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Application of NKT Cells in Immunotherapy

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Abstract: Invariant natural killer T (iNKT) cells are a conserved T cell sublineage and an important component of the innate immune system. The invariant T cell antigen receptor (TCR) α chain on iNKT cells interacts with glycolipid presented *via* CD1d on antigen-presenting cells (APCs), resulting in the production of a variety of cytokines, and thus bridging the innate and adaptive immune systems. In this review, we discuss two strategies of immune modulation that target iNKT cells using either liposomal α-galactosylceramide (α-GalCer) or α-GalCer-loaded APCs. Liposomal α-GalCer generates regulatory iNKT cells, which serve to induce regulatory T cells (Treg) and can be used to diminish immune responses as is seen in autoimmunity and allergic diseases. In contrast, α-GalCer-pulsed APCs generate stimulatory iNKT cells capable of releasing pro-inflammatory cytokines and leading to adaptive immune responses that can be used for treating malignancies. Here, we summarize the modalities used to manipulate the dual nature of iNKT cell function and their tremendous potential in treating both allergic and malignant disease.

Keywords: NKT cells, immunotherapy.

INTRODUCTION

Natural killer T (NKT) cells were originally defined as lymphocytes that had T-cell receptors (TCRs) and natural killer (NK) cell markers such as CD161 (NKR-P1) on their cell surface, and a capacity to show biological activities of both NK and T cells. However, a number of distinct types of NKT cells have been described. Invariant NKT (iNKT) cells have a single invariant TCRα chain encoding Vα14-Jα18 in mouse and Vα24-Jα18 in human and are restricted by CD1d, a non-classical major histocompatibility (MHC) molecule. Unlike classical MHC molecules which present peptides to T cells, CD1d binds lipids and presents them to iNKT cells [1, 2]. Another subclass of CD1d-restricted iNKT cells, type II NKT cells, are identified by particular TCRα chains, e.g. Vα3.2 and Vα8 [3]. And finally, some types of NKT cells having no CD1d-restriction had been reported, though these cells have not been fully characterized [4-6].

TCR on iNKT cells are composed of a single α-chain and a restricted number of β-chains, such as Vβ8, Vβ7, and Vβ2. Once assembled, the TCR of iNKT cells recognizes CD1d-loaded glycolipids [7, 8]. KRN7000 is the first synthetic ligand for iNKT cells, usually termed α-galactosylceramide (α-GalCer is used hereafter) [9-12]. Originally α-GalCer was discovered while screening compounds for anti-tumor effects [13], and Taniguchi *et al.* determined it as an iNKT cell ligand [14]. α-GalCer can be pulsed onto APC such as

dendritic cells (DCs) and used to activate iNKT cells. Once activated, iNKT cells produce various cytokines such as IL-2, IL-4, IL-10, IL-13, IL-17, IL-21 and IFN-γ [15, 16].

Because of the unique ability of iNKT cells to bridge the innate and adaptive immune system, these cells have been proven to be valuable targets for clinical applications. The role of iNKT cells in tumor surveillance has been widely investigated since the original studies of anti-tumor effects of α-GalCer [17, 18]. iNKT cells are activated by α-GalCer-pulsed DCs. This iNKT cell activation causes DC maturation and IL-12 production, which in turn causes iNKT cells to produce IFN-γ for the activation of NK cells and anti-tumor cytotoxic T cells (CTLs). Hence, the delivery of α-GalCer to DCs and the efficient production of IL-12 from DCs are critical for the application to a tumor immunotherapy using iNKT cells.

IL-12 production by DCs skews the differentiation of naïve helper T cells toward the inflammatory type I (Th1), not the allergic type 2 (Th2). For this reason, increased IL-12 secretion by DCs could attenuate Th2-dominant allergic diseases. iNKT cells activated by α-GalCer produce IL-4 as well as IFN-γ, so it was initially unclear whether *in vivo* treatment with α-GalCer would activate or suppress allergic responses. However, Cui *et al.* showed that Th2 differentiation and IgE antibody formation were suppressed in α-GalCer-treated wild type mice, but not in iNKT-deficient or IFN-γ-deficient mice, suggesting that Th1 type iNKT cells are most likely responsible for these findings [19].

In addition to Th1 cytokine production, Th2 cytokine production by iNKT cells could be clinically useful for its

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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
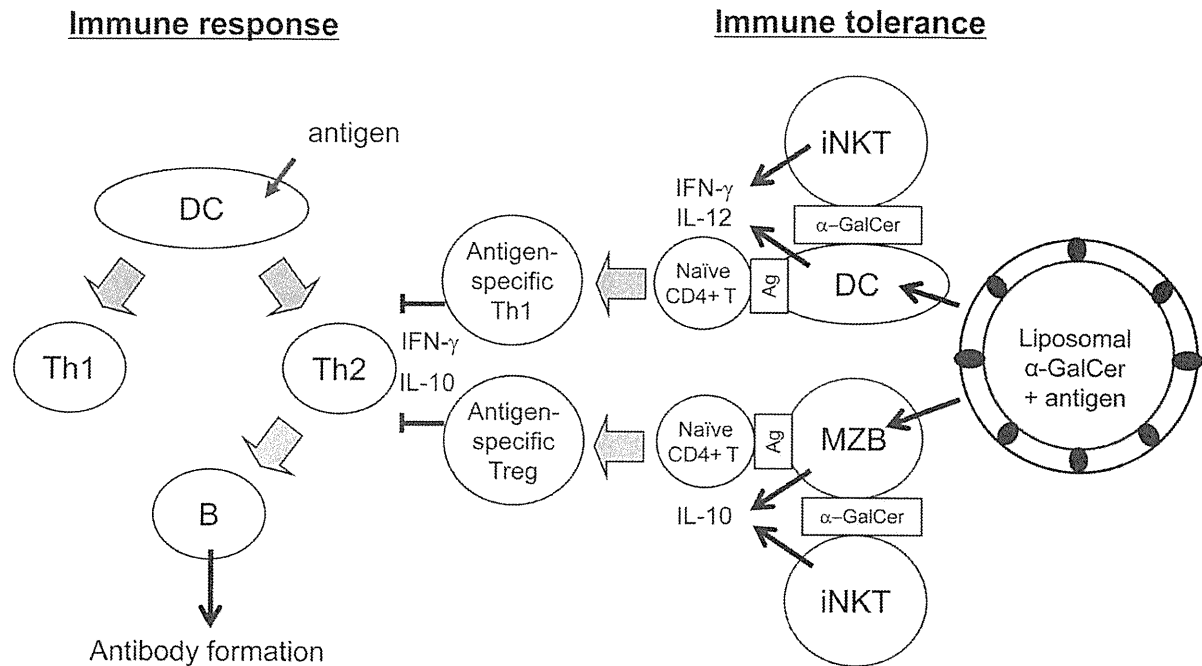
Fig. (1). Generation of antigen-specific Treg cells by liposomal α-GalCer encapsulating antigens. α-GalCer in liposomal formulation, but not aqueous formulation, preferentially enters into marginal zone B (MZB) cells. After uptake of liposomal α-GalCer encapsulating antigens, MZB cells present both α-GalCer 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, $5\times10^7/m^2$; level 2, $2.5\times10^8/m^2$; and level 3, $1\times10^9/m^2$) 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 ($1\times10^9/m^2$, 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
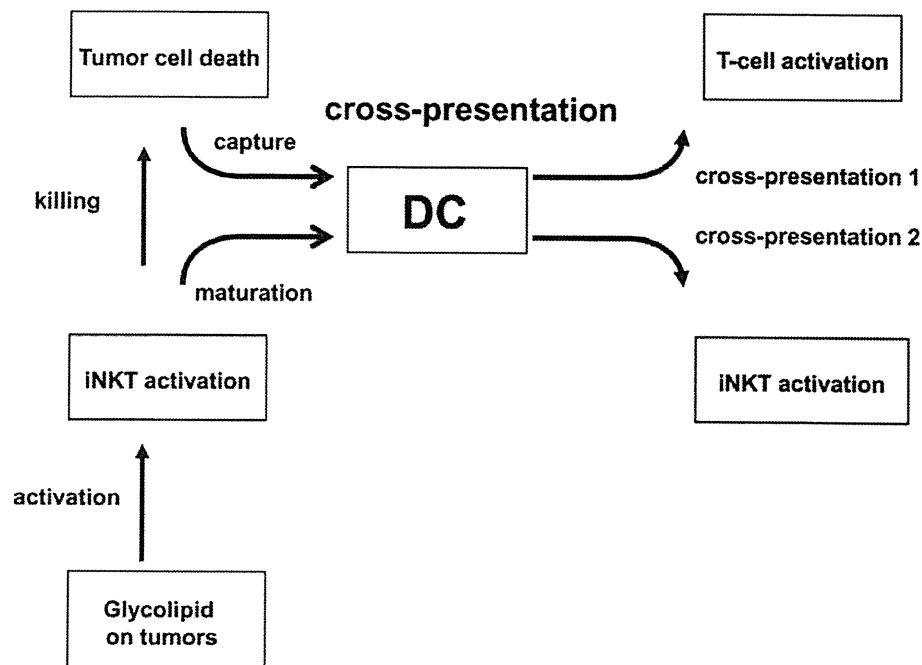
Fig. (2). Activation of iNKT cells to adaptive immunity as an adjuvant effect through DC cross presentation. After intravenous injection of a low dose of α-GalCer-loaded tumor cells, NK and NKT cells in the spleen become activated and kill tumor cells. Immature DCs then capture released tumor antigens 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-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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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

