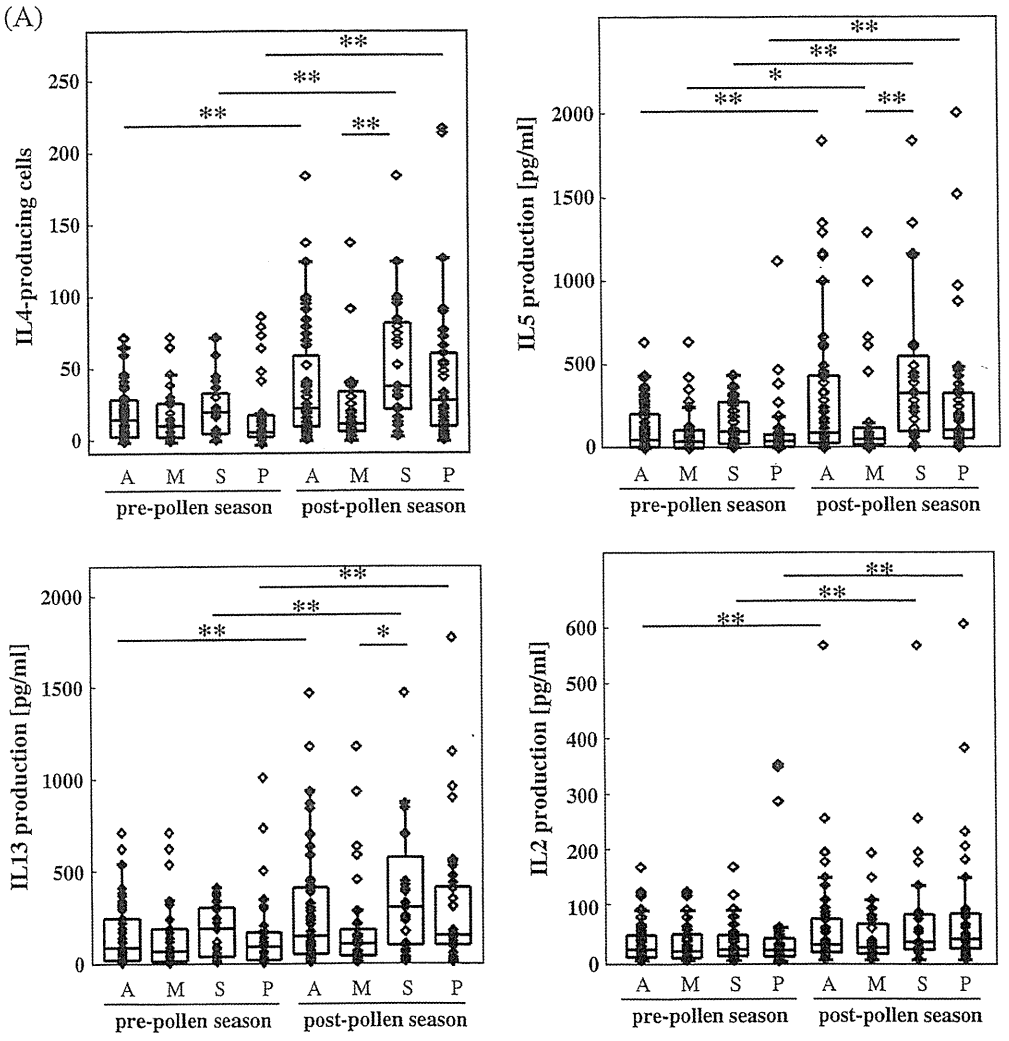

Figure 3 Cytokine production from PBMCs. (A) Number of Cry j 1-specific IL4-producing cells and Cry j 1-specific cytokine levels in the SLIT group (A; N=51), the mild subgroup of the SLIT group (M; N=28), the severe subgroup of the SLIT group (S; N=23), and the placebo group (P; N=37) at before and after the 2008 pollen season. Comparisons with a significant difference are indicated as * and **; otherwise, comparisons are not significantly different (P=N.S.). (B) Increases in the number of Cry j 1-specific IL4-producing cells and Cry j 1-specific cytokine levels occurred from before to after the 2008 pollen season in the SLIT group (Act.; N=51), the mild subgroup of the SLIT group (Mild; N=28), the severe subgroup of the SLIT group (Sev.; N=23), and the placebo group (Plc.; N=37). Each diamond shows the value for an individual. Two-group comparison was performed using a Mann–Whitney U-test. *P<0.05, **P<0.01.

