primary antibodies specific for Foxp3 (eBioscience, San Diego) overnight, followed by the additional incubation with Alexa Fluor 633 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. Sections were examined under Fluoview FV1000 laser scanning confocal microscopy (Olympus, Tokyo, Japan). The numbers of $Foxp3^{+}$ cells were counted in high power fields; five randomly chosen fields were evaluated.

Analysis of cytokine mRNA expression in mouse ears

At 6 h after the final challenge, the left ear skin was sampled. The specimen was homogenized and mRNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacture's instruction; 1 ml of homogenate was vigorously mixed with 200 μl of chloroform, and then centrifuged at 15,000 rpm for 15 min at 4°C. Aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 1 ml of 75% ethanol (Nacalai Tesque) and dried up. RNA was suspended in 50 μl of RNase-free water, the concentration was calculated based on the absorbance at 260 nm, and the quality was confirmed by electrophoresis. cDNA was synthesized from 10 μg of mRNA using archive kit (ABI, Foster City, CA, USA) according to the manufacturer's protocol.

Cytokine mRNA expression in skin

Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure transcriptional activity in the skin lesions. A 25-μl reaction mixture containing 1 μg total of cDNA, 900 nmol of each primer, and 250 nmol of TaqMan probe were mixed with 12.5 μl of TaqMan Master Mix (ABI, Foster City, CA, USA). The following primers and probes were used for the PCR reactions: mouse IL-4; forward: 5′-ACAGGAGAA GGGACGCCAT-3′, reverse: 5′-GAAGCCCTACAGAC GAGCTCA-3′, probe: 5′-TCCTCACAGCAACGAAGAA CACCACA-3′-TAMRA, IFN-γ; forward: 5′-TCAAGTG GCATAGATGTGGAAGAA-3′, reverse: 5′-TGGCTCT GCAGGATTTTCATG-3′, probe: 5′-TCACCATCCTTTT GCCAGTTCCTCCAG-3′-TAMRA, IL-10; forward: 5′-G GTTGCCAAGCCTTATCGGA-3′, reverse: 5′-ACCTGCT CCACTGCCTTGCT, probe: 5′-TGAGGCGCTGTCGTC ATCGATTTCTCCC-3′-TAMRA, TGF-β; forward: 5′-TG ACGTCACTGGAGTTGTACGG-3′, reverse: 5′-GGTTC ATGTCATGGATGGTGC-3′, probe: 5′-TTCAGCGCTC ACTGCTCTTGTGACAG-3′-TAMRA, β-actin; forward: 5′-AGAGGGAAATCGTGCGTGAC-3′, reverse: 5′-CAA TAGTGATGACCTGGCCGT-3′, probe: 5′-CACTGCCG CATCCTCTTCCTCCC-3′-TAMRA [25]. PCR was performed under the following conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 1 min were carried out. Fluorescence data were collected during each annealing-extension step and analyzed by using ABI Prism SDS software version 1.9.1. All samples were normalized for to the β-actin mRNA content.

Measurement of serum IgE

Blood was collected under anesthesia 6 h after the last challenge. Serum IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (BD PharMingen, CA, USA) according to the manufacturer's instructions. Optical density of each well was determined by using a microplate reader (Multiscan JX) (Thermo Electron, Yokohama, Japan). Standard curve was prepared using mouse anti-TNP IgE standard (BD PharMingen, CA, USA) diluted with PBS containing 10% FCS.

Statistical analysis

Differences in ear swelling and serum IgE levels were analyzed by the Kruskal–Wallis test. $P < 0.05$ was taken as significant.

Results

Effect of Ag85B on skin inflammation

We first examined whether Ag85B could modulate ear-swelling reaction in a mouse model of OX-induced AD like skin lesions. Repeated applications of OX cause Th2-mediated immediate type response. Ear swelling was measured with thickness gauge calipers before and 30 min after OX challenge on the pinna of the ear on day 32. In both prophylactic and therapeutic models, the administration of Ag85B significantly suppressed swelling compared to placebo-treated controls (Fig. 2a). The OX-challenged placebo-treated mice showed severe skin inflammation, however administration of Ag85B DNA reduced atopic inflammatory reactions (Fig. 2b).

Histological analysis

Histological examination in OX-challenged mice showed epidermal hyperplasia and strong intraepidermal and intradermal inflammatory cell infiltration including mononuclear cells, neutrophils, and granular cells (Fig. 3a). Both prophylactic and therapeutic administration of Ag85B DNA clearly reduced inflammatory cell infiltration and epidermal thickness. Skin sections stained with truidine blue showed decreased mast cell infiltration in Ag85B-treated mice (Fig. 3b).

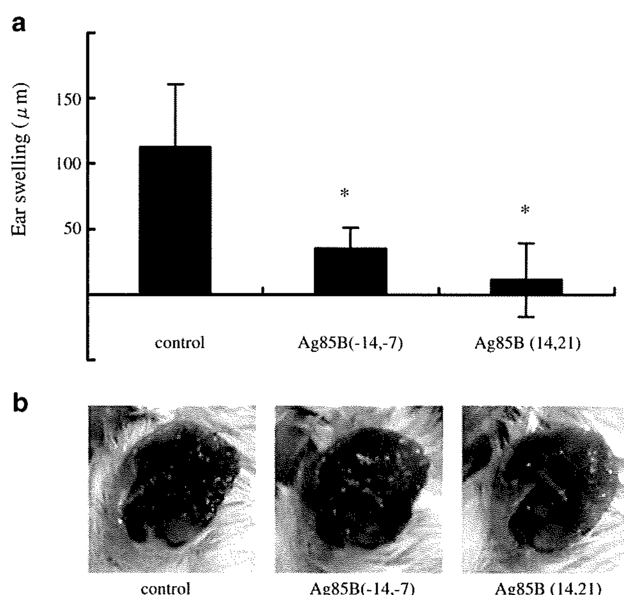


Fig. 2 **a** OX-induced ear swelling. The ear swelling response was expressed as the difference between ear thickness before and 30 min after each application on day 32. The columns and error bars represent mean ± SEM. $*P < 0.05$. Swelling was suppressed significantly in Ag85B-treated mice compared with those in placebo-treated mice. **b** Clinical features of ear skin on day 35. The OX-challenged mice showed severe skin eruption, however administration of the Ag85B DNA in both prophylactic and therapeutic models clearly reduced atopic inflammatory reactions in OX-sensitized mice

Ag85B treatment shifted the Th1/Th2 balance toward Th1

IFN-γ and IL-12 shift the Th1/Th2 balance toward Th1 condition; while IL-4 and IL-5 are key cytokines in Th2 response [24, 29]. To clarify the type of immune response in skin lesions after treatment with Ag85B, we analyzed the mRNA expression levels of IL-4 and IFN-γ by real time quantitative RT-PCR. The results were normalized to the β-actin mRNA content. As shown in Fig. 4, the expression of IL-4 mRNA was reduced in Ag85B-treated mice in both prophylactic and therapeutic models. On the contrary, the expression of IFN-γ was enhanced in Ag85B-treated mice. These results suggest that the application of Ag85B shifts the immune response toward Th1-predominace.

Total serum IgE levels

Atopic dermatitis is characterized by elevated IgE levels. Repeated applications of OX cause a gradual elevation of antigen-specific IgE level. We analyzed the degrees of IgE levels in sera collected from experimental mice. Administration of Ag85B significantly reduced the serum levels of IgE (Fig. 5).

Ag85B treatment induces regulatory T cells

TGF-β and IL-10 are important regulatory cytokines produced by Treg [11]. To investigate the mechanisms of the therapeutic effectiveness of Ag85B, we examined the mRNA levels of TGF-β and IL-10. As shown in Fig. 6a, TGF-β and IL-10 were significantly increased in Ag85B-treated mice in both prophylactic and therapeutic models. And then, we next looked at the induction of Treg in the inflamed skin. Naturally occurring $CD4^{+}CD25^{+}$ Treg are characterized by the expression of Foxp3 [10, 27]. Skin sections were stained with anti-Foxp3 mAb, and examined with a fluorescent microscope. As shown in Fig. 6b, $Foxp3^{+}$ cells were increased in the Ag85B-treated mice.

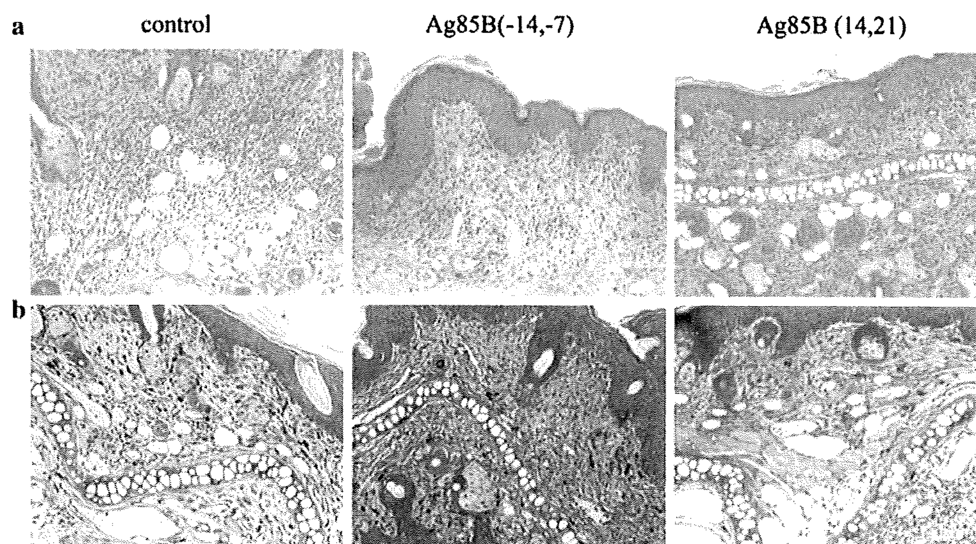
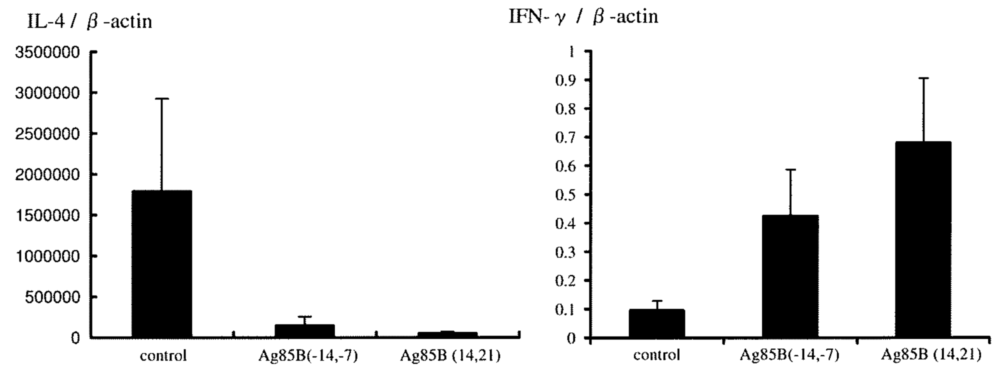


Fig. 3 Histopathological features of skin lesions. Skin was taken on day 35, paraffin embedded sections were stained with **a** hematoxylin and eosin or **b** truidine blue. OX-challenged mice showed epidermal hyperplasia along with strong intradermal inflammatory cell infiltration; whereas Ag85B DNA significantly reduced the inflammatory changes

Fig. 4 mRNA expression in the ear on day 35. In order to clarify the expression of cytokine mRNA, quantitative PCR was performed by using specific primers and probes for IL-4 and IFN-γ. The expression of IL-4 mRNA was reduced in Ag85B-treated mice compared with placebo-treated mice. On the other hand, mRNA expression of IFN-γ was significantly increased in Ag85B mice



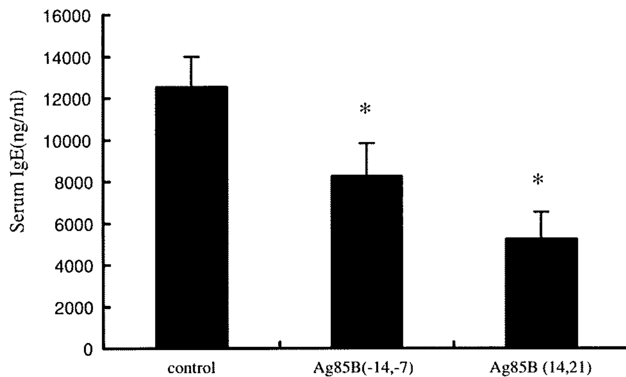


Fig. 5 Serum IgE concentrations. Serum IgE levels were measured on day 35 in control, Ag85B DNA IP (−14, −7), or Ag85B DNA IP (14, 21) mice. The columns and error bars represent mean ± SEM. $^*P < 0.05$. Administration of Ag85B reduced IgE level

Discussion

Human immune system responds to exogenous microorganisms for self-protection. These responses lead to Th1 and/ or Th2 type cytokine secretion depending on the nature of stimuli. AD is a chronic dermatitis characterized by a Th2-type immune responses that causes elevation of IgE. On the other hand, some bacterial infections including *Mycobacterium* species elicits strong Th1-type responses. Inducers of Th1 type immune response may be used as immuno-modulator having therapeutic effects against allergic disease elicited by Th2-type immune responses. Mycobacteria may affect atopic disorders by correction of the immune response from Th2 to Th1. Erb et al. reported that *M. bovis* (BCG) suppresses airway eosinophilia and associated local IL-5 production by inducing Th1-mediated response [9]. Furthermore, recent studies suggested that mycobacteria induce not only Th cells providing Th1 type immune responses but also Treg cells. In an animal model of allergy, the immunomodulatory effects of *M. vaccae* was found to be mediated by allergen-specific regulatory T lymphocytes [37], and oral administration of *M. vaccae* inhibited pulmonary allergic inflammation by induction of IL-10 [14].