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′-TAC-AGGCTTGTCACTCGAATT-3′); IFN-γ (forward primer, 5′-ATCTGGAG-GAACTGGCAAAA-3′; reverse primer, 5′-TTCAAGACTTCAAAGAGTC-TGAGGTA-3′); MIP-1α (forward primer, 5′-CAAGTCTTCTCAGCGCCA-TA-3′; reverse primer, 5′-GGAATCTTCCGGCTGTAGG-3′), MIP-1β (forward primer, 5′-GCCCTCTCTCTCCTTGTC-3′; reverse primer, 5′-GGAGG-GTCAGAGCCCATT-3′), RANTES (forward primer, 5′-TGCAGAGGACTC-TGAGACAGC-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 to 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. 4C and 5C, 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. 1A, 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. 1B, 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. 1C 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. 1D). As shown in Fig. 1E, 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. 2A). 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. 2B, second row, top and middle panels). In addition, TUNEL staining indicated Con A-induced apoptosis of hepatocytes (Fig. 2B, second row, bottom panels, green-stained cells). Preadministration of ApoA-II clearly suppressed the severe necrosis and apoptosis in the liver (Fig. 2B). 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. 2C). 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. 3A, 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. 3B). 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 the 12-h time point by the administration of ApoA-II (Fig. 3C). 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
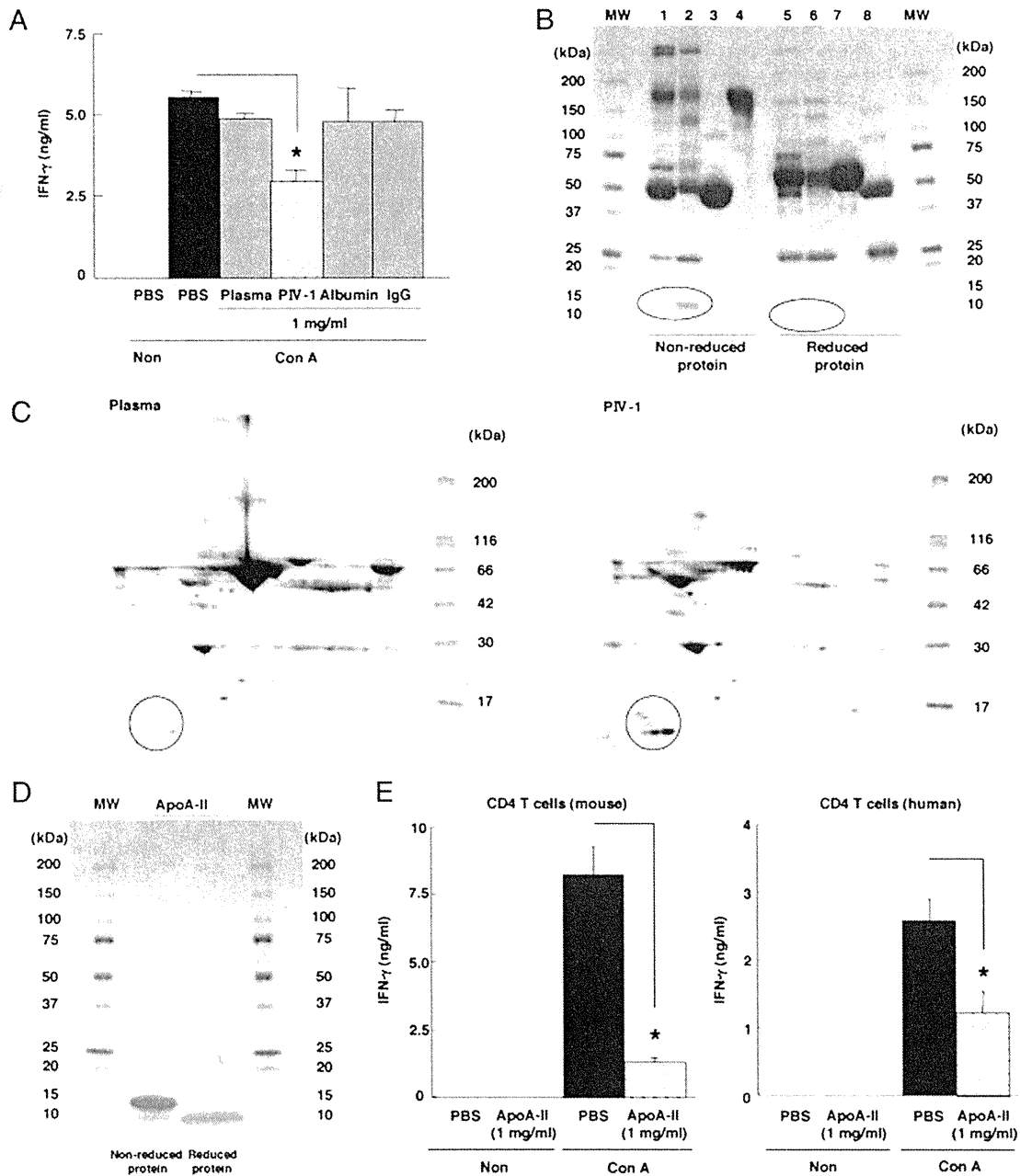
FIGURE 1. ApoA-II purified from human plasma inhibits IFN-γ production by Con A-stimulated mouse and human CD4 T cells. *A*, Purified mouse splenic CD4 T cells were stimulated with Con A (5 μg/ml) for 24 h in the presence of plasma, PIV-1, albumin, or IgG (1 mg/ml), and the amount of IFN-γ in the culture 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 [³H]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
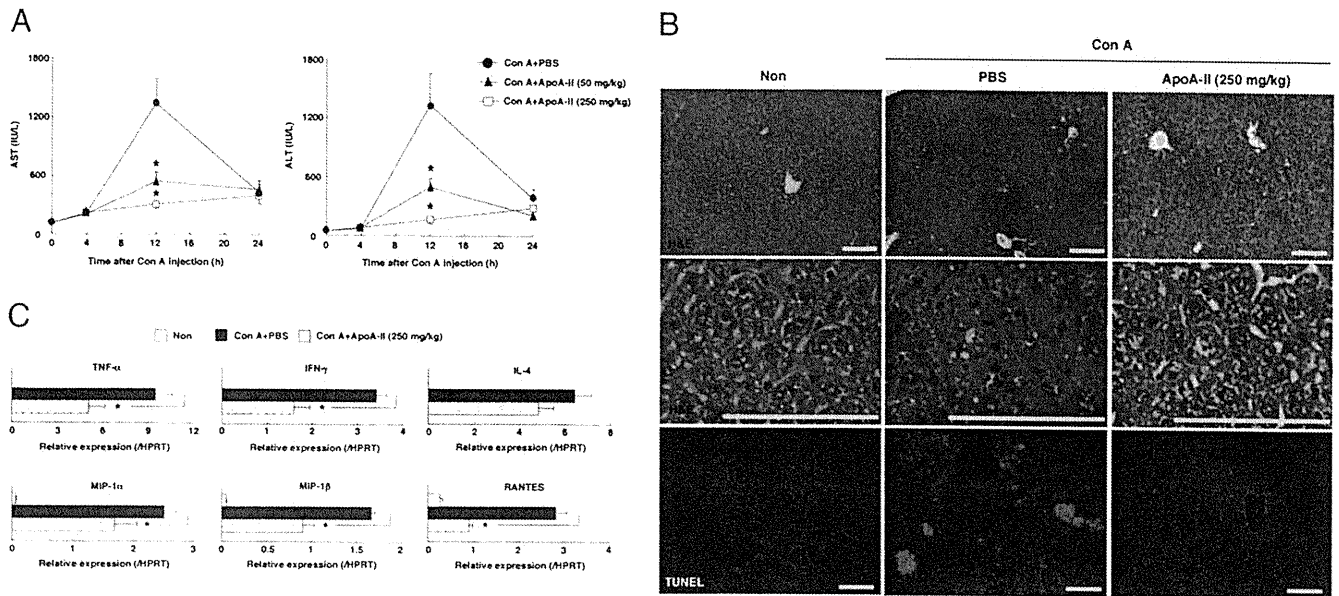
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FIGURE 2. Attenuation of Con A-induced hepatitis by ApoA-II administration. *A*, Plasma AST and ALT levels in Con A-induced hepatitis in mice. Con A (12.5 mg/kg, i.v.) and vehicle (PBS, i.v.) or Con A and ApoA-II (50 or 250 mg/kg, i.v.) were injected into BALB/c mice. The plasma was collected 4, 12, and 24 h after Con A injection. The results are expressed as mean ± SD ($n = 8$). *$p < 0.05$, compared with PBS-administered mice. *B*, Livers were collected 12 h after Con A injection, and liver damage was evaluated by H&E and TUNEL staining. Scale bars, 100 μm (*top* and *middle panels*, H&E staining) and 50 μm (*bottom panels*, TUNEL staining). *C*, Quantitative RT-PCR analysis for TNF-α, IFN-γ, IL-4, MIP-1α, MIP-1β, and RANTES mRNA expression in the liver tissue was measured 12 h after Con A injection and expressed as a ratio to HPRT. The results are expressed as mean ± SD ($n = 3$). *$p < 0.05$, compared with PBS-administered mice.