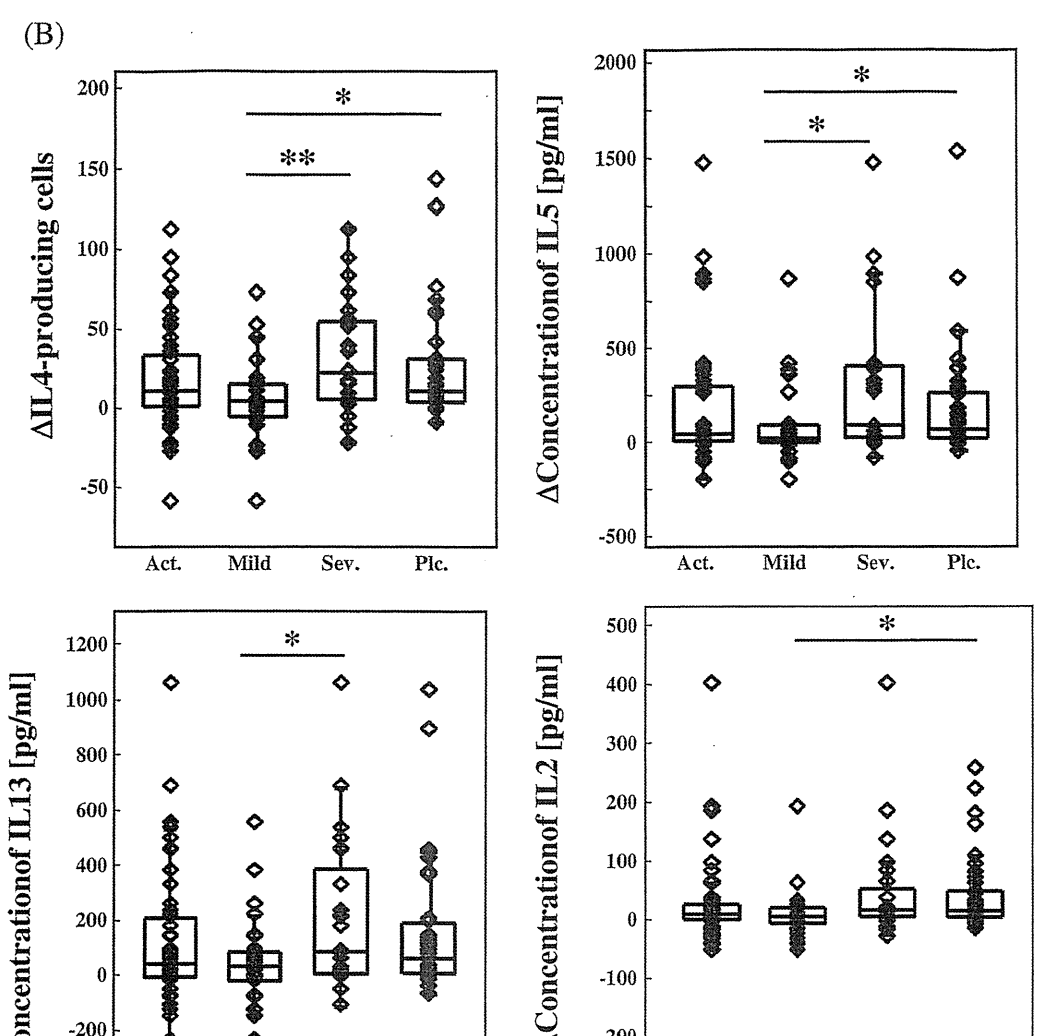

Figure 3 (*continued*).

significantly ameliorated compared to the placebo group in 2008. The percentage of mild subjects (SMS ≤ 4) in the SLIT group was 28% higher than that in the placebo group (SLIT, 55%; placebo, 27%), and the SMS was reduced by approximately 21% in the SLIT group compared with the placebo group (SLIT, 4.2; placebo, 5.3). This percentage of mild subjects differ significantly between the SLIT and placebo groups (P=0.009; 2×2 Chi-squared test). These effects following 2-year treatment were comparable to those in a trial of 1-year daily treatment using grass pollen tablets [12]. The low dose of the extract (about 1/40th of that used in Europe) may be one reason for the poor clinical outcome in the first year [13]. An extract of concentration >2000 JAU is not available for clinical use in Japan, and the clinical effects, safety, and optimum schedule for administration of an extract with a much higher allergen concentration remain unclear.

Positive clinical therapeutic effects were not obtained following 1-year treatment in our study, even in OT analysis (data not shown). In contrast, two previous reports demonstrated positive therapeutic effects after 1-year SLIT for Japanese cedar pollinosis [3,4]. However, in these studies, the annual pollen count (1154 grains/cm^2/season) [3] was less than in our study, and daily SMS was significantly attenuated on only 4 days in the pollen season [4]. The severity of SMS is affected by the amount of Japanese cedar pollen in the total and peak pollen season. Natural resolution and tolerance are not usually induced by natural exposure to Japanese cedar pollen, regardless of the amount of pollen [14].

Whether there are detectable alterations in peripheral T-cell responses after specific immunotherapy is still under debate [15–18]. The Cry j 1-specific cytokine profile from the SLIT group did not differ significantly from that in the placebo group. However, the increases in IL2, IL4, IL5, and IL13 production in the mild subgroup in the SLIT group were significantly attenuated (or showed a tendency to be attenuated) compared to the severe subgroup and the placebo