Alive BCG vaccination has been used for prevention of tuberculosis. The use of *Mycobacterium* for immunomodulation requires repeated exposures to the immune system. However, repeated alive BCG vaccination is contraindicated. For human therapeutic application, it needs intradermal or intramuscular injection for vaccination. Unfortunately, cutaneous vaccination with *Mycobacterium* species commonly produces granulomatous formation leading to recalcitrant ulcers. We need to develop Th1 type immunomodulating system that induces no granulomatous reaction, if species of mycobacteria are tried to use for human. The Ag85B protein is a main component of the cell wall of mycobacteria such as *M. tuberculosis and M. kansasii* [4]; this Ag85B is known as a strong Th1 inducer in vitro [17, 18]. Experiments using plasmid DNA encoding Ag85B has been previously reported. This Ag85B is able to protect against *M. tuberculosis* even in Balb/c mice [33]. Intraperitoneal administration of Ag85B DNA inhibits granulomatous changes or adhesive reaction of intraperitoneal organs in mice (data not shown). As a preliminary study, Ag85B DNA was intradermally injected in the skin of mice skin. No ulcerative changes were observed in vaccinated areas of the skin (data not shown).

In our present study, we evaluated the efficacy of DNA encoding Ag85B for inducing Th1- and Treg-type immune response in OX-induced acute phase dermatitis. Repeated applications of OX in mice ears caused Th2-type dominant dermatitis, which mimic most of the characteristic features of AD [16, 19, 20, 32]. We first investigated whether the application of Ag85B corrects the immune response from a type Th2 one to a type Th1 response. Our results showed that Ag85B successfully ameliorates Th2-cytokine dominant immediate type reaction in the skin lesions in both prophylactic and therapeutic models of the disease. In Ag85B-treated AD skin lesion, the ear swelling was significantly reduced compared to placebo-treated animals. Administration of Ag85B DNA suppressed histological abnormalities caused by atopic inflammations such as inflammatory cell infiltration, epidermal hyperplasia, and severe edema. The presence of mast cells in the skin lesion is closely associated with Th2-type dermatitis; the number of mast cells was

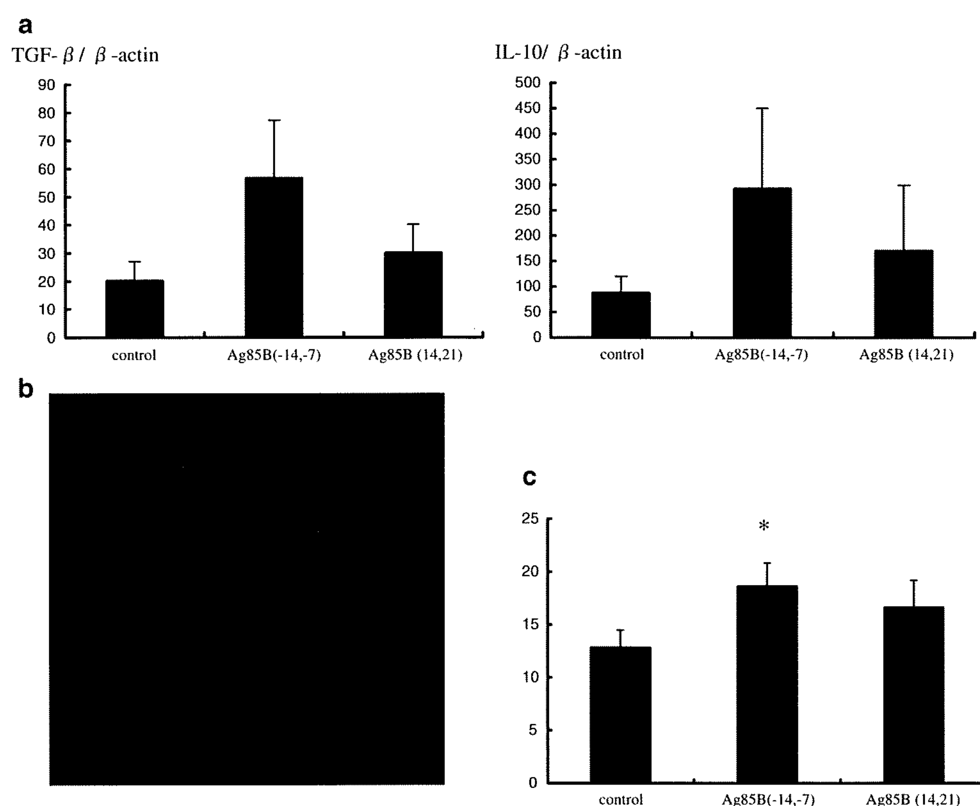


Fig. 6 **a** mRNA expression in the ear on day 35. Quantitative PCR was performed by using specific primers and probes for IL-10 and TGF-*β*. Both TGF-*β* and IL-10 were increased in the Ag85B-treated mice. **b** Foxp3$^+$ cells were clearly observed with confocal microscopy. **c** The number of Foxp3$^+$ cells per HPF was counted in five nonconsecutive fields, and Foxp3$^+$ cells were found to be increased in Ag85B-treated mice

increased in OX-treated control animals as expected; however, the number of mast cells was decreased in Ag85B-treated mice compared with controls. Enhancement of the expression of IFN-γ mRNA was significant in Ag85B-treated AD mice compared with placebo-treated animals. The expression of IL-4 mRNA were suppressed in Ag85B-treated mice compared to placebo-treated controls (Fig. 4). In addition, serum IgE levels were significantly suppressed in Ag85B treated mice compared with placebo-treated mice. These finding demonstrates that administration of Ag85B DNA significantly inhibited the development of Th2-cytokine dominant atopic inflammation by inducing Th1-type immune response.

We also examined the potential of Ag85B to induce Treg cell responses. TGF-*β* and IL-10 have been described as critical regulatory cytokines produced by Treg [11]. Heat-killed *M. vaccae* induces regulatory T cells that secrete IL-10 and TGF-*β* [37]. *M. vaccae* also induces a population of CD11$^+$ cells characterized by an increased expression of regulatory cytokines including IL-10 and TGF-*β*[1]. Treg cells are developed mainly in the presence of IL-10 and TGF-*β* [13]. More recently, Inoue and Aramaki reported that topical application of CpG-Oligodeoxynucleotides induces Foxp3$^+$ Treg in skin lesions of AD model mice in association with elevation of TGF-*β*[15]. Depletion of CD4$^+$CD25$^+$Treg from the peripheral blood of healthy individuals enhances proliferation of Th2 in response to various allergens [6, 23]. The mechanisms of the suppressive activity of Treg depend on cell-to-cell contact, and there is evidence for the involvement of IL-10 and TGF-*β*[2, 3, 26]. In this study, we have shown elevated expression of TGF-*β* and IL-10 in Ag85B-treated mice (Fig. 6a), and Foxp3$^+$ Treg was increased in the Ag85B-treated skin (Fig. 6b). We assume that the therapeutics capability of Ag85B is related to the induction of Foxp3$^+$Treg and Th1-type immune response.

In brief, in this study we have shown the usefulness of plasmid DNA of Ag85B for the amelioration of Thl/Th2 imbalance and for the generation of Treg cells. The observations suggest that Ag85B may be useful for the prevention and treatment of atopic disorders.

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Conflicts of interest statement None.

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IL-4/IL-13 antagonist DNA vaccination successfully suppresses Th2 type chronic dermatitis

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atopic dermatitis, contact hypersensitivity, DNA vaccine, IL-4 mutant

Conflicts of interest
None declared.

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Summary

Background Atopic dermatitis (AD) is a chronic disease with a Th2-type-cytokine dominant profile. Several cytokines and related peptides have been used for the treatment of AD but they were ineffective because of their limited biological half-life. We have recently developed a highly efficient mouse dominant negative interleukin (IL)-4/IL-13 antagonist (IL-4DM), which blocks both IL-4 and IL-13 signal transductions.

Objective To examine the effects of IL-4DM *in vivo* in an AD model induced by the repeated exhibition of oxazolone (OX).

Methods Plasmid DNA was injected intraperitoneally to cause an experimental AD-like dermatitis. The effect was evaluated by ear thickness, histological findings, and mast cells counts in the inflamed skin. The plasma IgE and histamine levels were measured. Cytokine production in skin and splenocytes were also analysed.

Results Mice treated with control plasmid developed marked dermatitis with mast cells and eosinophil infiltration, and had increased plasma IgE and histamine levels with a Th2 type splenocyte cytokine profile. Treatment with mouse IL-4 DNA augmented the ear swelling and thickness with an increased dermal eosinophil count, plasma histamine level, and production of splenocyte IL-4. However, IL-4DM treatment successfully controlled the dermatitis, decreased the mast cell and eosinophil count, and suppressed plasma IgE and histamine levels. Splenocytes produced an increased level of IFN-γ.

Conclusion These data showed that the simultaneous suppression of IL-4/IL-13 signals successfully controlled Th2-type chronic dermatitis. IL-4DM DNA treatment is a potent therapy for AD and related diseases.

Interleukin (IL)-4 plays a central role in Th2-cytokine-dominant inflammatory skin diseases such as atopic dermatitis (AD).[1–3] IL-4 is responsible for the differentiation of allergen-specific Th2 cells together with its closely related cytokine IL-13 for the class switching of activated B cells to IgE-producing cells. The effects of IL-13 are similar to IL-4 on B cells, monocytes, and other cell types, but T cells appear to lack an IL-13 binding receptor component and do not respond to IL-13.[4] The structural basis for the overlapping functions of IL-4 and IL-13 is a shared receptor subunit, and IL-4Rα organizes intracellular signals in response to both cytokines.[5,6] Signal transduction is induced by heterodimerization of the IL-4Rα with a second subunit; which may vary according to the cell types. The specific inhibition of IL-4 can be achieved by antagonistic IL-4 mutants. Variants of human IL-4 that bind to the receptor subunit IL-4Rα, but not to the other subunit γ-chain (γc) or IL-13Rα1 are competitive antagonists of IL-4.[7,8] IL-13 is inhibited by similar variants, which form unproductive complexes with IL-4Rα.[5,9] The single-site human IL-4 mutant Y124D has been used as an IL-4/IL-13 inhibitor in various studies,[7–17] but this variant retains some residual agonistic activity, which could be relevant for *in vivo* applications.[7,8] In contrast, IL-4 and IL-13 double mutant R121D/Y124D lacks detectable activity and appears to be an effective antagonist for human IL-4 and IL-13.[5,18]

We have recently developed a highly efficient murine IL-4 antagonist DNA (IL-4DM), in which the amino acids glutamine 116 and tyrosine 119 were changed for aspartic acid.[19] This murine mutant DNA is analogous to the R121D/Y124D double mutant. IL-4DM binds with high affinity to the murine

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IL-4Rα without inducing signal transduction, and has no detectable activity upon the proliferation or differentiation of murine cells. An appropriate amount of IL-4DM completely inhibits responses by wild-type IL-4.[19] Like its human analogue, the IL-4DM mutant is also an antagonist of IL-13 (B. Schnarr et al., unpublished data[37]). Recent experiments with monocytes from mice lacking a functional γc gene showed that IL-4DM is a complete inhibitor of IL-4 in the absence of γc as well.[20] In this study we have examined the effects of IL-4DM in vivo, using an AD model induced by the repeated exhibition of oxazolone (OX). The repeated application of a hapten such as OX on mice causes an initial delayed-type hypersensitivity that changes to an immediate-type response in the late phase with elevated IgE production and deviation of Th-cell responses. The skin lesions that appear in the late phase are compatible with the clinical findings as well as the cytokine profile observed in AD.[21–23] The inhibitory effect of IL-4DM on IL-4 and IL-13 on the immune response was comparable with that of knockout mice lacking either IL-4[24] or IL-4Rα. Treatment with IL-4DM prevented contact hypersensitivity responses with the increased production of interferon (IFN)-γ.