addition of ApoA-II. Increased phosphorylation of c-Jun was detected 3 h after Con A stimulation and was inhibited by ApoA-II but not ApoA-I. The phosphorylation of IκBα, an indicator of the activation of the NF-κB signaling pathway, was not affected by the addition of ApoA-II. We did not detect any increase in the phosphorylation of p38 MAPK after Con A stimulation. The nuclear translocation of NFATc1 was detected 5 min after Con A stimulation and was significantly inhibited by the addition of
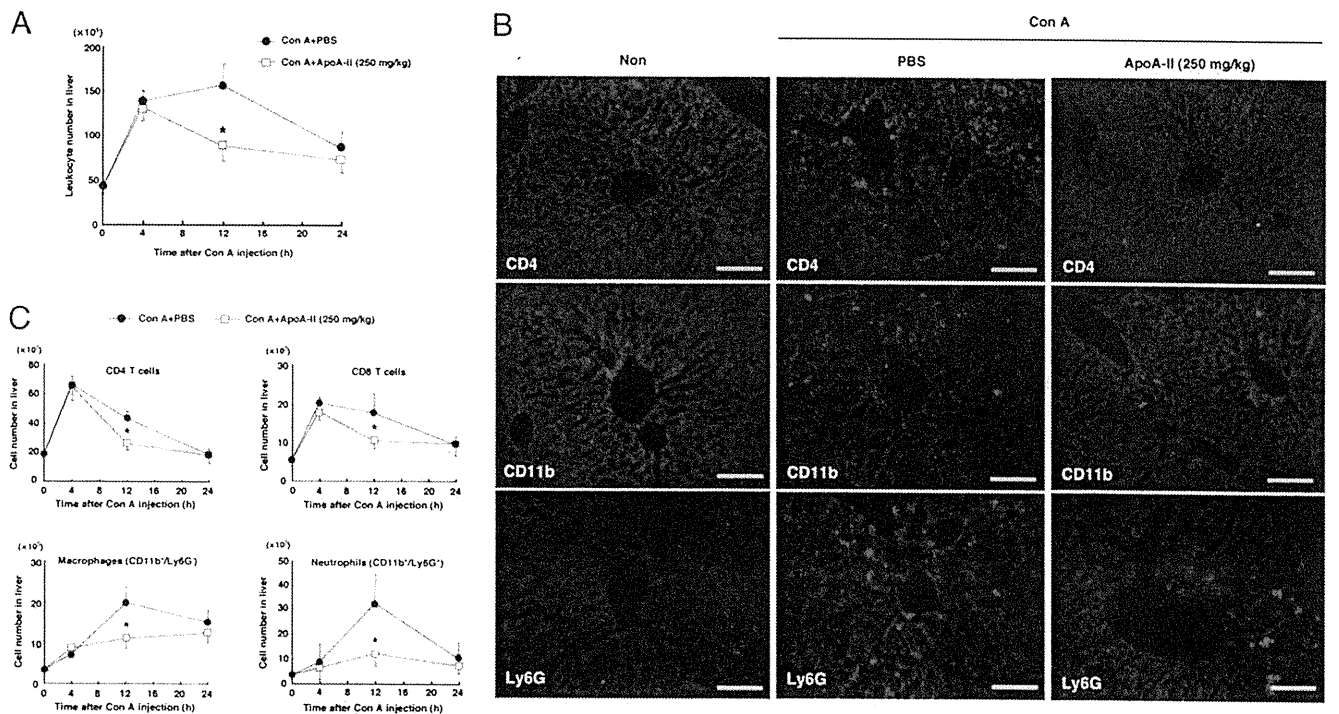