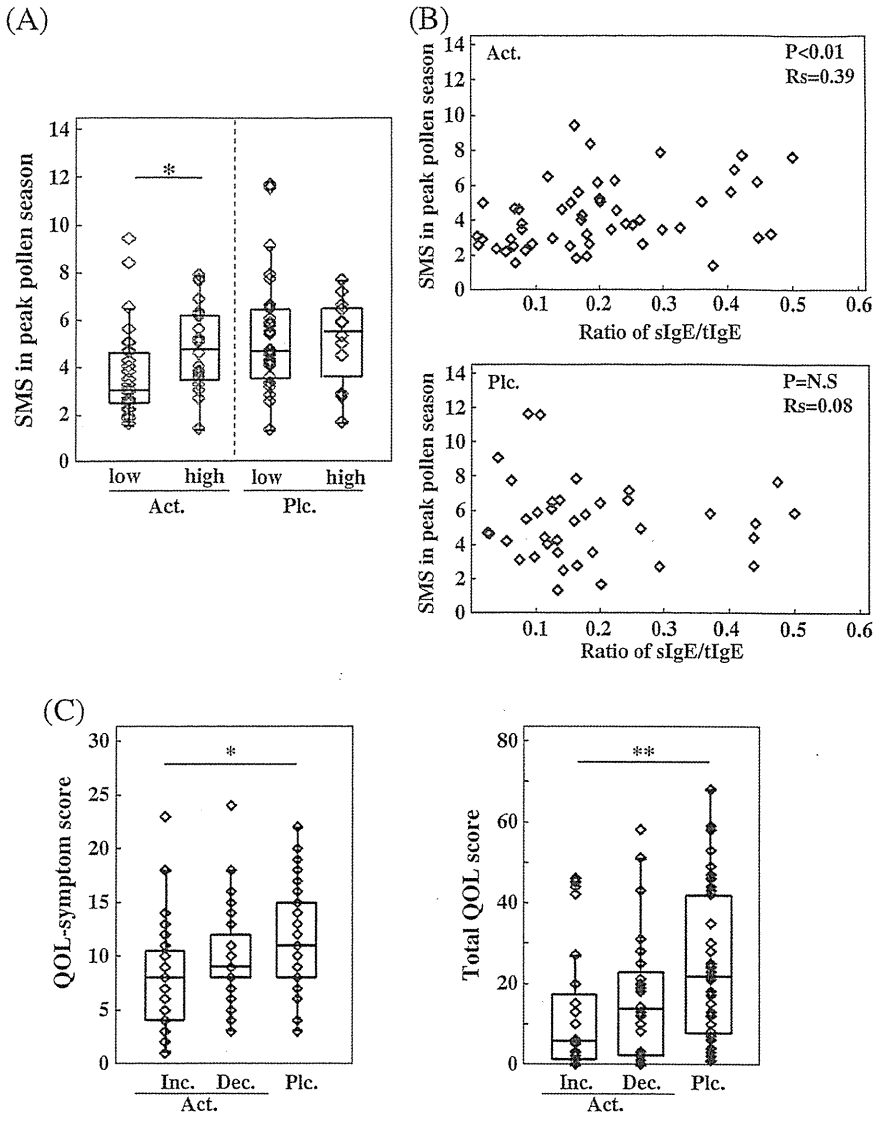

Figure 4 Biomarkers for positive therapeutic effects following SLIT. (A) SMSs in the 2008 peak pollen season for patients with low (low; $N=28$) and high (high; $N=23$) sIgE/tIgE ratios in the SLIT group (Act.), and for those with low ($N=25$) and high ($N=12$) sIgE/tIgE ratios in the placebo group (Plc.). *$P<0.05$. (B) Correlation between SMSs in the 2008 peak pollen season and sIgE/tIgE ratios before treatment in the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups. Statistical data were obtained with Spearman correlation analysis. (C) QOL-symptom and total QOL scores from the QOL questionnaire plotted for a subgroup with increased Cry j 1-iTreg in the SLIT group (Inc.; $N=24$), a subgroup with decreased Cry j 1-iTreg in the SLIT group (Dec.; $N=27$), and the placebo group (Plc.; $N=37$) in the middle of the 2008 pollen season. Each diamond shows the value for an individual. *$P<0.05$, **$P<0.01$.

group (Fig. 3B). The SMS in all patients in the study correlated with the seasonal increases in IL4 ($R=0.35$, $P<0.01$), IL5 ($R=0.35$, $P<0.01$), and IL13 ($R=0.36$, $P<0.01$). The discrepancy in our current results and the results of previous studies with regard to downregulation of cytokine production from PBMCs may depend on the extent of the therapeutic effects achieved in each clinical trial.

Cry j 1-specific IgE production was not changed by treatment, even in the mild subgroup, as also found in our preliminary study [6]. We speculate that more time is required for changing antibody production following the changes of antigen-specific T cell profiles, because the alteration of T cell profiles strongly influences subsequent class switch recombination of B cells and antibody production. Another possibility is that the dose for SLIT used in this study was not high enough to alter the antibody profiles.

The sIgE/tIgE ratio has been found to be significantly higher in responders than in non-responders following 4-year immunotherapy [19]. In our trial, this ratio did not differ significantly between responders and non-responders (P=N.S.; Mann–Whitney U-test). However, subjects with a low sIgE/tIgE ratio before treatment were more likely to be responders to 2-year SLIT, and the ratio correlated with the SMS only in patients treated with SLIT (Fig. 4A, B). This suggests that SLIT was more effective in patients with a low sIgE/tIgE ratio than in those with a high sIgE/tIgE ratio. The range of total IgE levels for the participants were relatively wide (6.8–2090 IU/ml in all patients); however, the change of the total IgE for each

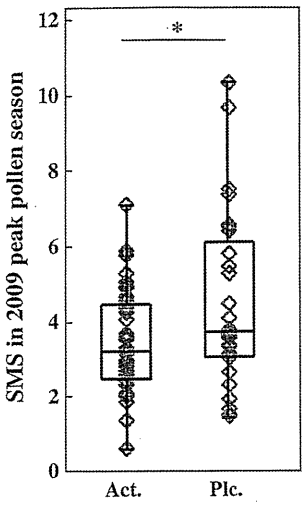

Figure 5 Carry-over effects following 2-year treatment with SLIT. SMSs in the 2009 peak pollen season were plotted for the SLIT (Act.; N=36) and placebo (Plc.; N=27) groups. Each diamond shows the value for an individual. Two-group comparisons were performed using an unpaired t-test.

individuals after 2-year treatment was not significantly different compared to before treatment (1.5±1.0 times higher, P=N.S.; paired t-test). Therefore, the wide range of total IgE levels was due to the variability on the allergic status for individuals, but not on method for measurement. The serum IgE level may affect the surface IgE level on effector cells such as mast cells and basophils, and Tregs can downregulate activation of mast cells and eosinophils [20,21]. We speculate that effector cells with a low specific IgE level are less likely to be activated by antigen crosslinking or are more susceptible to downregulation by Tregs than those with a high specific IgE level. It is also possible that the symptoms of patients with a low sIgE/tIgE ratio may be more readily attenuated by suboptimal potentiation of iTreg induced by SLIT.