Materials and methods

Animals

BALB/c male mice aged 5 weeks were purchased from Japan SLC Co. (Shizuoka, Japan) and were used at the age of 6 weeks. Age-matched wild-type BALB/c mice were used as controls. All animals were cared for according to the ethical guidelines approved by the Institutional Animal Care and Use Committee of Mie University.

Reagents

The cDNA coding region of mouse IL-4 was amplified by a polymerase chain reaction (PCR) based on the cDNA sequence of mouse IL-4. The mouse IL-4 fragment was inserted into BamHI and EcoRI-filled in pcDNA3.1+ (Invitrogen, San Diego, CA, U.S.A.) under the TPA leader sequence, and then digested by BamHI and SacI. A Quickchange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for the mutagenesis of mouse IL-4. The oligonucleotide primers used to prepare a mouse IL-4 double mutant (IL-4DM, Q116D/Y119D) were CTAAAGAGCATCATGGATATGGATGACTCGTAGTCTAGAG and CTCTAGACTACGAGTCATCCATATCCATGATGCTCTTTAG. The IL-4 mutant fragments were ligated into pcDNA3.1+.[25] Mouse IL-4, IL-4DM plasmid DNAs were purified using the Plasmid Mega kit (Qiagen, Chatsworth, CA, U.S.A.) and diluted with sterilized physiological saline. OX was purchased from Sigma (St Louis, MO, U.S.A.) and was dissolved in acetone/olive oil (4 : 1).

Administration of DNA

Mice were treated by intraperitoneal injection of 100 μg of IL-4DM DNA on days 0, 7, 14, 21 and 28. A control plasmid (pcDNA3.1+) vector and IL-4 DNA were also injected on the same day (Fig. 1).

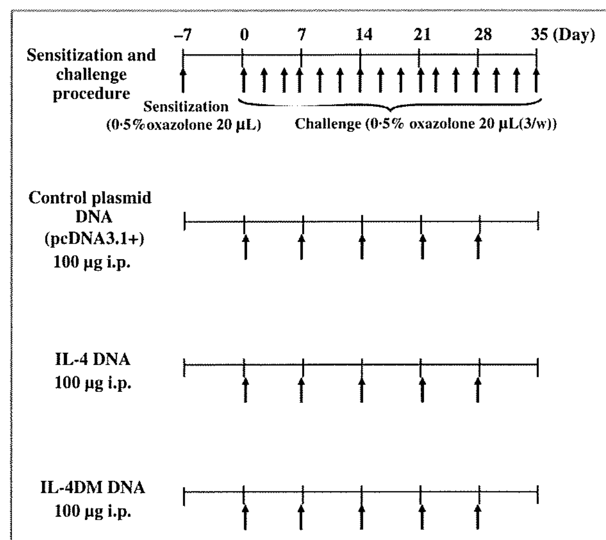

Fig 1. Schedule for induction of chronic contact hypersensitivity and administration of compounds. Mice received intraperitoneal (i.p.) injection of 100 μg of each plasmid DNA on days 0, 7, 14, 21 and 28.

Sensitization and challenge procedures

As shown in Figure 1, mice were initially sensitized by pasting 20 μL of 0·5% OX solution to their left ear 7 days before the first challenge (day 7) and then 20 μL of 0·5% OX solution was repeatedly applied on the left ear three times per week from day 0 as reported previously.[23] Ear swelling was measured with thickness gauge calipers before and 30 min after OX challenge to the pinna of the ear on day 35. The ear swelling response was expressed as the difference between the values taken before and 30 min after application.

Histological analysis

Ear skin specimens obtained 6 h after the final challenge on day 35 were fixed in 10% buffered neutral formaldehyde and embedded in paraffin wax. Histological sections were of 6 μm thickness and they were stained with haematoxylin and eosin. The sections were also stained with 0·5% toluidine blue for the identification of mast cells. The cell counts were performed in six consecutive microscopic fields at × 400 magnification.

Measurement of plasma IgE and plasma histamine

Blood was collected under ether anaesthesia 6 h after the last challenge. Plasma IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). In brief, 96-well immunoplates (Corning Inc., Corning, NY, U.S.A.) were coated with 100 μL of an antimouse IgE capture antibody (2 μg mL^{-1}) (BD PharMingen, San Diego, CA, U.S.A.) overnight at 4 °C. Plasma samples of 100 μL were diluted 60-fold with PBS

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containing 10% fetal calf serum (FCS) were placed in the wells. After incubation for 1 h at room temperature, 100 μL of biotin-conjugated antimouse IgE antibody (2 μg mL^{-1} in blocking buffer) (BD PharMingen) was added to each well. The plates were incubated at room temperature for 1 h, followed by six washes, incubated with 100 μL of horseradish peroxidase avidin D (FUNAKOSHI, Tokyo, Japan) 1 : 1000 in blocking buffer, and then incubated for 30 min at room temperature. A substrate solution of 100 μL containing 1·5 mg ABTS (Sigma-Aldrich, St Louis, MO, U.S.A.) in 5 mL of a 0·1 mol L^{-1} citric acid solution was added, and kept for 30 min at room temperature in a dark place. Thereafter the reaction was terminated by adding 50 μL of 2 mol L^{-1} H_2SO_4, and the optical density of each well at 405 nm was determined by using a microplate reader. A standard curve was prepared using mouse anti-TNP IgE standard (BD PharMingen). Plasma histamine levels were analysed using the commercial sandwich ELISA kit from Immunoteck (Marseille, France) according to the manufacturer's protocol.

Purification of mRNA from mouse ears

At 6 h after the final challenge, the skin of the left ear was sampled. The specimen was homogenized and the total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction; 1 mL of homogenate was vigorously mixed with 200 μL of chloroform, and centrifuged at 12 000 *g* for 15 min at 4 °C. The aqueous phase was separated and mixed with 0·5 mL of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 1 mL of 75% ethanol (Nacalai Tesque) and dried. RNA was suspended in 50 μL of RNase-free water, and the concentration was measured based on the absorbance at 260 nm, and the quality was confirmed by electrophoresis. cDNA was prepared from 10 μg of mRNA using archive kit (ABI, Foster City, CA, U.S.A.) according to the manufacturer's protocol.

Cytokine mRNA expression in skin

The transcriptional activity in the lesional skin samples was measured with a PCR. The amplification of cDNA was performed in 50 μL of a master mixture containing 0·5 μg of cDNA, 200 nmol deoxynucleotide triphosphate, 5 μL of PCR buffer, 2 U of Taq polymerase (ABI) and 2 μmol of each specific primer for the DNA of interest. The following primers were used for PCR reactions (5′–3′), mouse IFN-γ: TCAAGTGGCATAGATGTGGAAGAA and TGGCTCTGCAGGATTTTCATG; mouse IL-2: CCTGAGCAGGATGGAGAATAACA and TCCAGAACATGCCGCAGAG; mouse IL-4: CACTGACGGCACAGAGCTATTGATG and TCATGGTGCAGCTTTCGATGAATC; mouse IL-10: CTCTTACTGACTGGCATGAGGATCAGCAGG and TCTTCACCTGCTCCACTGCCTTGCTCTTAT; mouse IL-12: TCCTGCACTGCTGAAGACATC and TCTCGCCATTATAGATTCAGAGAC; mouse IL-13: AGACCAGACTCCCCTGTGCA and TGGGTCCTGTAGATGGCATTG; mouse β-actin: TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG.[26] PCR was performed under the following conditions: 95 °C for 5 min, followed by 35 or 40 cycles of 95 °C for 30 s, 56 °C (IFN-γ, IL-12) or 60 °C (IL-2, IL-4, IL-10, IL-13, β-actin) for 30 s, and 72 °C for 1 min were carried out. After the final cycle, the temperature was maintained at 72 °C for 7 min. PCR amplified fragments were electrophoresed through 1·5% agarose gels in tris-acetate EDTA buffer containing ethidium bromide, and the gels were scanned under ultraviolet light. The mRNA of β-actin was used as an internal control. The signal intensity of each reverse transcriptase (RT)-PCR product was estimated using an ATTO Lane & Spot Analyzer (ATTO, Shizuoka, Japan).

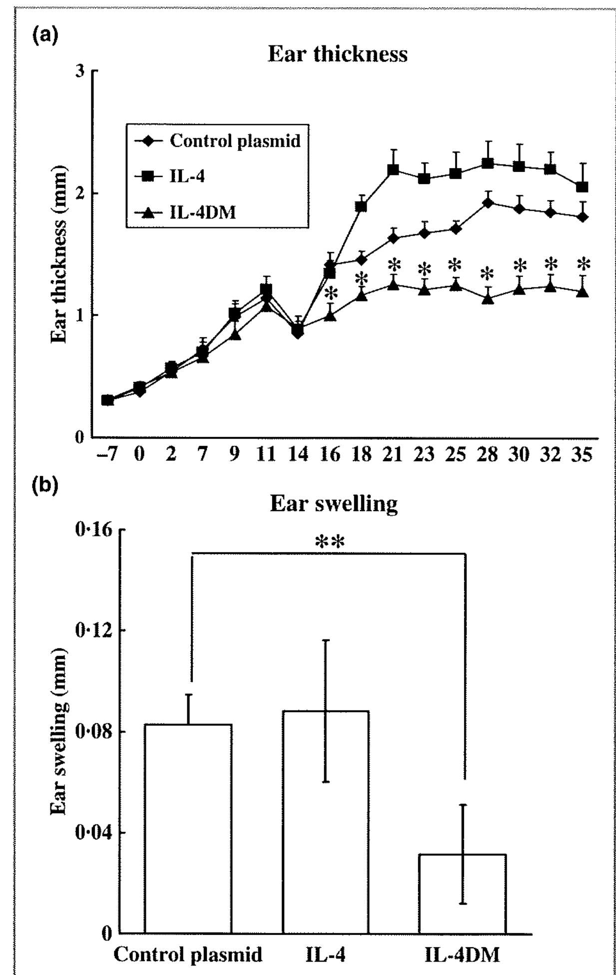

Fig 2. The effects of interleukin (IL)-4DM, IL-4, and control plasmid (pcDNA3.1) on ear swelling induced by repeated application of oxazolone (OX). (a) Ear thickness was measured before each OX challenge. Each point represents the mean ± SD of seven or eight mice. *$P < 0·05$: significantly different from the control group and IL-4 (Student's t-test). (b) Inhibition of the effecter phase of chronic hypersensitivity by IL-4DM, IL-4, and control plasmid DNA transfer. The ear swelling was measured 30 min after applying OX. The ear swelling in the IL-4DM groups was significantly suppressed compared with those in the IL-4 and control plasmid DNA groups. *Significant difference from the control by Student's t-test at $P < 0·05$.