FIGURE 3. Suppression of Con A-induced leukocyte infiltration into the liver by ApoA-II administration. *A*, Total leukocyte cell numbers in the liver. Con A (12.5 mg/kg, i.v.) and vehicle (PBS, i.v.) or Con A and ApoA-II (250 mg/kg, i.v.) were administered into BALB/c mice. Livers were collected 4, 12, and 24 h after Con A injection. The results are expressed as mean ± SD ($n = 8$). *$p < 0.05$, compared with PBS-administered mice. *B*, Livers were collected 12 h after Con A injection, and leukocyte migration into the liver was evaluated by staining with Abs specific for CD4, CD11b, and Ly6G. Scale bars, 50 μm. *C*, Flow cytometric analysis of leukocytes migrated in the liver was performed. The results are expressed as mean ± SD ($n = 8$). *$p < 0.05$, compared with PBS-administered mice.
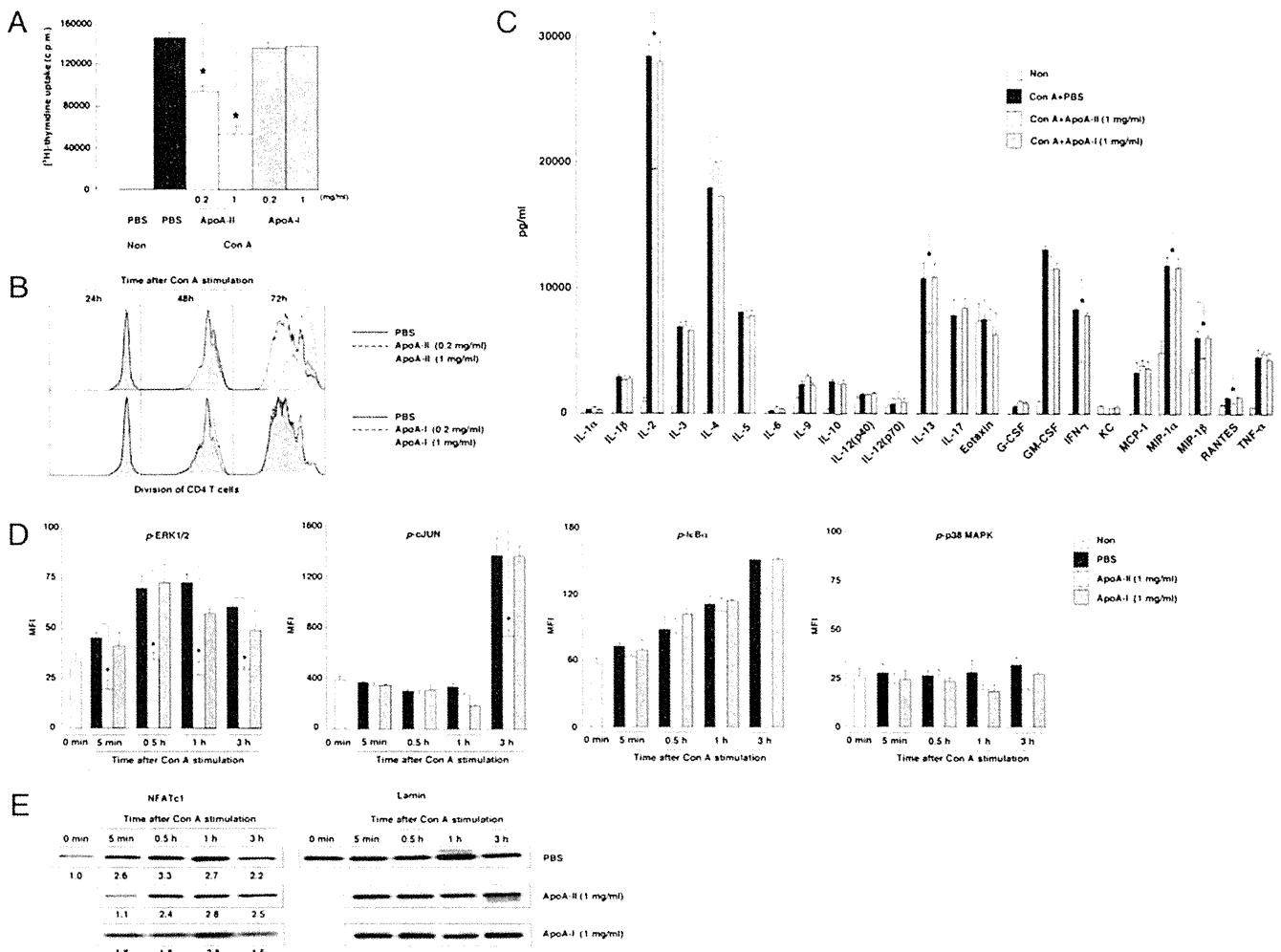
FIGURE 4. Suppression of the activation and function of mouse CD4 T cells. A, The proliferative response of mouse CD4 T cells was determined by [³H]thymidine uptake. Purified splenic CD4 T cells were stimulated with Con A (5 μg/ml) for 40 h in the presence of ApoA-II or ApoA-I (0.2 or 1 mg/ml). The results are expressed as mean ± SD (n = 5). *p < 0.05, compared with PBS-added CD4 T cells. B, Purified splenic CD4 T cells were labeled with CFSE and stimulated with Con A in the presence of ApoA-II or ApoA-I. C, Purified splenic CD4 T cells were stimulated with Con A for 24 h in the presence of ApoA-II or ApoA-I, and the amounts of 23 cytokines in the culture supernatant were assessed by Bio-Plex. The results are expressed as mean ± SD (n = 5). *p < 0.05, compared with PBS-added CD4 T cells. D, The expression of signaling molecules was assessed by Bio-Plex. The results are expressed as mean ± SD (n = 5). *p < 0.05, compared with PBS-added CD4 T cells. E, The protein expression of NFATc1 or lamin in the nuclear fraction of CD4 T cells stimulated with Con A in the presence of ApoA-II or ApoA-I was assessed. Arbitrary densitometric units were calculated by dividing the density of NFATc1 by the density of lamin and are shown under each indicated time point for the NFATc1 band. Similar data were obtained from three independent experiments.

ApoA-II (Fig. 4E). In contrast, ApoA-I had very little effect on the activation of signaling molecules. These results indicate that ApoA-II suppresses the activation and function of mouse CD4 T cells by inhibiting the ERK–MAPK pathway and NFAT signaling pathway activated by Con A stimulation.

Suppression of the activation of human CD4 T cells by ApoA-II

Next, we examined whether ApoA-II suppressed the activation of human CD4 T cells. As shown in Fig. 5A, ApoA-II, but not ApoA-I, significantly suppressed [³H]thymidine uptake by Con A-stimulated human CD4 T cells. The rate of cell division of Con A-stimulated human CD4 T cells was also suppressed by the addition of ApoA-II but not ApoA-I (Fig. 5B). In addition, we examined the effect of ApoA-II on the production of cytokines and chemokines by human CD4 T cells stimulated with Con A (Fig. 5C). Among the cytokines and chemokines tested, the production of IL-2, IL-9, IL-10, IL-17, IFN-γ, IFN-γ–inducible protein 10, and MIP-1α were significantly suppressed by ApoA-II. Again, no effect was observed by ApoA-I. These results indicate

that ApoA-II suppressed the activation of Con A-stimulated human CD4 T cells.