We previously reported that an increased count of Cry j 1-iTregs was a candidate biomarker that could be used to distinguish between responders and non-responders to SLIT, as evaluated by the QOL-symptom score. In this report, the subgroup with increased Cry j 1-iTregs showed significant amelioration of the QOL-symptom and total QOL scores compared to the placebo group, while the subgroup with decreased Cry j 1-iTregs did not show this response (Fig. 4C). However, there was no significant difference in Cry j 1-specific cytokine production from PBMCs among patients with increased iTregs and decreased iTregs, and those in the placebo group (data not shown). Foxp3-expressing $CD25^+CD3^+$ cells and IL10-expressing $CD3^+$ cells, which are induced in the nasal mucosa after subcutaneous immunotherapy, have been linked to the clinical efficacy and suppression of seasonal inflammation [22]. Immunotherapy using an Amb a 1-immunostimulatory oligodeoxynucleotide conjugate also induced $CD4^+CD25^+$ T cells and IL10-producing cells in the nasal mucosa after the pollen season [23]. These data suggest that iTregs may downregulate effector cells at local sites of inflammation to suppress clinical symptoms. Induction of iTregs in the nasal mucosa and functional analysis of these cells may be necessary to determine the regulatory mechanisms affected by SLIT. Mucosal biopsy in the peak pollen season is useful for evaluation of local induction of iTregs and downregulation of effector cells. However, nasal biopsy in the pollen season significantly influences the daily SMS in the peak pollen season. Mucosal biopsy outside the pollen season after exposure using an artificial pollen chamber may be a powerful tool for evaluation of local regulatory mechanisms induced by SLIT [24]. Upregulation of iTregs in nasal mucosa may be difficult to determine since the evaluation may be painful for patients. However, upregulation of iTregs in peripheral blood is simple to analyze and may be a useful biomarker because an increase of peripheral Cry j 1-iTregs is correlated with QOL and QOL-symptom scores in the pollen season, as discussed here and elsewhere [6].

Cry j 1-specific IgG4 production was not induced by SLIT in this study to the same extent as that in our previous study [6]. A clinical trial showing that daily 2500 SQ-T (14 μg Phl p 5 per 4 weeks) tablets failed to induce IgG production supports our current results [13]. A change in the immunoglobulin profile may require a higher allergen dose or longer duration of exposure. However, our study suggests that detectable quantitative changes in IgG4 are not essential for the amelioration of clinical symptoms.

In summary, we suggest that the sIgE/tIgE ratio and upregulation of iTregs may be considered as prognostic and response monitoring biomarkers, respectively, for SLIT. However, further investigation of induction of iTregs at local inflammatory sites and downregulation of inflammatory cells is needed. Furthermore, validation studies with larger sample size would be required before either biomarkers should be applied widely in the clinical management of pollinosis patients. Development of a more effective vaccine and better protocols may reveal more significant differences in the Cry j 1-specific cytokine profiles and iTreg induction, and these results may increase our understanding of the roles of iTregs or Tr1 in the therapeutic mechanisms underlying the efficacy of SLIT.

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Apolipoprotein A-II Suppressed Concanavalin A-Induced Hepatitis via the Inhibition of CD4 T Cell Function

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Con A-induced hepatitis has been used as a model of human autoimmune or viral hepatitis. During the process of identifying immunologically bioactive proteins in human plasma, we found that apolipoprotein A-II (ApoA-II), the second major apolipoprotein of high-density lipoprotein, inhibited the production of IFN-γ by Con A-stimulated mouse and human CD4 T cells. Con A-induced hepatitis was attenuated by the administration of ApoA-II. The beneficial effect of ApoA-II was associated with reduced leukocyte infiltration and decreased production of T cell-related cytokines and chemokines in the liver. ApoA-II inhibited the Con A-induced activation of ERK–MAPK and nuclear translocation of NFAT in CD4 T cells. Interestingly, exacerbated hepatitis was observed in ApoA-II–deficient mice, indicating that ApoA-II plays a suppressive role in Con A-induced hepatitis under physiological conditions. Moreover, the administration of ApoA-II after the onset of Con A-induced hepatitis was sufficient to suppress disease. Thus, the therapeutic effect of ApoA-II could be useful for patients with CD4 T cell-related autoimmune and viral hepatitis. *The Journal of Immunology*, **2011, 186: 3410–3420.**

Although nonspecific immunosuppressive drugs are widely used to inhibit autoreactive immune responses, these drugs show numerous side effects during prolonged usage and are therefore not an ideal treatment for autoimmune patients. Autoimmune hepatitis is a progressive chronic disease with occasional exacerbations (1, 2). A standard therapy with prednisolone sodium succinate (prednisolone) alone or in combination with azathioprine as immune suppressive drugs is used for nearly all autoimmune hepatitis patients. However, some patients are resistant to this standard therapy (3, 4), and thus the development of new therapeutic agents is warranted.

T cell-mediated immune responses play an important role in the development and progression of various liver diseases, including autoimmune hepatitis, viral infection, and alcoholic hepatitis (4–10). Con A-induced hepatitis is a murine experimental model of autoimmune or viral hepatitis that shares several pathological properties with the disease in humans (11). Con A-induced hepatitis has also been used as a model of T cell-mediated liver injury (12), and the infiltration of CD4 T cells into the liver is critical for the development of human autoimmune and viral hepatitis (6, 9, 10). The importance of T cells during the induction and effector phases of Con A-induced hepatitis has been well documented (12–15). Pretreatment with T cell-specific Abs or immunosuppressive agents, such as anti–thymocyte differentiation Ag 1, anti-CD4 mAb, FK506 (Tacrolimus), or cyclosporine A, inhibit Con A-induced hepatitis, indicating that CD4 T cells and their activation of TCR-mediated signaling are required for the induction of Con A-induced hepatitis (12). In addition, IFN-γ appears to be important for the development of Con A-induced hepatitis (13–15). Moreover, we reported previously that NKT cells and their production of IFN-γ play an important role in the development of Con A-induced hepatitis (16). Therefore, IFN-γ–producing CD4 T cells and NKT cells appear to be good therapeutic target cells in Con A-induced hepatitis.

High-density lipoprotein (HDL) has been implicated in several cardioprotective pathways and is thought to play a significant role in the removal of excess cholesterol from peripheral tissues and transport to the liver resulting in excretion into the bile (17, 18). HDL is classified according to its content of major apolipoproteins, apolipoprotein A-II (ApoA-II) and apolipoprotein A-I (ApoA-I). ApoA-II is synthesized in the liver and accounts for ~20% of HDL. The mean concentration of ApoA-II in human serum is ~30–35 mg/dl (19), although >20% of patients with coronary artery diseases showed a higher concentration between 40 and 60 mg/dl (19, 20). Studies of the physiological and pathophysiological effects of ApoA-II have focused on reverse cholesterol transport and antioxidant functions, mechanisms through which HDL are believed to protect against atherosclerosis (18, 21). ApoA-I is also the major apolipoprotein associated with HDL. There is abundant evidence indicating that the risk of coronary atherosclerotic cardiovascular disease is directly associated with the levels of plasma lipids and ApoA-I, as demonstrated by the analysis of ApoA-I transgenic mice (21, 22). However, the role

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALT, alanine aminotransferase; ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; ApoA-II$^{-/-}$, homozygous ApoA-II knockout; AST, aspartate aminotransferase; HDL, high-density lipoprotein; Ly6G, lymphocyte Ag 6G; PIV-1, precipitation of Cohn et al. fraction IV-1; prednisolone, prednisolone sodium succinate; Treg, regulatory T cell; WT, wild-type.