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Cytokine production from splenocytes

A suspension of 2×10^6 splenocytes were made in a solution of 200 μL RPMI-1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 UI penicillin, 50 μg mL^{-1} streptomycin, and 5 μg mL^{-1} soluble antimouse CD3 (BD Bioscience), and 10 μg mL^{-1} antimouse CD28 (BD Bioscience). Cells were dispensed in triplicate into 96-well flat-bottomed microplates (Sumitomo Bakelite, Tokyo, Japan). After incubation for 48 h at 37 °C in a humidified incubator (5% CO_2), culture supernatants were collected and analysed for IFN-γ (Quantikine; R&D Systems, Minneapolis, MN, U.S.A.) or IL-4 (Quantikine; R&D Systems) production with an ELISA according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using Student's t-test and Mann–Whitney U-test. Values are expressed as mean ± SEM. A 95% confidence limit was taken as significant ($P < 0{\cdot}05$).

Result

Ear thickness with the treatment of IL-4DM, IL-4, or control plasmid

In the control group, the ear thickness increased from the beginning of the challenge, and increased gradually through the experiments (Fig. 2a). The agonistic IL-4 DNA treatment augmented increase of the ear thickness after day 16. In contrast, IL-4DM DNA treatment significantly suppressed increase of the ear thickness compared with that of control plasmid or IL-4DNA-treated mice.

Effects of IL-4DM on the oxazolone-induced acute-phase ear swelling

The ear swelling was also measured 30 min after OX application on day 35, and the difference between before and 30 min after application was calculated. IL-4DM DNA treatment suppressed the ear swelling significantly compared with that of the control DNA-injected group (Fig. 2b). However, IL-4DNA showed no suppressive effects.

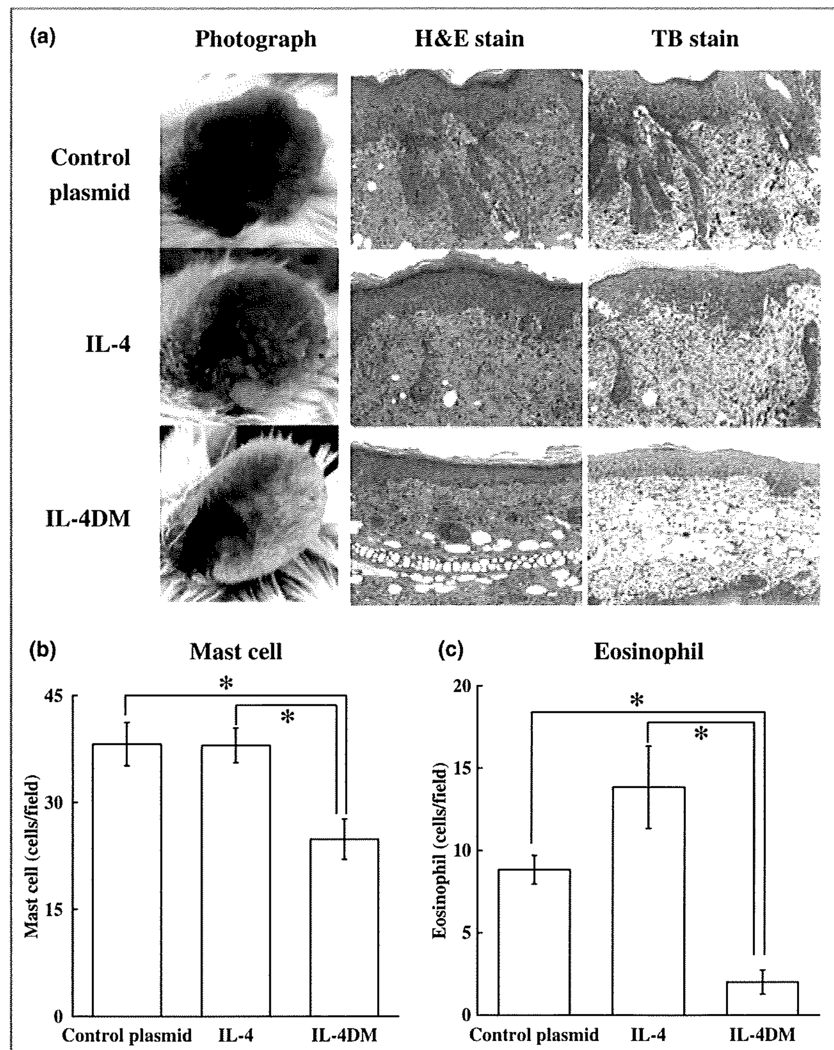

Fig 3. (a) Representative photographs and histological feature of oxazolone (OX)-treated skin lesion. OX-sensitized ear revealed hyperkeratosis, acanthosis, and parakeratosis in control and interleukin (IL)-4-treated mice. An increased number of infiltrating lymphocytes, macrophages and mast cells was observed in the skin lesions, all of which are typical histological findings observed in patients with atopic dermatitis. In contrast, acanthosis was clearly suppressed, and skin infiltration of granulocytes, eosinophils, and mast cells was decreased in the IL-4DM-treated mice as compared with control plasmid-treated mice (original magnification × 200). (b) The number of dermal mast cells was counted, and found to be decreased in the IL-4DM-treated mice. (c) The number of dermal eosinophils was also counted in 10 high power fields. The skin infiltration of eosinophils was significantly decreased in the IL-4DM-treated mice. Data are expressed as the mean ± SEM. *Significant difference by Student's t-test at $P < 0{\cdot}05$.

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Histological findings and mast cell counts in the inflamed skin

In control plasmid-treated mice and IL-4 DNA-treated mice, severe dermatitis was observed on the earlobe. A drastic decrease of inflammation was observed in IL-4DM DNA-treated mice (Fig. 3a). Histological examination on the OX-challenged ear skin revealed hyperkeratosis, acanthosis and parakeratosis in both of the control and IL-4-treated mice. An increased number of infiltrating lymphocytes, macrophages and mast cells was observed in the skin lesions in control DNA and IL-4 DNA-treated mice. These findings are comparable with those of AD skin lesions. In contrast, the acanthotic changes and infiltration of granulocytes, mast cells, and eosinophils were significantly suppressed in the IL-4DM DNA-treated mice compared with those of control DNA- or IL-4 DNA-treated mice (Fig. 3b,c). Interestingly, IL-4 DNA treatment increased eosinophil counts compared with control DNA treatment.

Plasma IgE and histamine levels

The total plasma IgE level was increased by repeated OX challenges (Fig. 4a). IL-4 DNA treatment showed no agonistic effects in the plasma IgE level; however, IL-4DM DNA treatment significantly suppressed the levels of plasma IgE. The plasma histamine level was also significantly increased in the control DNA- or IL-4 DNA-treated mice; however, IL-4DM DNA treatment significantly suppressed the plasma histamine levels (Fig. 4b).

Cytokine mRNA expression levels

To determine the effects of IL-4DM on cytokine production in the inflamed skin lesions, mRNA expression of Th1 and Th2 cytokines was analysed. The IFN-γ mRNA expression was significantly increased in IL-4DM DNA-treated mice ear compared with that of control DNA-treated samples (standardized by β-actin expression).

However, no remarkable difference in other cytokine mRNA expression was observed among three different DNA-treated samples (Fig. 5a,b).

Concentration of IFN-γ and IL-4 in splenocyte cell culture supernatants

To know the effects of IL-4DM DNA therapy in the systemic immune system, the concentration of IFN-γ in splenocyte cell culture supernatants was measured by ELISA. The IFN-γ level in the IL-4DM-treated samples was significantly higher than that in the control DNA- or IL-4 DNA-treated samples (Fig. 6a). We also measured the concentration of IL-4 in splenocyte cell culture supernatants by ELISA for mouse IL-4. The IL-4 level in the IL-4DM-treated samples was as high as the IL-4 DNA-treated samples. These were higher than that of the control DNA-treated samples (Fig. 6b).

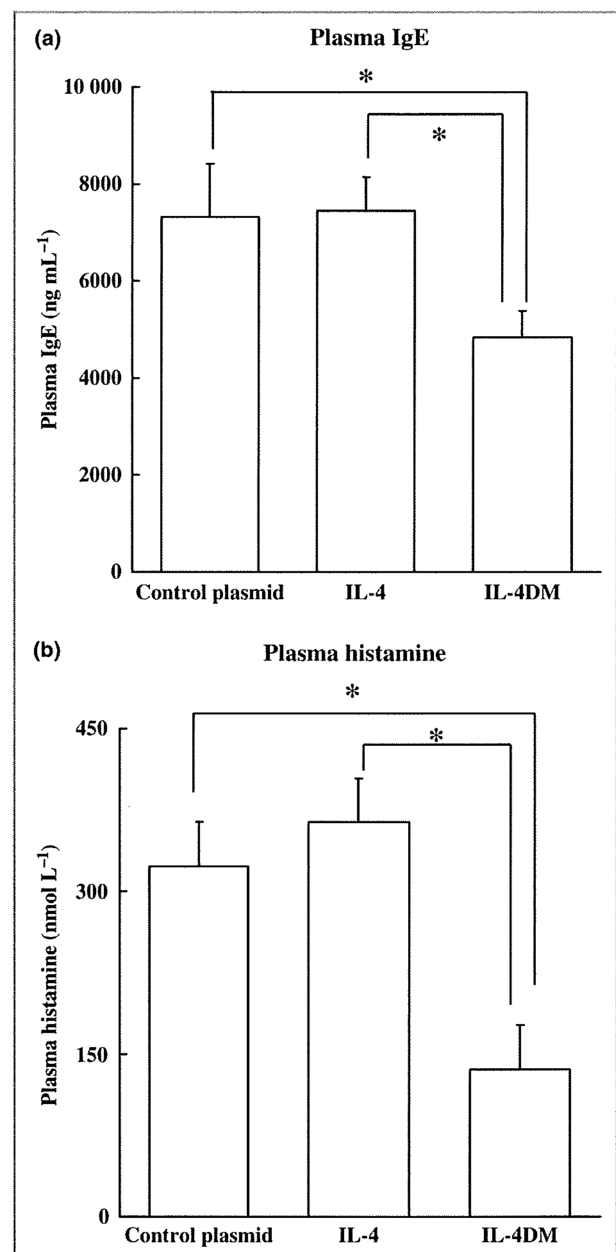

Fig 4. Plasma IgE and histamine levels. (a) Plasma IgE level was decreased in interleukin (IL)-4DM treated mice. (b) Inhibition of the production of plasma histamine was observed in IL-4DM DNA-treated mice. *Significant difference from the IL-4 and control by Mann–Whitney U-test at $P < 0.05$.

Discussion

Several previous studies have shown that AD is a chronic dermatitis with a predominance of Th2 cytokines in the lesional skin,[27–29] and that Th2 cytokines play a critical role in the pathogenesis of dermatitis.[28] IL-4 is one of the Th2 cytokines that affects the function of different cell types including T cells, B cells, mast cells, monocytes/macrophages, endothelial cells, fibroblasts, dendritic cells, Langerhans cells

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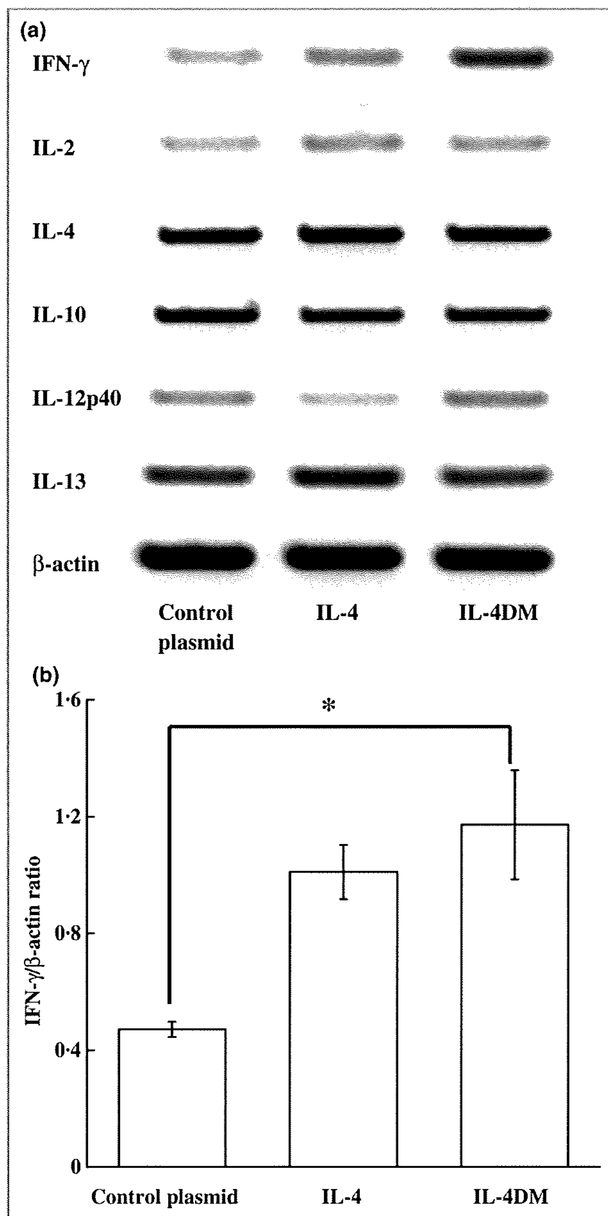

Fig 5. (a) Reverse-transcriptase polymerase chain reaction analysis of cytokine mRNA expression 6 h after oxazolone (OX)-sensitization. The cDNAs were amplified for respective cycles of six cytokines and β-actin, subjected to electrophoresis, and visualized with ethidium bromide. Representative results under optimal conditions are shown. Although almost all Th1 and Th2 cytokine levels were unchanged, mRNA expression for interferon (IFN)-γ was increased in IL-4DM-treated mice. (b) The level of mRNA expression of IFN-γ was expressed as the value relative to that for β-actin. The IFN-γ level in the IL-4DM group is significantly higher than that of control plasmid groups. *Significant difference from the control by Student's t-test at $P < 0{\cdot}05$.