Exacerbation of Con A-induced hepatitis in ApoA-II⁻/⁻ mice

We examined the physiological roles of ApoA-II in Con A-induced hepatitis using ApoA-II⁻/⁻ mice. No spontaneous pathological AST and ALT levels or leukocyte infiltration were observed in ApoA-II⁻/⁻ mice maintained under physiological conditions (Fig. 6A, 6C). However, once Con A was injected, ApoA-II⁻/⁻ mice showed dramatically increased levels of AST and ALT as compared with those of WT mice (Fig. 6A). We also performed histological analysis of the liver. Without Con A injection, the livers of ApoA-II⁻/⁻ mice showed slightly increased areas of glycogen accumulation (Fig. 6B, bottom row, left). After Con A injection, liver damage accompanied by increased numbers of apoptotic hepatocytes was apparently more severe in ApoA-II⁻/⁻ mice as compared with that observed in WT mice (Fig. 6B, right four panels). Moreover, leukocyte infiltration into the liver 4 h after Con A injection was significantly higher in ApoA-II⁻/⁻ mice (Fig.
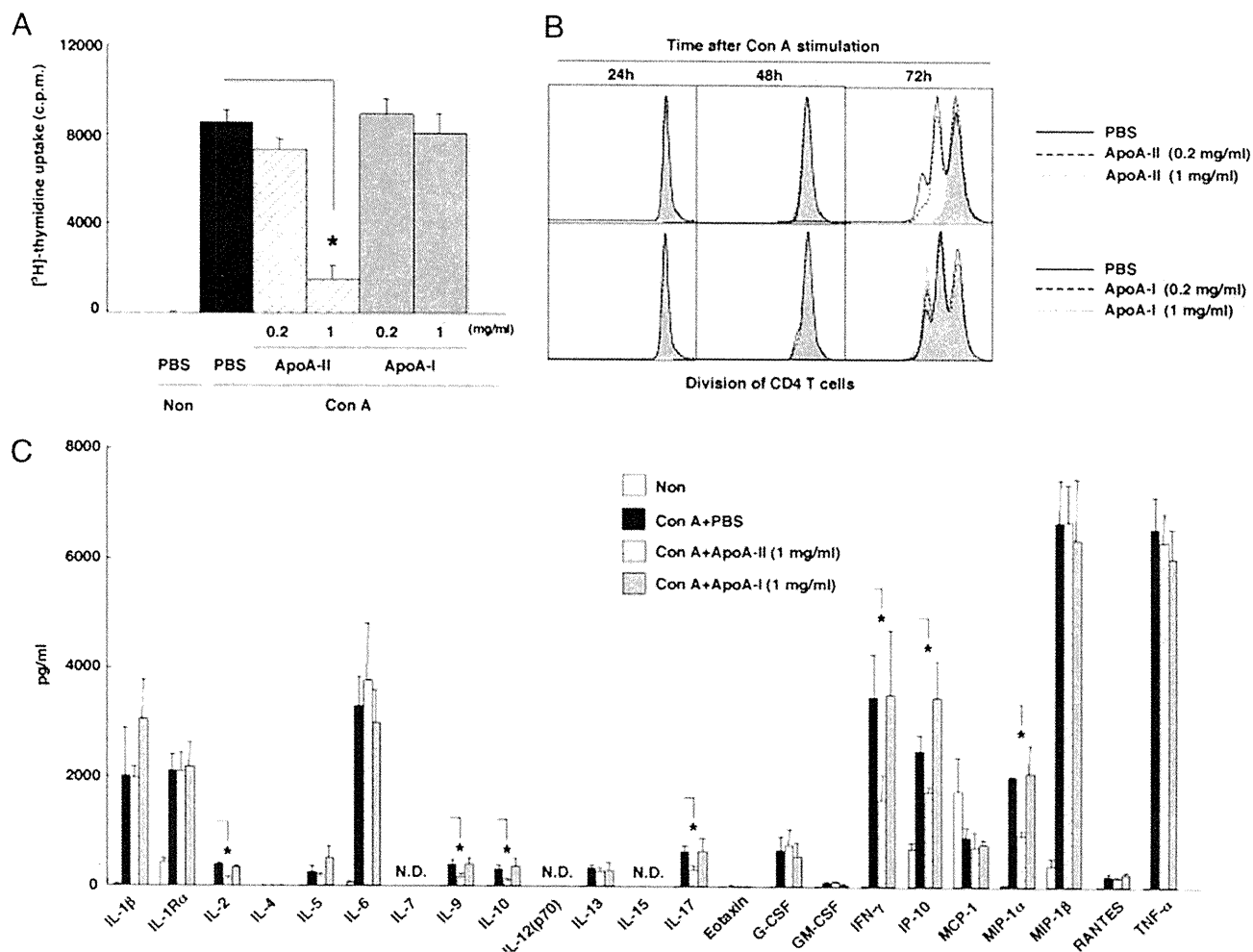
FIGURE 5. Suppression of the activation of human CD4 T cells. *A*, The proliferative response of human CD4 T cells was measured by [^{3}H]thymidine uptake. Purified human CD4 T cells were stimulated with Con A (5 μg/ml) for 40 h in the presence of ApoA-II or ApoA-I (0.2 or 1 mg/ml). The results are expressed as mean ± SD (*n* = 5). *p < 0.05, compared with PBS-added CD4 T cells. *B*, Purified human CD4 T cells were labeled with CFSE and stimulated with Con A in the presence of ApoA-II or ApoA-I. The division of CD4 T cells was assessed by flow cytometry. *C*, Purified human CD4 T cells were stimulated with Con A for 24 h in the presence of ApoA-II or ApoA-I, and the amounts of 23 cytokines in the culture supernatant were assessed by Bio-Plex. 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. N.D., not detected.

6*C*), and the increase in number of CD4 T cells in the liver of ApoA-II$^{-/-}$ mice was greater than that of WT mice (Fig. 6*D*). There was no significant difference in infiltration of macrophages or neutrophils into the liver 12 h after Con A injection between ApoA-II$^{-/-}$ and WT mice (Supplemental Fig. 2). The levels of IFN-γ production by Con A-stimulated ApoA-II$^{-/-}$ CD4 T cells were significantly higher than those in WT CD4 T cells, and the production of IFN-γ was equivalently inhibited by the addition of ApoA-II in WT and ApoA-II$^{-/-}$ groups (Fig. 6*E*). No significant difference in the production of IL-2 or the proliferation of CD4 T cells was detected between ApoA-II$^{-/-}$ and WT mice, whereas similar inhibition by ApoA-II was observed in CD4 T cells prepared from WT and ApoA-II$^{-/-}$ mice (Supplemental Fig. 3). Next, to confirm the critical role of IFN-γ– or IL-17–producing CD4 T cells in vivo in contributing to the liver injury in ApoA-II$^{-/-}$ mice during Con A-induced hepatitis, we depleted CD4 T cells, neutralized IFN-γ or IL-17 in ApoA-II$^{-/-}$ mice by the administration of anti-CD4, anti–IFN-γ, or anti–IL-17 mAb, and assessed the liver damage in response to Con A injection. Con A-induced hepatitis was protected almost completely by the injection of either anti-CD4 or anti–IFN-γ mAb in both WT and ApoA-II$^{-/-}$ mice, although no obvious protection after Con A injection was

mediated by the injection of anti–IL-17 mAb (Fig. 6*F*). These results indicate that IFN-γ–producing CD4 T cells play an important role in the development and progression of Con A-induced hepatitis in both WT and ApoA-II$^{-/-}$ mice.