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of apolipoproteins in the immune response and in the pathogenesis of inflammation remains unidentified.

In this study, we demonstrate that ApoA-II has a suppressive effect on IFN-γ production by Con A-stimulated mouse and human CD4 T cells and attenuates Con A-induced hepatitis. ApoA-II could be used as an effective new therapeutic agent for CD4 T cell-dependent autoimmune or viral hepatitis in humans.

Materials and Methods

Mice

Female BALB/c mice (6-wk-old) were purchased from Charles River Laboratories. Heterozygous ApoA-II knockout mice, which were purchased from The Jackson Laboratory, were bred to produce wild-type (WT) controls and homozygous ApoA-II knockout (ApoA-II$^{-/-}$) mice (23, 24). All of the mice used in this study were maintained under specific pathogen-free conditions. All of the animal care and experimental protocols were conducted in accordance with the guidelines of Chiba University.

Purification of ApoA-II from human plasma

Human plasma was fractionated by the cold ethanol method according to Cohn et al. (25). Precipitation of Cohn et al. fraction IV-1 (PIV-1) was used as the starting material, and ApoA-II was further purified from PIV-1 by an additional ethanol precipitation (26). Briefly, PIV-1 was resuspended in buffer, 100 mM Tris-HCl (pH 8) containing 6 M urea. A first extraction was performed with a 1:1 (v/v) mixture of chloroform and ethanol with stirring, and the phases were separated by centrifugation. The lower organic phase contained most of the lipids of PIV-1. For the supernatant, which contains ApoA-II and ApoA-I, ethanol was added with stirring, and the mixture was incubated. After centrifugation, the pellet was discarded, and the supernatant, now depleted of proteins with molecular masses >28 kDa, was fractionated to separate ApoA-II from ApoA-I. This was achieved by further addition of ethanol and an additional incubation. After centrifugation, the supernatant and pellet contained ApoA-II (17 kDa) and ApoA-I (28 kDa), respectively. These were dialyzed against PBS.

SDS-PAGE and two-dimensional electrophoresis

SDS-PAGE (5–20% gels) was used for separation of proteins. After electrophoresis, the gels were stained with bromphenol blue. Two-dimensional electrophoresis was performed as described (27) with slight modifications. Briefly, protein samples were mixed with rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 0.2% ampholine (pH 3–10), 20 mM DTT, and 0.001% bromphenol blue] and loaded onto Immobiline Drystrip (pH 3–10, nonlinear; Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed for a total of 30,000 Vh on an IPGphor apparatus (Amersham Biosciences). After a standard SDS equilibrating step, proteins were further separated by SDS-PAGE.

In vivo experimental design

For the Con A-induced hepatitis experiments, 12.5 mg/kg Con A was injected into BALB/c mice i.v. as a single dose. ApoA-II (50 or 250 mg/kg, i.v.) or PBS (30 ml/kg, i.v.) was slowly administered into the mice via the tail vein 10 min before the injection of Con A. Four, 12, and 24 h after the ApoA-II or PBS treatment, heparinized blood was collected from the heart. The plasma fraction was then separated by centrifugation. These samples were used to measure activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using an automatic analyzer (Fujifilm Medical). In addition, the livers were excised after the ApoA-II (250 mg/kg, i.v.) or PBS treatment for flow cytometry analysis. In separate experiments, the livers were excised 12 h after the ApoA-II (250 mg/kg, i.v.) or PBS treatment for histological analysis, immunohistochemistry, and detection of mRNA expression of cytokines and chemokines.

For assessment of the physiological role of ApoA-II, ApoA-II$^{-/-}$ and WT mice were injected with 20 mg/kg, i.v., Con A as a single dose. Twelve hours after the injection of Con A, heparinized blood was used to measure levels of AST and ALT, and the livers were excised for histological analysis. Where indicated, the livers from ApoA-II$^{-/-}$ and WT mice were excised 4 h after Con A injection and used for flow cytometry analysis. In addition, anti-CD4 mAb (GK1.5; 200 μg) was i.p. injected into ApoA-II$^{-/-}$ and WT mice 5, 3, and 1 d before Con A injection. Essentially no CD4 T cells were detected in the liver by either direct or indirect method of CD4 staining on the day of Con A injection (data not shown). Anti–IFN-γ mAb (R4-6A2; 250 μg) was i.v. injected into ApoA-II$^{-/-}$ and WT mice 30 min before Con A injection. Anti–IL-17 mAb (50104; 100 μg) was i.p. injected into ApoA-II$^{-/-}$ and WT mice 30 min before Con A injection. Twelve hours after the injection of Con A, heparinized blood was used to measure levels of AST.

For the experiment analyzing in vivo treatment with ApoA-II and prednisolone (Shionogi), ApoA-II (250 mg/kg, i.v.) or prednisolone (4 mg/kg, i.v.) were slowly administered via the tail vein (pretreatment, 10 min before the injection of Con A; posttreatment, 2 h after the injection of Con A). Twelve hours after ApoA-II or prednisolone treatment, heparinized blood was used to measure activities of AST and ALT, and livers were excised for histological analysis.