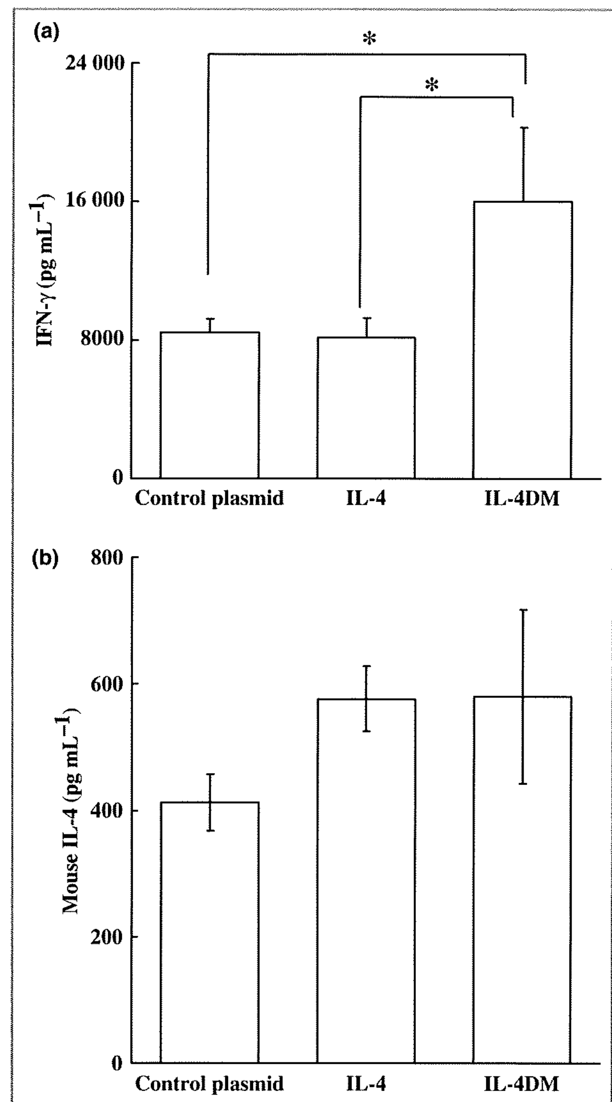

Fig 6. Cytokine production from splenocytes in chronic hypersensitivity mice. Interferon (IFN)-γ and interleukin (IL)-4 production from splenocytes was measured. (a) Actual IFN-γ protein production was increased in the IL-4DM-treated mice. (b) IL-4 levels did not reach the significance, but showed a tendency to increase in the IL-4 and IL-4DM mice. *Significant difference from the control by Mann–Whitney U-test at $P < 0{\cdot}05$.

and keratinocytes. Because of this broad-spectrum action, IL-4 is believed to play a crucial role in the pathogenesis of AD.[30,31] In the present study, we employed a contact hypersensitivity model by the repeated application of OX, which mimics the histological phenotype of AD in humans; this model also showed increased levels of Th2 cytokines in the lesional skin as reported by Kitagaki et al.[21]

Immunotherapy such as the direct blocking of Th2 responses with neutralizing antibody against Th2 cytokines, the soluble form of IL-4 receptor (IL-4R), or antagonistic IL-4 mutant proteins have been used for the treatment of asthma.[32–34] These proteins directly inhibit IL-4 binding thereby inhibiting host immune responses. A previous study by Nishikubo et al.[25] showed inhibition of immune responses by using IL-4 mutant protein for at least 50 weeks. However, results from these experimental animals have shown that the application of these trials to humans is difficult. Because the pharmacokinetic half-life of IL-4 mutant and sIL-4R protein

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are very short in vivo (IL-4 mutant: $t_{1/2}$ = 0·83 h; sIL-4R: $t_{1/2}$ = 4·6 h),[35,36] huge amounts of these molecules are required in plasma to maintain a long period of inhibitory action on allergic inflammation. In fact, administration of these molecules was required many times in high doses from the sensitization to the challenge periods.[35–37] In the present study, we demonstrated a remarkable antagonistic effect of IL-4 mutant DNA applied in a form of vaccination, as a potent new type of immunogene therapy for AD. In previous studies in which gene therapy and DNA vaccines were used in combination with a cytokine gene for tumours or pathogens, effective immune responses to antigen were recognized even in the absence of detectable plasma levels of cytokines. Recently, we also reported that administration of plasmid DNA coding IL-4 cDNA completely inhibited the development of insulitis, which is one of the Th1-type autoimmune diseases, although no IL-4 was detected in plasma.[38] These results suggest that genes applied as a DNA vaccine express and supply products to the host continuously. To occupy the IL-4/IL-13 receptors, a continuous supply of IL-4DM is needed but not bolus application. Therefore, IL-4DM applied as a DNA vaccine might inhibit the allergic inflammation by persistent secretion of mutant IL-4 over a long period in a limited amount.

As we had expected, IL-4DM mitigated phenotypical and histological changes such as severe oedema, inflammatory cell infiltration and epidermal hyperplasia. IL-4DM also significantly decreased the number of dermal mast cells. IL-4 is known to be a potent activator of mast cells. Mast cells, which participate in the inflammatory cascade, serve as an abundant source of Th2 cytokines as well as inflammatory mediators.[39–41] Therefore, inhibition of mast cell activation is another possible mechanism through which IL-4DM ameliorates inflammatory responses in the present model of dermatitis. Eosinophil infiltration into the dermis has been well documented in AD.[42] In this study, an increased number of eosinophils was observed in contact hypersensitivity skin lesions, and was dramatically inhibited by IL-4DM treatment. Inhibition of cellular infiltration in IL-4DM mice may be due to suppression of IL-4-mediated immunological events such as a decreased expression of cellular adhesion molecules on endothelial cells.[43]

Injected IL-4DM and IL-4 DNA are trapped by monocytes/macrophages by phagocytosis. They may migrate to lymph nodes or spleen and show systemic effects. In fact, we could observe a high concentration of IL-4 in cultured splenocytes from IL-4DM DNA injected mice by ELISA. Unfortunately, there is no specific anti-IL-4DM antibody or anti-IL-4DM ELISA. The standard ELISA used in this study could not differentiate the natural mouse IL-4 and mutant IL-4 protein; these findings are consistent with the previous report.[25] The plasma IL-4 levels in the agonistic IL-4DNA-treated mice were consistent with those of the IL-4DM DNA-treated mice. Therefore, we speculate that exogenously applied IL-4DM DNAs were expressed the same as IL-4 DNAs, and showed systemic immunological effects.

IFN-γ production increased systemically and locally in mice treated with IL-4DM DNA. Repeated OX treatments cause expansion both of Th1 and Th2 cells. IL-4DM DNA therapy interfered with the development of the Th2 milieu. Subsequently, IFN-γ production and mRNA expression might become abundant locally and systemically.

Tissue-specific gene transfer could be achieved naturally and effectively through the cell specificity of virus receptors.[44] However, there may be a risk of vector toxicity through viral infection of host cells. Also, the limited size of transgenes is often a serious obstacle. Moreover, immune responses to viral vectors are also induced, and the effects of transgenes are eliminated by immune responses to the vectors. For human applications, the efficacy and safety of any delivery system for gene transfer are always of major concern. Nonviral approaches are advantageous in immunogene therapy. DNA vaccines are capable of inducing potent biological effects in a variety of experimental systems.[45] One of the characteristic features of DNA vaccines is their ability to induce long-lasting immunity. The animals that had been treated with IL-4DM DNA did not develop severe allergic inflammation even before or after antigen sensitization.

In the present study, we showed the beneficial effects of immunogene therapy with IL-4 mutant DNA in an experimental model for AD. An IL-4 mutant DNA vaccination is a potent new tool for the systemic treatment of AD.

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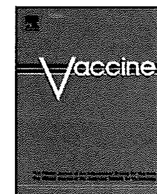

T cell-based functional cDNA library screening identified SEC14-like 1a carboxy-terminal domain as a negative regulator of human immunodeficiency virus replication

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ABSTRACT

Genome-wide screening of host factors that regulate HIV-1 replication has been attempted using numerous experimental approaches. However, there has been limited success using T cell-based cDNA library screening to identify genes that regulate HIV-1 replication. We have established a genetic screening strategy using the human T cell line MT-4 and a replication-competent HIV-1. With this system, we identified the C-terminal domain (CTD) of SEC14-like 1a (SEC14L1a) as a novel inhibitor of HIV-1 replication. Our T cell-based cDNA screening system provides an alternative tool for identifying novel regulators of HIV-1 replication.

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1. Introduction

The molecular interaction between HIV-1 and the host is not fully understood. A systematic genome-wide approach provides the critical information for the completion of the HIV-1-host interactome. Many experimental genome-wide screening systems have been established to identify the cellular genes required for HIV-1 replication (Table 1, [1–8]). More than a hundred genes have been identified as being cellular factors that regulate HIV-1 replication. However, different screening systems do not identify the same set of genes, and many systems yielded non-overlapping candidates. These discrepancies are assumed to be due to differences in the experimental approaches, such as the virus, the cell line, or the genetic materials used.

For viruses, the wild-type HIV-1 [1,3–6] or a replication-incompetent HIV-1 pseudotyped with vesicular stomatitis virus (VSV)-G is used [2,7,8]. The VSV-G-pseudotyped "HIV-1-based vector" has been used to identify factors associated with the viral entry processes. However, in reality, it covers the events from post-membrane fusion to translation. One of the potential caveats in the use of the VSV-G-pseudotyped vector is that it enters cells via the VSV-G-restricted route, which is fundamentally different from the HIV-1 *Env*-mediated entry pathway [9–12]. The replication-competent HIV-1 should be ideal to cover the entire viral replication cycle; however, this may raise biosafety concerns.

For cells, non-T cells, such as a genetically engineered HeLa cells that ectopically express luciferase or beta-galactosidase (TZM-bl cells), are often used, since they are efficiently transduced with genetic materials [2,5–8]. Not many studies employ a T cell-based system, partly because genetic materials are not efficiently transduced into T cells [1,3,4]. To identify HIV-1 replication regulatory factors, it is preferable to perform the functional analysis in the natural targets of HIV-1 including T cells. The gene expression profile of non-T cells is apparently different from that of T cells as exemplified by the absence of T cell specific markers on non-T cells such as CD4. It is possible that a candidate gene isolated in the non-T cell-based system might not be expressed in T cells. It is impossible to identify T cell-specific factors in the non-T cell-based screening using the siRNA library or in the screening using cDNA libraries derived from non-T cells. Also, the effect or functions of some genes may not be identical in distinct cell types. The potential risk of a non-T cell-based assay is that we may falsely score a gene as a regulator of HIV-1 replication, although many genes have been discovered using non-T cell-based screening systems including the viral receptors. Ideally, the primary CD4-positive T cells, dendritic cells, macrophages, or NK/T cells should be used.