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

Finally, we assessed whether ApoA-II inhibits Con A-induced hepatitis even after the onset of hepatitis. We administered ApoA-II 2 h after injection of Con A, because approximately one and a half times the number of CD4 T cells was observed in the liver by this time (data not shown), and compared the efficacy of ApoA-II and a clinically used dose of prednisolone (4 mg/kg, i.v.) that is a standard treatment for autoimmune hepatitis patients. Preadministration of ApoA-II or prednisolone significantly suppressed the Con A-induced increase in the levels of AST and ALT, and the efficacy of prednisolone given before Con A injection was more potent than ApoA-II. Interestingly, postadministration of ApoA-II but not prednisolone significantly suppressed the Con A-induced increase in the levels of AST and ALT (Fig. 7*A*). We also observed no improvements using a higher dose (20 mg/kg, i.v.) of postadministration of prednisolone (data not shown). In addition,
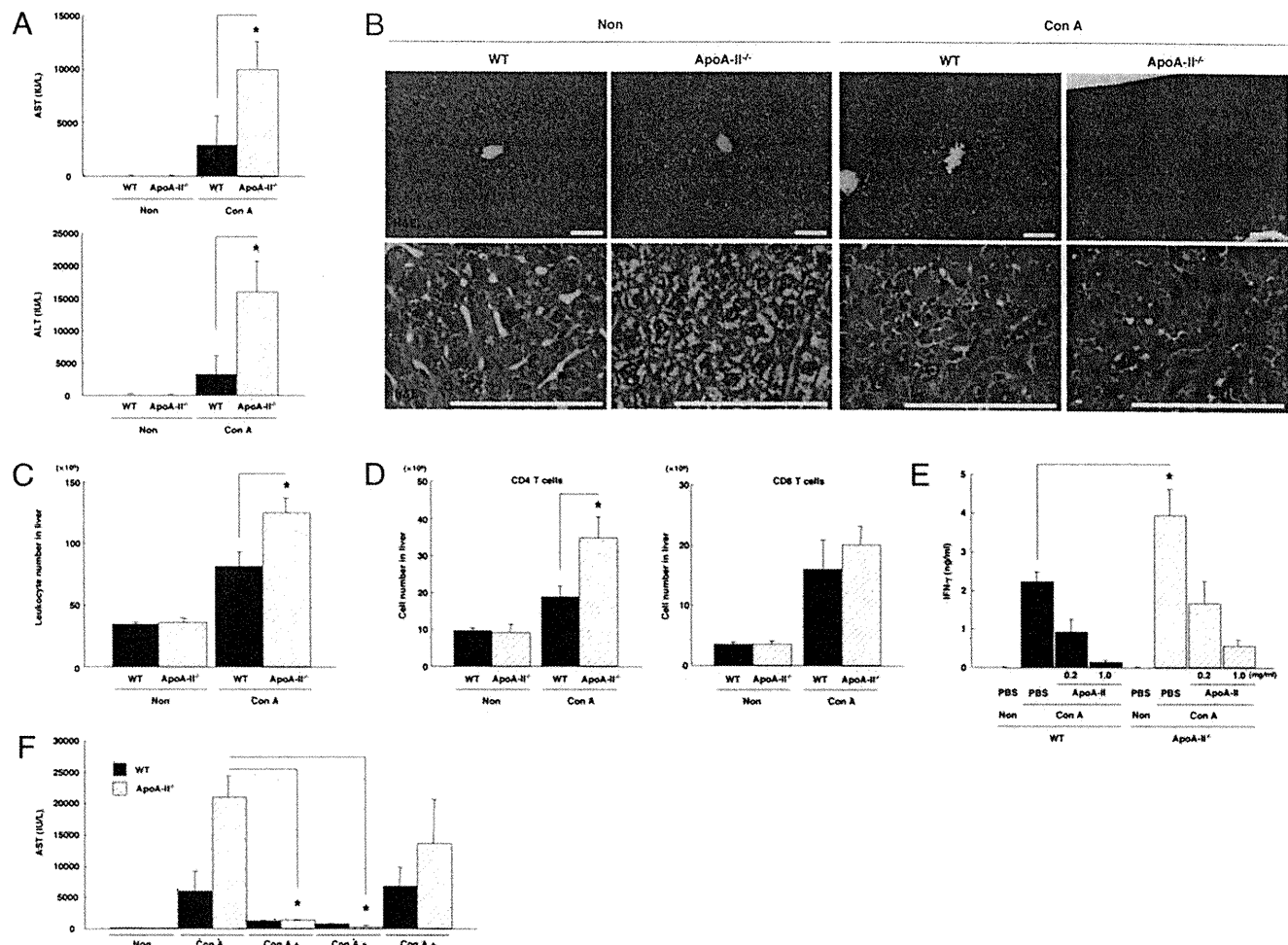
FIGURE 6. Exacerbation of Con A-induced hepatitis in ApoA-II$^{-/-}$ mice. A, Plasma AST and ALT levels in ApoA-II$^{-/-}$ and WT mice 12 h after Con A (20 mg/kg, i.v.) injection. The results are expressed as mean ± SD (n = 7). *p < 0.05, compared with WT mice. B, The livers were collected 12 h after Con A injection, and liver damage was evaluated by H&E staining. Scale bars, 100 μm (upper and lower panels). C, Total leukocyte cell numbers in the liver 4 h after the Con A injection into ApoA-II$^{-/-}$ or WT mice. The results are expressed as mean ± SD (n = 6). *p < 0.05, compared with WT mice. D, Flow cytometric analysis of liver mononuclear cells 4 h after Con A injection. The results are expressed as mean ± SD (n = 6). *p < 0.05, compared with WT mice. E, Purified splenic CD4 T cells from ApoA-II$^{-/-}$ and WT mice were stimulated with Con A (5 μg/ml) for 24 h 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 from WT mice. Similar data were obtained from three independent experiments. F, Plasma AST levels in ApoA-II$^{-/-}$ and WT mice 12 h after Con A injection. Anti-CD4 mAb was i.p. injected into ApoA-II$^{-/-}$ and WT mice 5, 3, and 1 d before Con A injection. Anti–IFN-γ mAb was i.v. injected into ApoA-II$^{-/-}$ and WT mice 30 min before Con A injection. Anti–IL-17 mAb was i.p. injected into ApoA-II$^{-/-}$ and WT mice 30 min before Con A injection. The results are expressed as mean ± SD (n = 3). *p < 0.05, compared with ApoA-II$^{-/-}$ mice.

Con A-induced histological damage such as severe necrosis and apoptosis of hepatocytes in the liver was also suppressed by postadministration of ApoA-II but not of prednisolone (Fig. 7B). The increased infiltration of leukocytes (Fig. 7C) and CD4 and CD8 T cells (Fig. 7D) into the liver was significantly suppressed by the postadministration of ApoA-II but not prednisolone (Fig. 7C, 7D). These results indicate that Con A-induced hepatitis is inhibited by ApoA-II administration even after the onset of hepatitis.