Histological and immunohistochemical analysis

For histological analysis, the livers from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Specimens were examined under a light microscope. For immunohistochemical analyses, livers were fixed in 4% paraformaldehyde, and tissue was embedded in Tissue-Tek OCT compound (Sakura Finetechnical). Ten-micrometer cryostat sections were treated with 3% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Sections were preblocked with anti-CD16/CD32 mAb (2.4G2; BD Pharmingen) in PBS containing 2% FCS and stained with biotin-conjugated Ab for deoxyuridiine triphosphate by using MEBSTATIN Apoptosis Kit II (Medical & Biological Laboratories) according to the manufacturer's protocol or with biotin-conjugated mAbs (BD Pharmingen) for anti-CD4 (RM4-5), anti-CD11b (M1/70), and anti-lymphocyte Ag 6G (Ly6G) (1A8). After being washed with TNT buffer [0.1M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20], sections were treated with HRP-conjugated streptavidin (Endogen) in TNT buffer. After another washing step, amplification of the fluorescent signal with FITC-tyramide was performed by TSA direct kit (PerkinElmer). The specimens were analyzed using a fluorescence microscope (Biorevo; Keyence).

Quantitative PCR analysis

Total RNA was isolated from the liver (three mice in each group) with TRIzol reagent (Sigma-Aldrich). Reverse transcription was carried out with Superscript II Reverse Transcriptase (Invitrogen). Samples were then subjected to real-time PCR analysis on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) as described (28). The primers and TaqMan probes for the detection of mouse IL-4 and hypoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems. The probes for the detection of the other genes in this study were purchased from Roche Diagnostics (Basel, Switzerland). Primers for the Roche Diagnostics probes were as follows: TNF-α (forward primer, 5′-ATGAGCACAGAAAGCATGATC-3′; reverse primer, 5′-TACAGGCTTGTCACTCGAATT-3′); IFN-γ (forward primer, 5′-ATCTGGAGGAACTGGCAAAA-3′; reverse primer, 5′-TTCAAGACTTCAAAGAGTCTGAGGTA-3′); MIP-1α (forward primer, 5′-CAAGTCTTCTCAGCGCCATA-3′; reverse primer, 5′-GGAATCTTCCGGCTGTAGG-3′), MIP-1β (forward primer, 5′-GCCCTCTCTCTCCTTGTC-3′; reverse primer, 5′-GGAGGGTCAGAGCCCATT-3′), RANTES (forward primer, 5′-TGCAGAGGACTCTGAGACAGC-3′; reverse primer, 5′-GAGTGGTGTCCGAGCCATA-3′), and HPRT (forward primer, 5′-TCCTCCTCAGACCGCTTTT-3′; reverse primer, 5′-CCTGGTTCATCATCGCTAATC-3′). Gene expression was normalized using the HPRT signal.

Preparation of liver leukocyte cells

Liver leukocyte cells were isolated as previously described (29, 30). Briefly, the liver was pressed through a stainless steel mesh (#200) and suspended in PBS. After being washed once, the cells were resuspended in 33% Percoll solution and centrifuged. The pellet was subjected to flow cytometric analysis. For surface staining, one million cells were incubated with anti-CD16/CD32 mAb (2.4G2) and stained with the appropriate staining reagents, according to a standard method. The reagents used in this study were anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), and anti-Ly6G (1A8) mAbs.

Preparation of mouse and human CD4 T cells

For the preparation of mouse CD4 T cells, splenic CD4 T cells were purified from splenocytes using FITC-conjugated anti-CD4 mAb (RM4-5; BD Pharmingen), anti-FITC magnetic beads (Miltenyi Biotec), and autoMACS cell sorting (Miltenyi Biotec). For the preparation of human CD4 T cells, PBMCs from healthy volunteers were collected after obtaining informed consent. Peripheral blood was diluted with PBS and applied on Ficoll-Paque (Amersham Biosciences). After centrifugation, mononuclear cells in the interface were harvested. Human CD4 T cells were purified from mononuclear cells using FITC-conjugated anti-CD4 mAb (RPA-T4; BD Pharmingen), anti-FITC magnetic beads, and autoMACS cell sorting.

Measurement of cytokines

Mouse splenic CD4 T cells (1×10^6 cells per well) or human CD4 T cells (5×10^5 cells per well) from PBMCs were stimulated with 5 μg/ml Con A (Sigma-Aldrich) for 24 h in the presence or absence of ApoA-II or ApoA-I. The amounts of IL-2 and IFN-γ in the culture supernatants were measured by ELISA as previously described (31). In Figs. 4*C* and 5*C*, the amounts of 23 cytokines from mouse and human CD4 T cells in the culture supernatants were detected by Bio-Plex Pro Mouse Cytokine Standard Group I 23-plex (Bio-Rad Laboratories) or Bio-Plex Pro Human Cytokine Standard Group I 23-plex according to the manufacturer's protocol, respectively. Data from the reaction was then acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories.

Proliferation and cell division assay

To assay proliferation of CD4 T cells, mouse splenic CD4 T cells or human CD4 T cells were stimulated with Con A for 24 h in the presence or absence of ApoA-II or ApoA-I. [^{3}H]Thymidine (37 kBq per well) was added to the stimulation culture for the last 16 h, and the incorporated radioactivity was measured on a β plate (32). For cell division, mouse splenic CD4 T cells or human CD4 T cells were labeled with CFSE (Molecular Probes) as described (31) and stimulated with Con A for 24, 48, and 72 h in the presence or absence of ApoA-II or ApoA-I. Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences), and the results were analyzed using the FlowJo software program (Tree Star).

Phosphoprotein assay

Mouse splenic CD4 T cells were stimulated with Con A for 0, 5 min, 0.5, 1, and 3 h in the presence or absence of ApoA-II or ApoA-I. Protein lysates were prepared with a cell lysis kit (Bio-Rad Laboratories) on samples collected at the indicated time points. The presence of phosphorylation of ERK1/2, c-Jun, IκBα, and p38 MAPK was detected by Bio-Plex 4-plex Phosphoprotein assay kit (Bio-Rad Laboratories) and the Phosphoprotein Testing Reagent kit (Bio-Rad Laboratories) according to the manufacturer's protocol. Data from the reaction was then acquired and analyzed using the Bio-Plex suspension array system.