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Table 1
Summary of genome-wide screening strategies to identify regulatory factors of HIV-1 replication.

Genetic material	Transduction approach	Cell line	Replication competency of HIV-1	Reference
cDNA library	Retroviral, stable	TE671	Incompetent	[2,8]
siRNA library	Transfection, transient	HeLa or 293T	Competent or incompetent	[5,6,7]
cDNA library	Lenti- or retroviral, stable	MT-4	Competent	[1,3,4]

Given technical limitations, this is currently unrealistic for genetic screening experiments.

As for the genetic material, cDNA libraries are often used [1–4,8]. Recent studies utilized siRNA libraries [5–7]. The cDNA approach is advantageous for providing genetic diversity. Expression of the full-length open reading frame of a gene can upregulate the function of the gene, whereas cDNA fragments can function in a diverse fashion. The gene silencing approach downregulates gene expression; however, the silencing efficiency of a gene varies in different cell types and at different time points in the assay (reviewed in [13]). As mentioned above, the gene silencing approach is unable to score the contribution of genes that are not expressed in the cells used in the assay.

The screening can be performed in cells that are either transiently [5–7] or stably [1–4,8] transduced with genetic materials. In the transient transfection assays, it is possible that the dysregulation of a gene function can damage the physiology of the cells. In such a case, the inhibition of HIV-1 replication can be observed, but may not be a direct inhibitory effect of the gene of interest. Such a risk can be minimized by using cells stably transduced with the genetic materials.

We conducted a phenotype cDNA screen using a T cell line-based assay to identify cellular genes that render cells resistant to HIV-1 replication [3]. The advantage of our functional screening system is that cDNA libraries are stably transduced into cells, and that a replication-competent HIV-1 and a human T cell line MT-4 are used. With this system, we have successfully identified the SEC14-like 1a (SEC14L1a) C-terminal domain (CTD) as an inhibitor of HIV-1 replication that targets the late phase of the viral life cycle.

2. Materials and methods

2.1. Cells, transfection, cDNA selection

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The method of selecting human cDNAs that confer resistance to HIV-1 has been described previously in detail [3].

2.2. Plasmids

The SEC14L1a CTD1 was amplified from MT-4 polyA RNA by reverse transcriptase PCR (RT-PCR) using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACCCTGCGTGCGCCGCCAGCAGC-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. The SEC14L1a CTD2 was amplified by PCR from human lymph node cDNA library (Takara, Otsu, Japan) using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACTGCGAAGTGCCAGAGGGTGGAC-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. Full length (FL) SEC14L1a was amplified by PCR from a plasmid containing the SEC14L1a open reading frame (ORF, CSODL004YN18, Invitrogen), using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACGTGCAGAAATACCAGTCCCCAG-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. The AgeI-MfeI fragments of the PCR products were cloned into the XmaI-MfeI sites of the pEGFP-C3 plasmid (Clontech, Palo Alto, CA), generating pEGFP-SEC14L1a-CTD1, -CTD2, and -FL. The XhoI-MfeI fragments from the resulting plasmids were cloned into the corresponding restriction sites of the pCMMP KRAB vector, creating pCMMP GFP-SEC14L1a-CTD1, -CTD2, and -FL. The HIV-1 *tat* was amplified by PCR using the primers 5′-AACCGGTCTCGAGCCACCATGGAGCCAGTAGATCCTAGAC-3′ and 5′-GGATCCTCAGTCGTCATCGTCTTTGTAGTCTTCCTTCGGGCCTGTCGGGTC-3′. A Tat expression vector pCMMP Tat was constructed by cloning the AgeI-BamHI fragment of the PCR product into the corresponding restriction sites of the pCMMP KRAB vector. The HIV-1 *Env* and GFP expression vectors (pIIIex and pCMMP GFP, respectively) are described previously [3,12,14]. To construct the pCMMP GFP-FLAG (GFPf), pCMMP CXCR4 d-10 [15] was digested with AgeI and XhoI to remove CXCR4 d-10 ORF and self-ligated after blunting with T4 DNA polymerase. The HIV-1 *gag-pol*, *tat*, and *rev* expressing plasmid pCMVR8.91 was a generous gift from Dr. Trono's group [16].

2.3. Western blotting

Western blotting was performed according to techniques described previously [17]. The following reagents were used: anti-FLAG (rabbit polyconal, 600-401-383, Rockland, Gilbertsville, PA), anti-p24 (183-H12-5C, NIH AIDS Research and Reference Reagent Program), anti-gp120 (vA-20 and vT-21 antibodies, Santa Cruz Biotech, Santa Cruz, CA), biotinylated anti-goat antibody (GE Healthcare Bio-Sciences, Piscataway, NJ), horseradish peroxidase-conjugated streptoavidin (GE Healthcare Bio-Sciences), and EnVision+ system (Dako, Glostrup, Denmark). Signals were visualized with an LAS3000 imager (Fujifilm, Tokyo, Japan) and quantified by Multi Gauge ver 3.0 software (Fujifilm).

2.4. Confocal microscopy

293T cells transiently transfected with expression vectors for SEC14L1a derivatives were grown on glass plates, fixed in 4% formaldehyde in phosphate buffer saline (PBS) for 5 min at 24 h post-transfection, stained with Hoechst 33258 (Sigma), mounted (Vectorshield, Vector Laboratories, Burlingame, CA), and imaged using a confocal microscope META 510 (Carl Zeiss, Tokyo, Japan). For MT-4 cells, live cells were incubated with Hoechst 33258 and imaged unfixed. Image brightness and contrast were processed by META510 software (Carl Zeiss).

2.5. Immunoprecipitation

Cells expressing FLAG-tagged proteins were harvested and washed twice with PBS and then lysed in the lysis buffer (50 mM Tris–HCl, pH 8.0, 0.5% IGEPAL CA630, protease inhibitor cocktail from Sigma) on ice for 30 min. The soluble fraction was obtained by centrifugation at 15,000 rpm for 30 min at 4 °C, and was incubated with 20 μl of Red-Anti-FLAG M2 Affinity Gel (Sigma) with gentle mixing overnight at 4 °C. After washing the agarose beads for five times with the lysis buffer, the bound complexes were eluted with the FLAG peptide, and analyzed by Western blotting.

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171 *2.6. Flow cytometry*

172 Cells were labeled with PE-Cy5-conjugated anti-CD4 antibody
173 or PE-conjugated anti-CXCR4 antibody (Beckton Dickinson, San
174 Jose, Calif.) for 30 min at 4 °C. Cells were washed once with PBS
175 supplemented with 1% FBS and analyzed by FACS Aria (Beckton
176 Dickinson). The GFP-positive cells were sorted using FACS Aria.

177 *2.7. Monitoring HIV-1 replication*

178 For HIV-1 infection, 1×10^5 cells were incubated at the room
179 temperature with the HIV-1_{HXB2}-containing culture supernatant,
180 which had approximately 1.0 ng of $p24^{CA}$, for approximately
181 30 min. The culture supernatants were collected at 4 d post-
182 infection and subjected to ELISA to measure the p24 ^{CA} antigen,
183 using a Retro TEK p24 Antigen ELISA Kit according to the manu-
184 facturer's protocol (Zepto Metrix, Buffalo, NY). The signals were
185 measured with an ELx808 microplate photometer (BIO-TEK®,
186 Winooski, VT).

187 *2.8. PCR analysis*

188 The cellular DNA and RNA were extracted from cells infected
189 with VSV-G-pseudotyped HIV-1 vector produced by using pNL-
190 Luc plasmid, as described previously [17]. The Alu-LTR PCR and
191 RT-PCR were performed as described previously [3,17] using
192 the following primers: for the first Alu-LTR PCR reaction, 5'-
193 AACTAGGGAACCCACTGCTTAAG-3' and 5'-TGCTGGGATTACAGGC-
194 GTGAG-3'; and for the second Alu-LTR PCR reaction, 5'-AACT-
195 AGGGAACCCACTGCTTAAG-3' and 5'-CTGCTAGAGATTTTCCACA-
196 CTGAC-3'. For amplification of HIV-1 mRNA, 5'-ATGGAGCCAGTAG-
197 ATCCTAGAC-3′ and 5′-CTATTCCTTCGGGCCTGTCGGG-3′ primers
198 were used. For the control, we amplified beta-globin and cycloph-
199 ilin A using the following primers: beta-globin, 5'-TATTGGTCT-
200 CCTTAAACCTGTCTTG-3' and 5'-CTGACACAACTGTGTTCACTAGC-3';
201 and cyclophilin A, 5'- CACCGCCACCATGGTCAACCCCACCGTGTTCT-
202 TCGAC-3′ and 5′- CCCGGGCCTCGAGCTTTCGAGTTGTCCACAGTCA-
203 GCAATGG-3′. The amplicons were separated in a 2% agarose gel,
204 stained with ethidium bromide, and imaged with a Typhoon
205 scanner (GE Healthcare Bio-Sciences).

206 *2.9. Collection of virus-like particle*

207 Tissue culture supernatants containing virus-like particles (VLP)
208 were passed through nitrocellulose filters (0.45 μm, Millipore,
209 Tokyo, Japan) and the virions were collected by centrifugation
210 (Optima™ TL, TLA 100.3 rotor, 541 k × g for 1 h; Beckman Coulter,
211 Miami, FL).

212 **3. Results**

213 *3.1. Identification of SEC14L1a as a potential regulator of HIV-1*
214 *replication*

215 We prepared MT-4 cells that constitutively express cDNA trans-
216 duced by a lentiviral vector or an MLV-based retroviral vector
217 (Fig. 1A). The cDNAs were derived from human peripheral blood
218 mononuclear cells (PBL) and *Oryctolagus cuniculus* (European rab-
219 bit) kidney-derived cell line RK13 cells. MT-4 cells transduced with
220 cDNA were collected by FACS sorter using the green fluorescence
221 as a marker since viral vectors encoded the GFP expression cas-
222 sette. Then, cells were infected with HIV-1. Surviving cells were
223 propagated and the genomic DNA was extracted to recover the
224 transduced cDNA by PCR as previously described [3]. We iso-
225 lated two clones encoding the carboxy terminal domain (CTD) of
226 SEC14L1a (Gene ID 6397, Fig. 1B and C); one from the PBL cDNA
library (1/65 independent clones, 1.5%), and one from the RK13 cDNA library (1/42 independent clones, 2.4%). The fact that the SEC14L1a CTD was successfully identified from two independent cDNA libraries strongly suggests that it is a negative regulator of HIV-1 replication. It is important to note that previous genome-wide screenings for HIV-1 regulators have not identified SEC14L1a CTD. This clearly suggests that our T cell-based cDNA screening system is unique, and should be able to complement the other genome-wide screening systems.

SEC14L1a belongs to the widely-expressed SEC14-superfamily that is involved in membrane trafficking and phospholipid metabolism [18–21]. The function of SEC14L1a is not well understood. The C-terminus of SEC14L1a encodes a Golgi dynamics (GOLD) domain (amino acids (aa) 523–674; Fig. 1C) that mediates the protein-protein interaction possibly involved in the maintenance of Golgi apparatus function and vesicular trafficking [22]. The only reported biological activity of SEC14L1a is to interact with cholinergic receptors AchT and CHT1 [23]. The GOLD domain is responsible for the physical interaction between SEC14L1a and cholinergic receptors. However, the functional significance of these interactions remains to be clarified. The conserved SEC14 domain directly interacts with lipid molecules [17–21]. However, the lipid ligand of SEC14L1a (aa 319–490, Fig. 1C) has yet to be identified.

3.2. Construction of expression vectors for SEC14L1a derivatives

The longest SEC14L1a cDNA recovered from the PBL cDNA library spanned nucleotides (nt) 2045–2492 of SEC14L1a mRNA (NM_003003.3), covering the CTD of the SEC14L1a open reading frame (ORF; Fig. 1B). We detected a potential translational start codon at nt 2188-2190 within the GOLD domain (asterisk, Fig. 1B). We speculated that the isolated cDNA might have expressed the carboxy half of the GOLD domain (aa 641–715) in MT-4 cells, leading to the inhibition of HIV-1 replication.