Discussion

In this report, we demonstrate clear evidence indicating that ApoA-II, which is the second major HDL in human plasma, has a suppressive effect on Con A-induced hepatitis. Exacerbated hepatitis was observed in ApoA-II$^{-/-}$ mice, indicating a physiological role for ApoA-II in the protection of Con A-induced hepatitis. The suppressive effect of ApoA-II was observed even after the onset of Con A-induced hepatitis. ApoA-II showed a potent suppressive effect on both mouse and human CD4 T cells. Therefore, ApoA-II

could be used as a new relatively safe therapeutic agent for CD4 T cell-dependent autoimmune or viral hepatitis in humans.

Activated T cells and subsequent production of cytokines play a critical role in the pathogenesis of hepatitis. Upregulation of proinflammatory cytokines such as IFN-γ and TNF-α by Con A injection directly induce hepatocellular apoptosis and necrosis (13, 15, 35), with relatively more critical roles for IFN-γ having been suggested (36, 37). Although significantly decreased expression of both IFN-γ and TNF-α in the liver was detected (Fig. 2C), the in vitro experiments revealed that ApoA-II suppressed the production of IFN-γ but not TNF-α in Con A-stimulated mouse and human CD4 T cells (Figs. 4C, 5C). Activated CD8 T cells, which produce a high amount of IFN-γ, also contribute to the development of Con A-induced hepatitis, however less so as compared with CD4 T cells (12). ApoA-II also inhibited the production of IFN-γ in Con A-activated CD8 T cells (Supplemental Fig. 4). It is also known that macrophages and neutrophils are involved in the induction of Con A-induced hepatitis (38, 39),
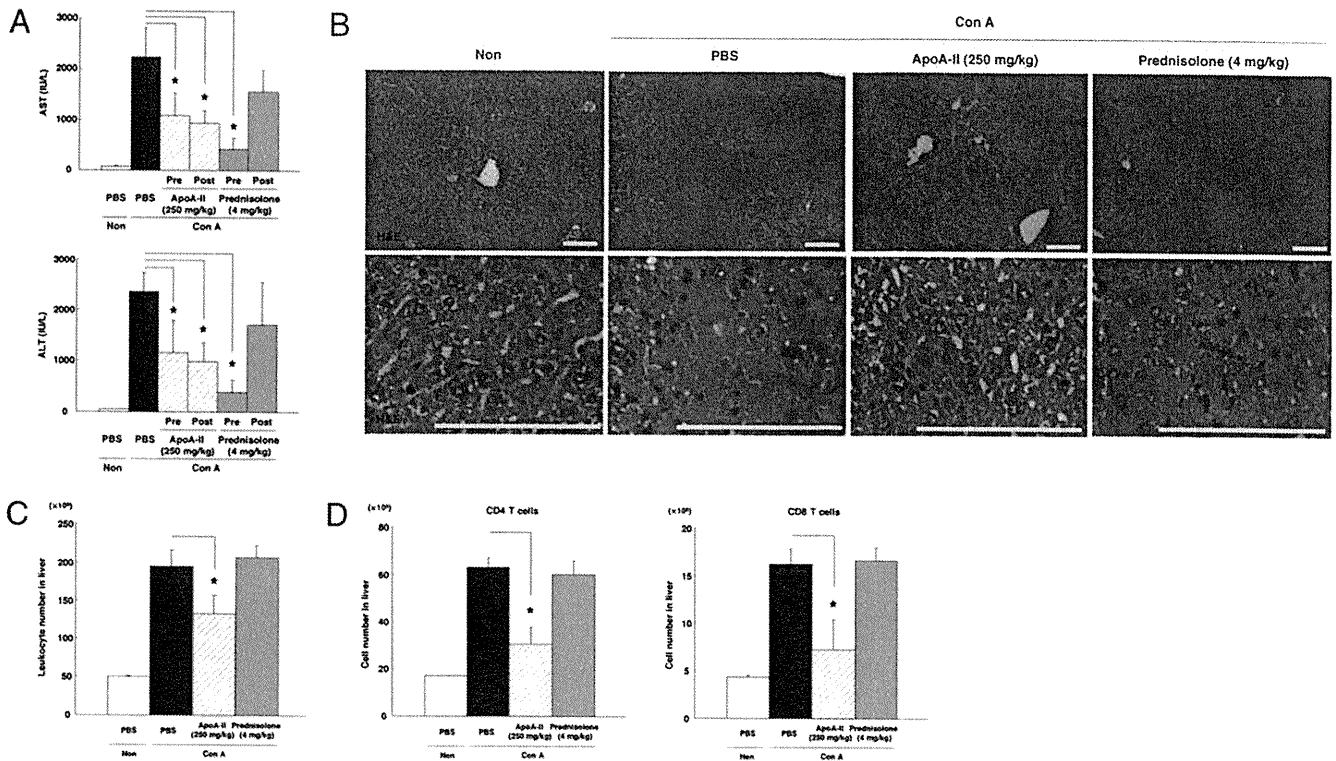
FIGURE 7. Attenuation of Con A-induced hepatitis by ApoA-II administration after the onset of hepatitis. *A*, Plasma AST and ALT levels in Con A-induced hepatitis in mice. Con A (12.5 mg/kg, i.v.) and PBS, Con A and ApoA-II (250 mg/kg, i.v.), or Con A and prednisolone (4 mg/kg, i.v.) were injected into BALB/c mice. Plasma was collected 12 h after Con A injection (pretreatment, 10 min before the injection of Con A; posttreatment, 2 h after the injection of Con A). The results are expressed as mean $\pm$ SD ($n = 6$). $*p < 0.05$, compared with PBS-administered mice. *B*, The livers were collected 12 h after the posttreatment of ApoA-II and prednisolone, and liver damage was evaluated by H&E staining. Scale bars, 100 μm (*upper* and *lower panels*). *C*, Total leukocyte cell numbers in the liver after Con A injection. Livers were collected 12 h after Con A injection. The results are expressed as mean $\pm$ SD ($n = 6$). $*p < 0.05$, compared with PBS-administered mice. *D*, Flow cytometric analysis of leukocyte infiltration into the liver 12 h after Con A injection. The numbers of CD4 or CD8 T cells are expressed as mean $\pm$ SD ($n = 6$). $*p < 0.05$, compared with PBS-administered mice.

because these cells can produce various cytokines and chemokines, leading to liver injury. However, the production of TNF-α from IFN-γ–stimulated peritoneal macrophages was not changed by ApoA-II (Supplemental Fig. 5*A*). ApoA-II also did not suppress TNF-α–induced activation of peritoneal neutrophils (Supplemental Fig. 5*B*), which was evaluated by the expression of activation markers for neutrophils, such as CD62L and CD11b (40). Previously, it was reported that IL-17–producing CD4 T cells also contributed to the induction of Con A-induced hepatitis (41). ApoA-II was capable of suppressing IL-17 production in activated CD4 T cells (Supplemental Fig. 6). However, no obvious protection in both WT and ApoA-II$^{-/-}$ mice after Con A injection was observed by the injection of anti–IL-17 mAb (Fig. 6*F*). In addition, ApoA-II injection did not alter the number of Foxp3^{+} regulatory T cells (Tregs) among CD4 T cells infiltrating in the liver even after Con A injection, and also the number of Foxp3^{+} Tregs was not reduced in the ApoA-II$^{-/-}$ mice (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Thus, Th17 cells and Tregs may not play a major role in the attenuation of Con A-induced hepatitis by ApoA-II. Taken together, ApoA-II appears to attenuate Con A-induced hepatitis largely by the suppression of IFN-γ production by CD4 T cells.