Immunoblot assay

Mouse splenic CD4 T cells were stimulated with Con A for 0, 5 min, 0.5, 1, and 3 h in the presence or absence of ApoA-II or ApoA-I. Nuclear extracts for the detection of NFAT2 (NFATc1) or lamin were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) on samples collected at the indicated time-points. Immunoblotting was performed with anti-NFATc1 mAb (BD Pharmingen) or anti-lamin polyclonal Ab (Santa Cruz Biotechnology). Protein levels were visualized by ECL (Amersham Biosciences) using HRP-conjugated anti-mouse IgG Ab or anti-rabbit IgG Ab (Amersham Biosciences).

Statistical analysis

All of the data are expressed as the mean ± SD. Statistical analysis was performed using GraphPad Prism, and differences were determined by unpaired two-tailed Student *t* test or one-way ANOVA with Dunnett's multiple comparison tests. A p value <0.05 was considered statistically significant.

Results

Inhibition of IFN-γ production in Con A-stimulated mouse and human CD4 T cells by ApoA-II

To identify new therapeutic agents to suppress T cell-dependent inflammatory responses with better safety profiles than the currently used drugs, we purified the protein fraction from healthy human plasma using an ethanol precipitation method as reported by Cohn et al. (25), designated PIV-1. The albumin and IgG fraction were also purified from plasma by ethanol precipitation. As shown in Fig. 1*A*, the PIV-1 fraction significantly suppressed IFN-γ production by Con A-stimulated mouse CD4 T cells. No significant change was observed after the addition of the albumin or IgG fraction. Next, we compared the protein content of whole plasma and PIV-1 using SDS-PAGE. A spot corresponding to a 17-kDa protein (indicated by circles) showed increased intensity in PIV-1 compared with plasma (Fig. 1*B*, compare *lanes 1* and *2* and *lanes 5* and *6*). Furthermore, the two-dimensional electrophoresis revealed that the 17-kDa protein appears to contain several proteins. The spots circled in Fig. 1*C* were identified as apolipoproteins, ApoA-II and ApoA-I, using the databases of SWISS-2DPAGE Viewer. To examine the inhibitory effect of apolipoproteins on IFN-γ production, ApoA-II was further purified from PIV-1 by an additional ethanol precipitation (Fig. 1*D*). As shown in Fig. 1*E*, ApoA-II (17 kDa) purified from the PIV-1 fraction showed a potent suppressive effect on IFN-γ production from Con A-stimulated mouse and human CD4 T cells.

Attenuation of Con A-induced hepatitis by ApoA-II administration

Next, we examined whether the administration of ApoA-II inhibits liver injury induced by i.v. injection of Con A. Hepatitis was evaluated by measuring the levels of AST and ALT in plasma 4, 12, and 24 h after Con A injection (12.5 mg/kg). In the Con A-induced hepatitis model, the levels of AST and ALT increased 4 h after Con A injection and thereafter increased rapidly, reaching peak values at the 12-h time point (Fig. 2*A*). The administration of ApoA-II (50 or 250 mg/kg) before injection of Con A significantly and dose-dependently suppressed the increased levels of AST and ALT. We then evaluated the histological and immunohistochemical changes in the liver 12 h after Con A injection. Histological examination of the liver by H&E staining revealed focal or massive severe necrosis in the area between the central veins and the portal tracts of Con A-injected mice (Fig. 2*B*, *second row*, *top* and *middle panels*). In addition, TUNEL staining indicated Con A-induced apoptosis of hepatocytes (Fig. 2*B*, *second row*, *bottom panels*, green-stained cells). Preadministration of ApoA-II clearly suppressed the severe necrosis and apoptosis in the liver (Fig. 2*B*). We then examined the expression of cytokines and chemokines in the liver. The mRNA expression of TNF-α, IFN-γ, IL-4, MIP-1α, MIP-1β, and RANTES in the liver 12 h after Con A injection was significantly lower in the mice administered ApoA-II (Fig. 2*C*). Programmed death 1 and T cell Ig mucin 3 expression was not upregulated by the administration of ApoA-II (data not shown). The treatment of normal mice with ApoA-II alone had no effects on the levels of AST or ALT (data not shown).

Suppression of Con A-induced leukocyte infiltration into the liver by ApoA-II administration

ApoA-II suppressed the mRNA expression of MIP-1α, MIP-1β, and RANTES that attract several types of leukocytes including CD4 T cells into the liver (33, 34). We thus evaluated the number of leukocytes infiltrating the liver. As shown in Fig. 3*A*, the administration of ApoA-II significantly suppressed leukocyte infiltration into the liver 12 h after Con A injection. Immunohistochemical analyses revealed a massive infiltration of $CD4^+$, $CD11b^+$, and $Ly6G^+$ cells 12 h after Con A injection, and this was inhibited by the administration of ApoA-II (Fig. 3*B*). The flow cytometry analysis revealed that the number of CD4 T cells, CD8 T cells, $CD11b^+/Ly6G^-$ macrophages, and $CD11b^+/Ly6G^+$ neutrophils in the liver increased after Con A injection, and these increases were significantly suppressed at the12-h time point by the administration of ApoA-II (Fig. 3*C*). In normal mice, treatment with ApoA-II had no effects on the proportion or the absolute numbers of these cells in the liver (data not shown).