To test this, we constructed an expression plasmid for FLAG-tagged CTD (aa 642-715) fused to the carboxy terminus of GFP (CTD1; Fig. 1C). We also constructed GFP fusion proteins spanning the GOLD domain (CTD2, aa 493–715) or the full-length SEC14L1a (FL; Fig. 1C). Expression of these proteins was verified by Western blotting of transient transfected 293T cells (Fig. 1D). The confocal microscopy analysis indicated that the FL localized mainly in the cytoplasm, with some accumulation in the perinuclear regions (Fig. 1E), consistent with a previous report [23]. CTD1 was distributed in the cytoplasm and the nucleus, with a slight preference for the cytoplasm. CTD2 was evenly distributed to the nucleus and cytoplasm. When MT-4 cells constitutively expressing FL, CTD1, and CTD2 were analyzed, the subcellular distribution was less clear, due to the small cytoplasm (Fig. 1F). However, FL was distributed evenly to the nucleus and cytoplasm in MT-4 cells. In contrast, CTD1 was excluded from the nucleus in MT-4 cells (Fig. 1F). The distribution of CTD2 in MT-4 cells was similar to that in 293T cells (Fig. 1F). The differences of protein distribution in two cell types may be caused by the cell type-dependent regulation of protein trafficking and/or the effect of protein expression levels.

3.3. Verification of anti-HIV-1 activity associated with SEC14L1a CTD1

We introduced FL, CTD1, or CTD2 into MT-4 cells using the MLV vector, and isolated cells constitutively expressing FL, CTD1, or CTD2. Expression of SEC14L1a derivatives in MT-4 cells was verified by Western blotting (Fig. 2A). FL expression was verified by immuno-precipitation assay (Fig. 2A). The detection of FL by Western blotting was inefficient considering the fact that all the SEC14L1a derivatives are GFP-tagged, and the GFP intensity of FL-expressing MT-4 cells was not lower than that of CTD1-expressing

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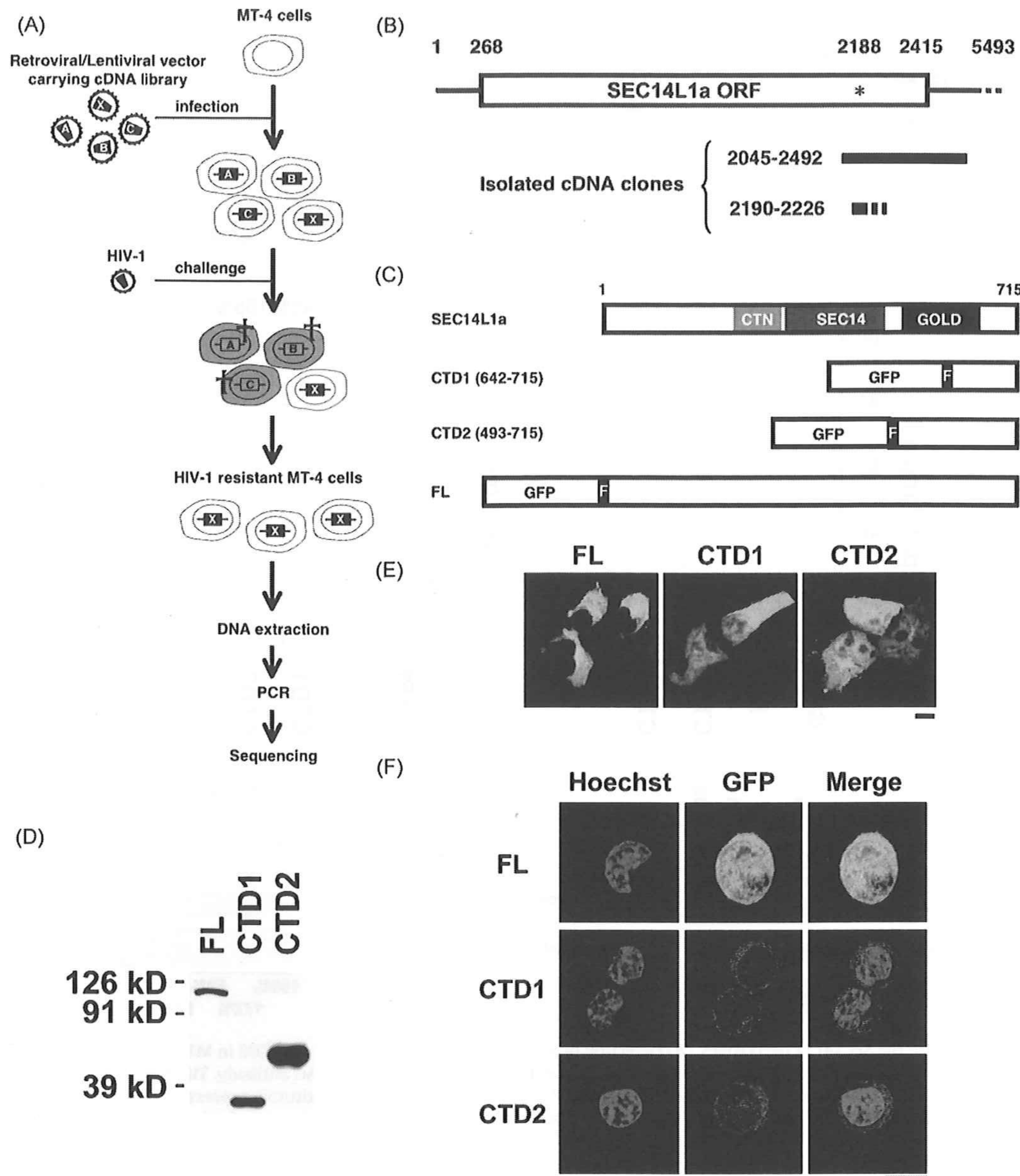

Fig. 1. Identification of SEC14L1a CTD as a potential regulator of HIV-1 replication. (A) The experimental strategy used to screen a cDNA library for genes rendering cells resistant to HIV-1. MT-4 cells were infected with a retroviral or lentiviral vector carrying cDNA libraries and were challenged with wild-type HIV-1_{HXB2}. The HIV-1-infected cells (gray with cross) quickly undergo cell death. The surviving cells were propagated, collected, and the transduced cDNA labeled X was determined. (B) Schematic representation of SEC14L1a mRNA (NM_00303.3) and the isolated gene fragments. The open reading frame (ORF) is assigned from nucleotides (nt) 268 to 2415. The potential internal translational initiation codon is marked with an asterisk. (C) Schematic representation of the SEC14L1a protein (NP_002994). SEC14L1a has a CRAL_TRIO_N domain (CTN, amino acids 241–313), a SEC14p-like lipid-binding domain (SEC14, amino acids 319–490), and a Golgi dynamics domain (GOLD, amino acids 523–674). The cloned fragments (CTD1 and CTD2) and full-length (FL) gene were tagged with a FLAG epitope (indicated with an "F") on their N-termini, and fused to the C-terminus of GFP. (D) Verification of FL, CTD1, and CTD2 expression in 293T cells by Western blotting using anti-FLAG antibody. (E) Confocal microscopy images of 293T cells expressing FL, CTD1, or CTD2. The green signal represents GFP fluorescence. Magnification, 630×; scale bar, 10 μm. (F) Confocal microscopy images of MT-4 cells constitutively expressing FL, CTD1, or CTD2. The blue signal represents the Hoechst-stained nucleus, and green represents GFP fluorescence. Magnification, 630×; scale bar, 5 μm.

cells (Fig. 1F). The MLV vector expressing GFP alone was used as a control. The cell proliferation, morphology, and cell surface levels of HIV-1 receptors were unaltered by any of the SEC14L1a derivatives (Fig. 1F, 2B, and data not shown). HIV-1 replication was tested in these cells. The level of HIV-1 replication was significantly inhibited in CTD1- and CTD2-expressing cells (69.1% and 69.8% on the average from seven independent experiments, respectively, $P < 0.05$, two-tailed Student's t-test), but was hardly inhibited in FL-expressing cells (86.4%, not statistically significant; Fig. 2C). This observation was reproducible in independently established MT-4 cells and SupT1 cells (data not shown). These data verified the original screening results, and suggest that the C-terminal half of GOLD domain of SEC14L1a serves as an inhibitor of HIV-1 replication. In contrast, it is suggested that FL is not a potent negative regulator of HIV-1 replication.

3.4. SEC14L1a CTD1 and CTD2 target the late phase of the HIV-1 life cycle

We analyzed the viral entry and production phases to determine which step of the HIV-1 life cycle CTD1 and CTD2 target.

The Alu-LTR PCR assay was performed to examine the effect of SEC14L1a derivatives on the viral entry phase. The MT-4 cells stably expressing GFP, FL, CTD1, or CTD2 were infected with VSV-

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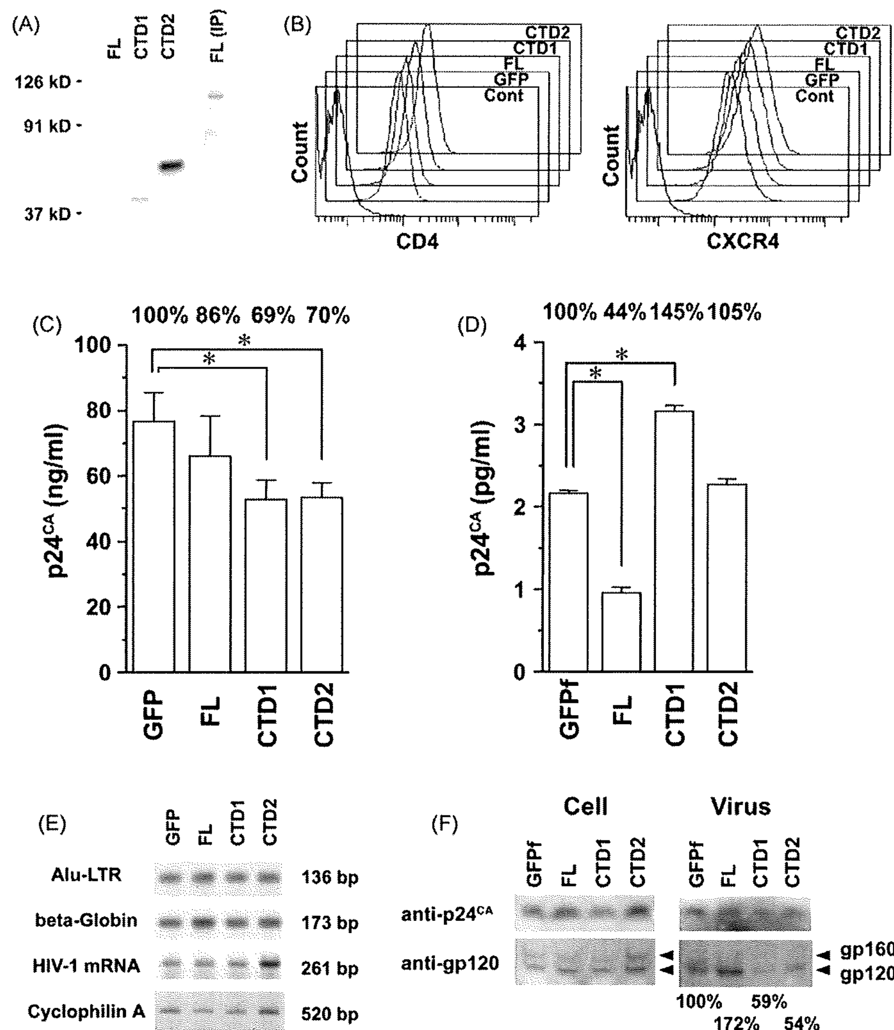