The administration of ApoA-II suppressed the migration of CD4 T cells, CD8 T cells, macrophages, and neutrophils into the liver after Con A injection (Fig. 3*B*, 3*C*). We measured the expression of several chemoattractant factors and found that ApoA-II significantly suppressed the mRNA expression of MIP-1α, MIP-1β, and RANTES that attract CD4 T cells, CD8 T cells, and macrophages in the liver after Con A injection (Fig. 2*C*). Indeed, the increased

production of MIP-1α, MIP-1β, and RANTES was reported in chronic hepatitis C (42–44), alcoholic hepatitis and cirrhosis (45), and transplanted liver (46, 47) in humans. In the mouse model, these chemokines also have an important role in the induction of hepatitis (33, 34). The infiltration of CD4 or CD8 T cells into the liver after Con A injection was more rapid as compared with that of macrophages and neutrophils (Fig. 3*C*). Therefore, the suppression of chemokine production from CD4 T cells by ApoA-II at the early stage of hepatitis could reduce the migration of macrophages and neutrophils, both of which are known to be involved in the pathogenesis of Con A-induced hepatitis. Previously, we and others reported that Vα14iNKT cells are rapidly activated, produce large amounts of IFN-γ after the injection of Con A, and can contribute to the development of Con A-induced hepatitis (16, 48, 49). Although we observed a decrease in the number of Vα14iNKT cells in the liver after Con A injection, the levels were equivalent between ApoA-II– and PBS-treated groups (Supplemental Fig. 7*A*). In addition, no obvious difference in the number of IFN-γ–producing Vα14iNKT cells in the spleen between ApoA-II– and PBS-treated mice was observed (Supplemental Fig. 7*B*). Thus, Vα14iNKT cells may not be involved in the process of inhibition of hepatitis by ApoA-II.

The activation of nuclear transcription factors such as AP-1, NFAT, and NF-κB are essential for the activation of T cells and the transcriptional upregulation of the various cytokine genes (50–53). ApoA-II inhibited the phosphorylation of ERK1/2 and c-Jun, a member of the AP-1 transcription factor family, and suppressed the nuclear translocation of NFATc1 in Con A-stimulated CD4 T cells (Fig. 4*D*, 4*E*). Thus, this could be the mechanism by which

ApoA-II inhibited the activation and IFN-γ production in CD4 T cells. In fact, cyclosporine A and FK506 (Tacrolimus) inhibited Con A-induced hepatitis through the inhibition of the activation of calcineurin, the upstream signaling molecule of NFAT activation and nuclear translocation (12).

We demonstrate a physiological role for ApoA-II in the protection from Con A-induced hepatitis using ApoA-II$^{-/-}$ mice (Fig. 6). CD4 T cells may play a more important role in this protection as compared with CD8 T cells, because selectively increased infiltration of CD4 T cells and their enhanced IFN-γ production were observed in ApoA-II$^{-/-}$ mice (Fig. 6D, 6E). In the liver of ApoA-II$^{-/-}$ mice, increased areas of glycogen accumulation were observed (Fig. 6B). Thus, the changes in lipoprotein metabolism in ApoA-II$^{-/-}$ mice could induce the malfunction of CD4 T cells leading to enhanced Con A-induced hepatitis. However, the up-regulated CD4 T cell function, such as enhanced IFN-γ production, observed in ApoA-II$^{-/-}$ CD4 T cells was normalized by the addition of ApoA-II (Fig. 6E). Con A-induced production of IL-2 and proliferation were not significantly altered in ApoA-II$^{-/-}$ CD4 T cells and were equivalently inhibited by ApoA-II (Supplemental Fig. 3). Therefore, the basic function of CD4 T cells developed in ApoA-II$^{-/-}$ mice appeared to be normal. In any event, CD4 T cells appear to be the major target cells for the inhibitory effect of ApoA-II in Con A-induced hepatitis under physiological conditions.

Patients with autoimmune hepatitis usually require immunosuppressive therapy for many years. The immunosuppressive drugs, primarily glucocorticoids, serve as the standard therapy for autoimmune hepatitis (3, 4, 54, 55). Therefore, we compared the ability of ApoA-II and prednisolone to suppress Con A-induced hepatitis. Interestingly, postadministration of ApoA-II attenuated Con A-induced hepatitis, whereas the effect of prednisolone was less impressive (Fig. 7A, 7B). The postadministration of ApoA-II but not of prednisolone reduced leukocyte infiltration including CD4 and CD8 T cells into the liver (Fig. 7C, 7D). Thus, the mechanisms underlying the inhibition of Con A-induced hepatitis appeared to be distinct between ApoA-II and prednisolone. Because ApoA-II is a component of normal human plasma, side effects induced by the administration of ApoA-II could be marginal. In fact, preliminary preclinical experiments suggest that only marginal side effects are observed (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Therefore, a combination therapy of ApoA-II with a low dose glucocorticoids and/or other immunosuppressive agents may prevent the severe side effects and consequently may prove that ApoA-II is an effective therapeutic agent for autoimmune hepatitis.

In summary, we showed that ApoA-II protected mice from Con A-induced hepatitis by suppressing the function of activated CD4 T cells and reducing the intrahepatic infiltration of inflammatory cells. Although we used ApoA-II prepared from human plasma in this study, we recently found that rApoA-II also attenuated Con A-induced hepatitis (J. Yamashita, K. Kaneko, and T. Nakayama, unpublished observations). Hence, our study offers new perspectives for the treatment of CD4 T cell-related autoimmune or viral hepatitis with ApoA-II in humans.

Acknowledgments

Disclosures

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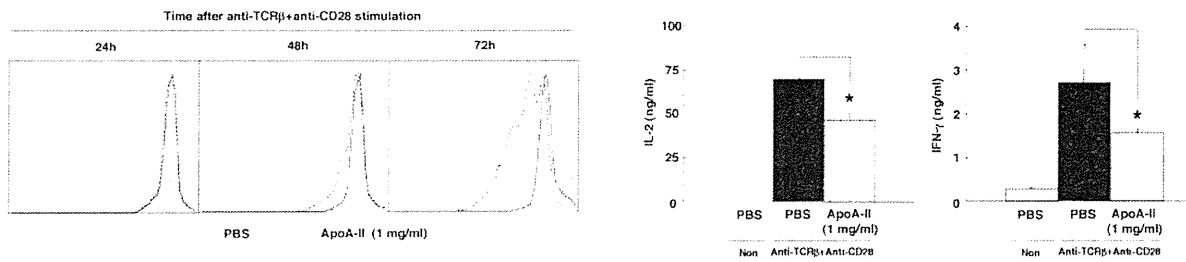
Supplemental Figure 1. ApoA-II suppresses the function of mouse CD4 T cells stimulated with anti-TCRβ plus anti-CD28. Purified mouse splenic CD4 T cells were labeled with CFSE and stimulated with immobilized anti-TCRβ mAb plus anti-CD28 mAb in the presence of ApoA-II (1 mg/ml) for 24, 48 and 72 h, and the amounts of IL-2 and IFN-γ in the culture supernatants after 24 h stimulation were assessed by ELISA. The results are expressed as mean ± SD. *P < 0.05, compared with PBS-added CD4 T cells.

Supplemental Figure 1. Yamashita et al