Suppression of the activation and function of mouse CD4 T cells by ApoA-II

To clarify the mechanisms underlying the ApoA-II–induced inhibition of Con A-induced hepatitis, we assessed the effect of human ApoA-II on the activation and function of mouse CD4

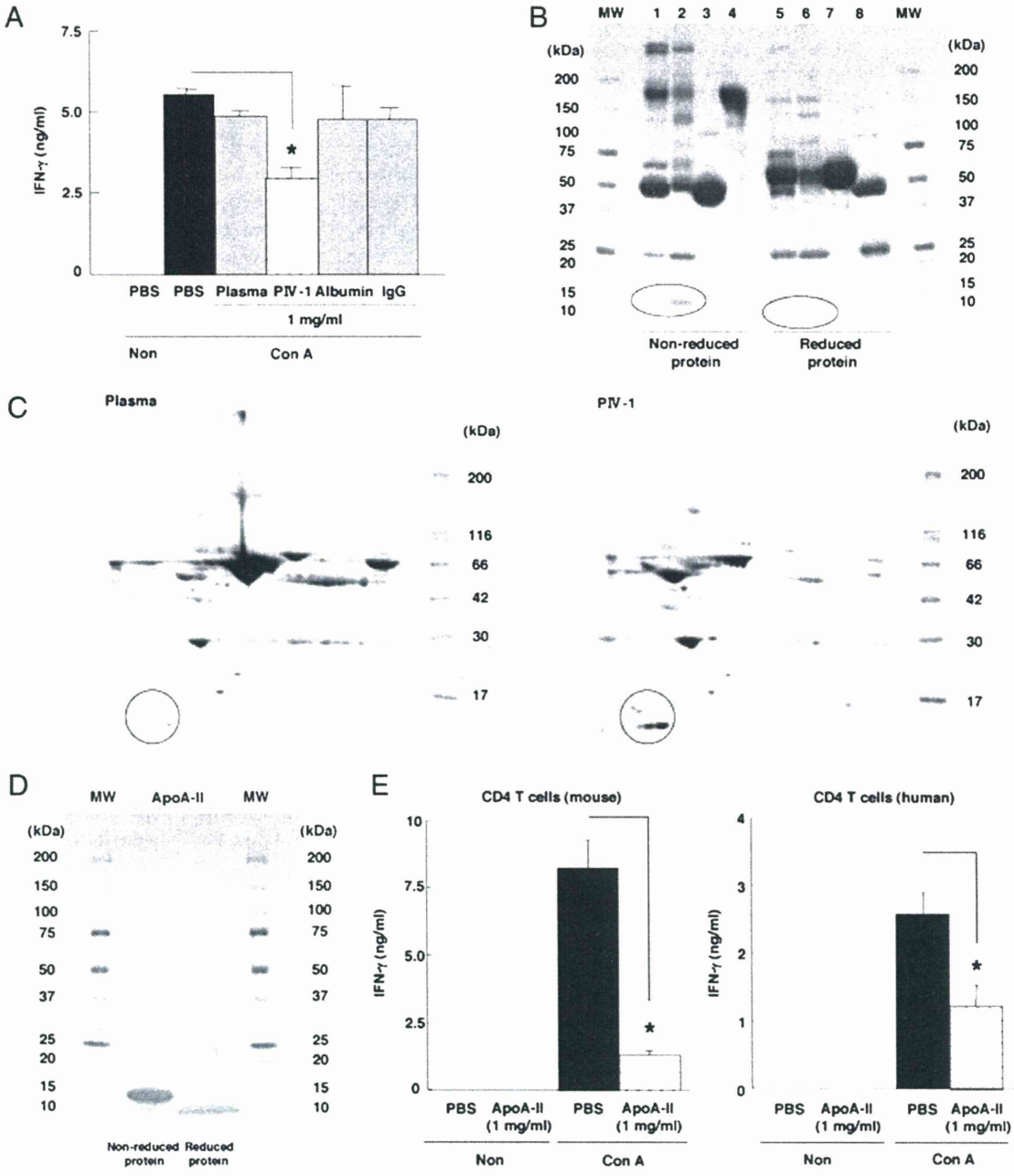

FIGURE 1. ApoA-II purified from human plasma inhibits IFN-γ production by Con A-stimulated mouse and human CD4 T cells. *A*, Purified mouse splenic CD4 T cells were stimulated with Con A (5 μg/ml) for 24 h in the presence of plasma, PIV-1, albumin, or IgG (1 mg/ml), and the amount of IFN-γ in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD ($n = 5$). *$p < 0.05$, compared with PBS-added CD4 T cells. *B*, SDS-PAGE gel stained with bromphenol blue. *Lanes 1* and *5*, plasma; *lanes 2* and *6*, PIV-1; *lanes 3* and *7*, albumin; *lanes 4* and *8*, IgG. Circles indicate the increased spots of biologically active proteins in PIV-1. *C*, A representative two-dimensional electrophoresis pattern of plasma and PIV-1. The circle indicates the increased spots of biologically active proteins in PIV-1. *D*, SDS-PAGE gel stained with bromphenol blue showing ApoA-II purified from human plasma. *E*, Purified mouse splenic CD4 T cells or human CD4 T cells were stimulated with Con A in the presence of ApoA-II, and the amount of IFN-γ in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD ($n = 5$). *$p < 0.05$, compared with PBS-added CD4 T cells. Similar data were obtained from three independent experiments.

T cells in vitro. We used ApoA-I, which is the major apolipoprotein associated with HDL, purified from human plasma as a control. As shown in Fig. 4*A*, ApoA-II, but not ApoA-I, significantly and dose-dependently suppressed [^{3}H]thymidine uptake by Con A-stimulated CD4 T cells. Moreover, the rate of cell division of Con A-stimulated CD4 T cells was clearly and dose-dependently suppressed by the addition of ApoA-II but not ApoA-I (Fig. 4*B*). In addition, ApoA-II also suppressed the proliferation of CD4 T cells stimulated with anti-TCRβ mAb plus anti-CD28 mAb (Supplemental Fig. 1). Next, we examined whether ApoA-II suppressed the production of cytokines and chemokines from CD4 T cells stimulated with Con A (Fig. 4*C*). Among the cytokines and chemokines tested, the production of IL-2, IL-13, IFN-γ, MIP-1α, MIP-1β, and RANTES was significantly suppressed by the addition of ApoA-II. However, ApoA-I did not suppress the production of any cytokines or chemokines by Con A-stimulated CD4 T cells. Moreover, we examined whether the treatment of CD4 T cells with ApoA-II inhibited the signal transduction pathways downstream of the TCR. As shown in Fig. 4*D*, the phosphorylation of ERK1/2 induced by Con A was selectively inhibited by the