Fig. 2. Functional characterization of the SEC14L1a derivatives. (A) Detection of stable expression of FL, CTD1, and CTD2 in MT-4 cells by Western blotting using anti-FLAG antibody. FL was detected by the immunoprecipitation (IP) assay using agarose beads conjugated with anti-FLAG antibody. The flow cytometric analysis of the cell surface expression of HIV-1 receptors CD4 and CXCR4 in MT-4 cells stably expressing GFP, FL, CTD1, and CTD2. (C) Constitutive expression of CTD1 and CTD2 limited the replication of HIV-1 in MT-4 cells. The concentration of viral $p24^{CA}$ antigen in the culture supernatant was measured at 4 d post-infection. The results represent the average of seven independent experiments ± the standard error of the mean. The reduction of viral $p24^{CA}$ concentration relative to GFP was shown on the top. Asterisks indicate the statistical significance compared to GFP ($P<0.05$ by two-tailed Student's *t*-test). (D) The PCR-based assay to examine the effect of SEC14L1a derivatives on the early phase of viral life cycle (top two panels) and the transcription from LTR promoter (bottom two panels). The HIV-1 entry efficiency was examined by Alu-LTR PCR. Beta globin was used as an internal control. The HIV-1 transcription efficiency was examined by RT-PCR targeting spliced viral mRNA. Cyclophilin A was used as a control. The expected length of each PCR amplicon was indicated. (E) The effect of SEC14L1a derivatives on the HIV-1 production. The 293T cells grown in a well of a 6-well plate were transfected with 200 ng of HIV-1 proviral DNA and 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The culture supernatant was recovered at 2 d post-transfection and the $p24^{CA}$ concentration was measured. The representative data from five independent experiments was shown. The results indicate the average ± the standard deviation. The relative $p24^{CA}$ concentration compared to GFPf was shown on the top. Asterisks indicate the statistical significance compared to GFPf ($P<0.001$ by two-tailed Student's *t*-test). The *Env* incorporation onto the virus-like particles (VLP) produced by 293T cells expressing SEC14L1a derivatives. The 293T cells grown in a well of a 6-well plate were transfected with 1 μg of *gag-pol* (pCMVR8.91) and *Env* (pIIIex) expression vectors along with 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The cell lysates (Cell) and VLP fractions (Virus) were subjected to Western blot analysis detecting gp120 and $p24^{CA}$ harvested at 2 d post-transfection. The *Env* incorporation levels normalized to $p24^{CA}$ relative to GFPf were shown at the bottom.

311 G-pseudotyped HIV-1 vector, and the cellular genomic DNA was
312 recovered at 4 d post-infection. The amount of Alu-LTR PCR prod-
313 ucts from FL-, CTD1-, or CTD2-expressing MT-4 cells was almost
314 equal to that from GFP-expressing cells, suggesting that the early
315 phase of the viral life cycle is not inhibited by any of the SEC14L1a
316 derivatives (Fig. 2D). To examine the viral production phase, we
317 examined the LTR-driven viral gene transcription by RT-PCR. Cel-
318 lular RNA was extracted from the same MT-4 cells infected with
319 VSV-G-pseudotyped HIV-1 vector, and RT-PCR was conducted to
320 amplify LTR promoter-driven spliced HIV-1 mRNA. The amount of
321 viral RNA expressed in FL-, CTD1-, or CTD2-expressing cells was
322 not lower than that in GFP-expressing cells when the levels of the
323 internal control was taken into account (Fig. 2D). Given that the
324 similar number of viral genome was integrated as indicated by the

Alu-LTR PCR, these data suggest that viral transcription is not inhibited by any of the SEC14L1a derivatives, and that the action point of CTD1 and CTD2 should be at post-transcriptional levels of the viral production phase.

Next, the FL, CTD1, or CTD2 expression vector was co-transfected with HIV-1 proviral DNA into 293T cells, and viral production was quantified by $p24^{CA}$ ELISA. The FLAG-tagged GFP (GFPf) was used as a control hereafter. We found that the FL expression significantly reduced the production of HIV-1 (44.2%, $P<0.001$, two-tailed Student's *t*-test) compared to the GFPf control (Fig. 2E). In contrast, the CTD1 enhanced the production of HIV-1 (145.9%, $P<0.001$, two-tailed Student's *t*-test; Fig. 2E). However, CTD2 did not measurably affect the HIV-1 production (105.1%, not statistically significant; Fig. 2E). As the ELISA assay examines the effect

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of CTDs on *Gag* functions, we next tested the functional interaction between CTDs and *Env*. The *Env* incorporation onto the virion was examined by tripartite-transfection of expression vectors for *Env*, *gag-pol*, and SEC14L1a derivatives into 293T cells, and the VLP was collected by centrifugation. The immunoblotting against gp120 was performed on the cell lysate and the VLP fraction. The cellular *Env* and *Gag* expressions were not detectably affected by any of the SEC14L1a derivatives (Fig. 2F, left panel). The *Env* incorporation onto the VLP was slightly enhanced by FL (157%; Fig. 2F, right panel). In contrast, the VLP produced from CTD1- or CTD2-expressing cells incorporated substantially fewer *Env* than those from GFPf-expressing cells (59% or 54%, respectively; Fig. 2F, right panel). These data were reproducible in independently performed experiments. The densitometric analysis of Western blot image showed that the average ± the standard error of the mean of Env incorporation onto the virion was 129.7 ± 39.9%, 54.8 ± 24.7%, and 25.5 ± 10.3% for FL, CTD1, and CTD2 compared to GFPf, respectively (3–4 independent experiments). The *Env*-mediated cell-to-cell fusion assay indicated that SEC14L1a derivatives did not limit the cell surface targeting and function of *Env* (data not shown). In addition, the *Gag* processing in virion was unaffected by any of the SEC14L1a derivatives (data not shown). Collectively, these data suggest that the HIV-1 replication is inhibited by CTD1 and CTD2 due to the inefficient *Env* incorporation onto the virion. To test this possibility, we infected fresh MT-4 cells with the equal amount of HIV-1 propagated in CTD1- or CTD2-expressing MT-4 cells (1–2 ng $p24^{CA}$), and the viral replication was monitored at 3–4 days post-infection by measuring the $p24^{CA}$ concentration. The infectivity of HIV-1 propagated in CTD1- or CTD2-expressing cells was attenuated to 83.1 ± 17.9% or 82.4 ± 5.5% relative to the virus recovered from GFP-expressing cells, respectively (the average ± the standard error of the mean of 3 independent experiments). Altogether, these data suggest that the inhibition of HIV-1 replication by CTD1 and CTD2 is attributed to the attenuation of viral infectivity by lowering the *Env* incorporation onto the virion.

4. Discussion

In the present study, we provide the first evidence that the C-terminal fragment of SEC14L1a functions as an inhibitor of HIV-1 replication. The advantage of this system is that, since MT-4 cells are stably transduced with a cDNA library, the anti-HIV-1 function of a candidate gene is not due to a perturbed cell physiology. This system has been successful in identifying CD14, CD63, and Brd4-CTD as regulators of HIV-1 replication [1,3,4], and more candidates are being analyzed. Among the candidates, SEC14L1a CTD appeared to be one of the relatively modest inhibitors of HIV-1 replication. However, of note, the SEC14L1a derivatives have not been identified in other genetic screening systems. These facts point that our T cell-based system is sensitive in detecting the modest anti-HIV-1 activity of a gene, and is a unique tool in the pursuit of HIV-1 regulatory factors to complete the HIV-1-host interactome.

SEC14L1a may affect the Golgi-mediated vesicular trafficking since SEC14L1a lowers the cell surface levels of cholinergic transporters [23]. However, we do not have any data to suggest that SEC14L1a and its derivatives affect the cell surface targeting of membrane proteins including CD4, CXCR4 and *Env*. These data suggest that SEC14L1a's effect on cholinergic receptor expression is specific, and that the CTD's ability to inhibit HIV-1 replication is independent from SEC14L1a's regulatory functions on vesicular trafficking. The action point of CTD1 and CTD2 was shown to be the late phase of the viral life cycle. Given that CTD1 and CTD2 did not inhibit the biogenesis and the cell surface targeting of *Gag* and *Env*, the major mechanism of CTD1 and CTD2 to inhibit HIV-1 replication was to reduce the infectivity of HIV-1 by limiting the *Env* incorporation onto the virion. Consistent with this idea, the viral infectivity of virions produced in CTDs-expressing cells was attenuated. Then, how do CTDs block the *Env* incorporation onto the virion? We detected a weak interaction between *Gag* and CTD1 or CTD2 by immuno-coprecipitation analysis. Thus, we speculate that the interaction between *Env* and *Gag* at the plasma membrane is interfered by *Gag*-CTDs interaction, resulting in the reduction of *Env* incorporation onto the virion.

The CTD1 was an inhibitor of HIV-1 replication. While the CTD1 negatively affected the *Env* incorporation onto the virion, it positively affected the HIV-1 production. These observations may be seemingly controversial. However, the SEC14L1a derivatives' effect on HIV-1 replication is a summation of their effects of on each step of the viral life cycle. Therefore, it is conceivable that CTD1 can serve as a negative regulator of HIV-1 replication as well as a positive and negative factor on distinct steps of the viral life cycle. These seemingly controversial findings may be in part due to the cells in which the biological functions of SEC14L1a derivatives were examined. The effect of SEC14L1a derivatives on HIV-1 replication was investigated in MT-4 cells, whereas those on the HIV-1 production and *Env* incorporation onto the virion were examined in 293T cells. Although the basic biological features are largely shared among different cell types, it is possible that the SEC14L1a derivatives may function slightly differently in MT-4 cells from 293T cells given that the intracellular distribution of SEC14L1a derivatives in MT-4 cells was not identical to that in 293T cells (Fig. 1E and 1F).

Elucidating the molecular mechanism underlying CTDs' activity not only provides a hint to understand how the HIV-1 virion actively uptakes *Env* through the *Gag-Env* interaction, but also leads to the development of a novel anti-retroviral drug that lowers the infectivity of the virus by preventing *Env* incorporation onto the virion. This is the strength of our T cell-based assay since CTDs inhibit HIV-1 replication specifically. In the previous study, we proposed that a small portion of Brd4 may serve as a therapeutic molecular target for HIV-1 infection, since the constitutive expression of Brd4-CTD limited HIV-1 replication specifically [3], akin to the SEC14L1a CTDs. However, it remains to be examined whether the SEC14L1a and Brd4 derivatives inhibit HIV-1 replication in primary HIV-1 target cells.

The genome-wide screening has potential caveats, including a cDNA bias and a cell line bias. A cDNA library is not a perfect representation of mRNA expressed in the cells from which the library is constructed. For example, the longer the mRNA, the less efficiently the full-length cDNA is synthesized. In fact, we isolated Brd4-CTD from the PBL cDNA library as a potent inhibitor of HIV-1 replication [3]. However, although Brd4 (approximately 5000 nt mRNA in length) is expressed in MT-4 cells, we were unable to recover Brd4-CTD from the MT-4 cDNA library [3]. This clearly demonstrates the cDNA bias in the genetic screening. A cDNA library derived from non-T cells does not contain genes specifically expressed in T cells. Thus, we have to explore many more cDNA libraries to completely cover the genetic diversity of human cells. The cDNA libraries isolated from long-term non-progressors of HIV-1-seropositive individuals or from elite controllers might be of particular interest, considering that a dominant innate HIV-1 resistance gene, such as CCR5 delta 32, may partly account for the slow progression of AIDS. Similarly, use of a particular cell line and/or virus strain may bias the results. MT-4 cells are positive for HTLV-1, and are able to support robust HIV-1 replication. MT-4 cells do not express CCR5, and are unable to support R5-tropic HIV-1 strains. What if other T cell lines and R5-tropic viral strains are used? What if we assay the same cDNA library in TZM-bl cells? We plan to address these issues in the future studies.

In conclusion, genome-wide genetic screening is a powerful tool for identifying the regulatory factors of HIV-1 replication and innate HIV-1 resistance factors that limit HIV-1 infection and AIDS progression. The HIV-1-host interactome should also reveal poten-

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469 tial therapeutic molecular targets that may be used to develop
470 novel anti-AIDS drugs to tackle the emerging drug resistant viruses.
471 However, the fact that different experimental systems often yield
472 non-overlapping candidates suggests that we have to explore more
473 experimental systems to fully understand the HIV-1-host inter-
474 actome. Our T cell-based system provides an alternative tool for
475 identifying novel HIV-1 regulatory factors, and should help us
476 understand the HIV-1-host interaction in more detail.

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