Table 2 Primers for RT-PCR analysis

Gene		Sequence (5' to 3')	Parameter (° C)	Expected size (bp)
human CAR	Forward	AGCCTTCAGGTGCGAGATGTTACG	65	366
	Reverse	TAGGACAGCAAAAGATGATAAGAC		
human αv integrin	Forward	GCCTATCTCCACGCACACTG	65	287
	Reverse	TTGGACCATTGTTTCTCAGC		
human β3 integrin	Forward	GAGGATGACTGTGTCGTCAG	65	232
	Reverse	CTGGCGCGTTCTTCCTCAAA		
human β5 integrin	Forward	GCCTATCTCCACGCACACTG	58	454
	Reverse	AGACTCCGACCCTTCCTGAC		
human β̃actin	Forward	CAAGAGATGGCCACGGCTGCT	60	275
	Reverse	TCCTTCTGCATCCTGTCGGCA		
rat CAR	Forward	ATGGATCCTACACCCGAACAGAGGATCG	53	280
	Reverse	GCGAATTCGCGTCGCCAGACTTGACAT		
rat αv integrin	Forward	GCCTATCTCCACGCACACTG	57	273
	Reverse	CGGGTGCTATCTGTCTTATG		
rat β3 integrin	Forward	TAATGATGGGCGCTGCCACA	58	279
	Reverse	CGTAAGCATCAACGATGAGC		
rat β5 integrin	Forward	TGTGTCTCTGCGGTGTTTGC	53	312
	Reverse	CCACGAGAACACCACAACAA		
rat GAPDH	Forward	ACCACAGTCCATGCCATCAC	60	452
	Reverse	TCCACCACCCTGTTGCTGTA		

Table 3 Primer sequences and reaction parameter used for PCR amplification.

Gene		Primer sequence (5' to 3')	Denaturation	Annealing	Extension	Cycle No.	Product size (bp)
Mouse CCR7	**F**	ACAGCGGCCTCCAGAAGAACAGCGG	for 30 s at 95℃	for 30 s at 60℃	for 30 s at 72℃	20	345
	R	TGACGTCATAGGCAATGTTGAGCTG					
Mouse β-actin	**F**	TGTGATGGTGGGAATGGGTCAG	for 30 s at 95℃	for 30 s at 60℃	for 30 s at 72℃	20	514
	R	TTTGATGTCACGCACGATTTCC					

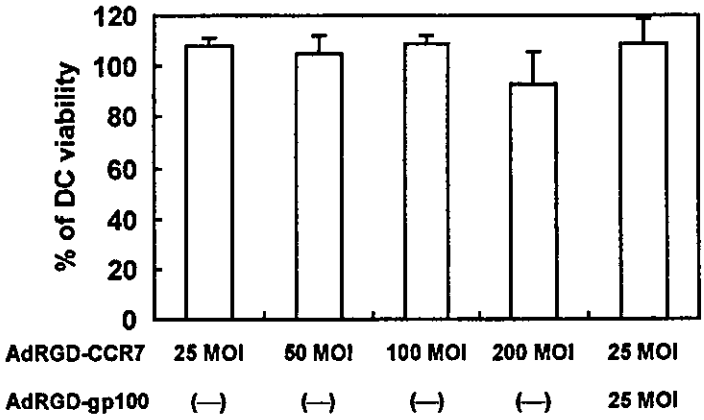


Fig. 16 Cytopathic effect of AdRGD-CCR7 and AdRGD-gp100 on gene transduction into DCs.
DCs were transduced with AdRGD-CCR7 or co-transduced with AdRGD-CCR7 and AdRGD-gp100 at the indicated MOI. These cells were cultured on 96-well plates at 1×10^5 cells/100 μl/well in GM-CSF-free culture medium. At 24 h after transduction, cell viability was assessed by WST-8 cell counting kit. Data represents the mean ± SD of triplicate cultures.

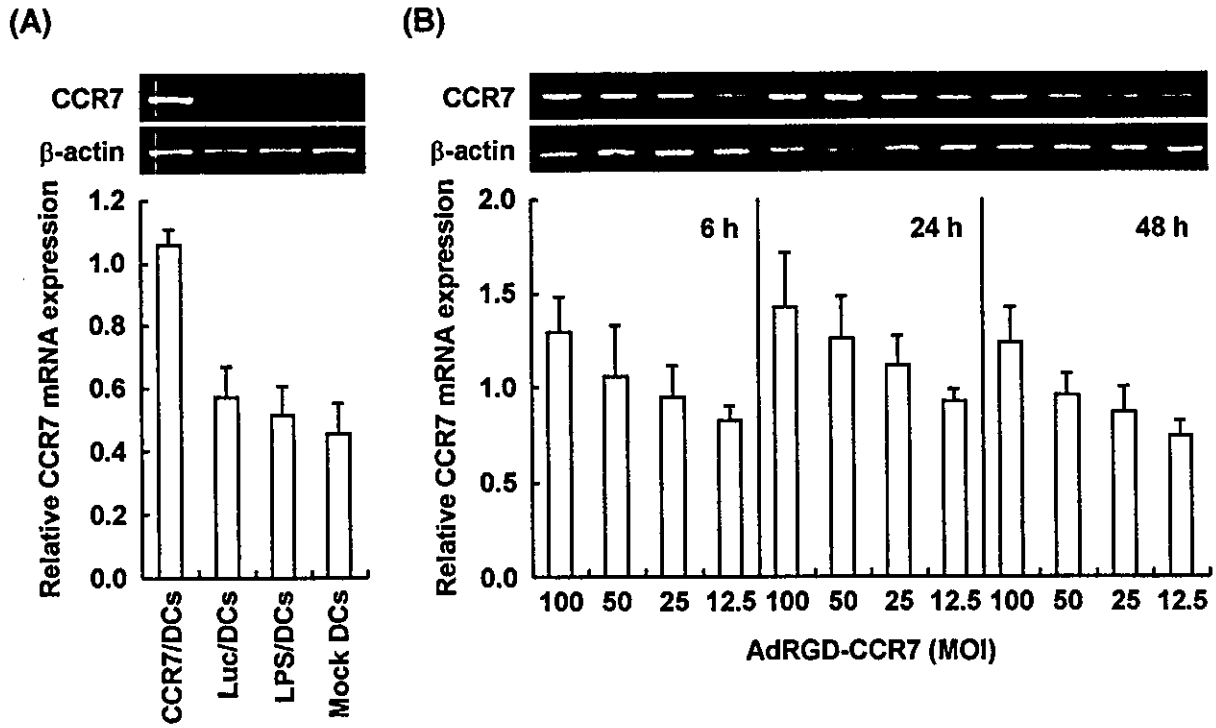


Fig17 RT-PCR analysis for mouse CCR7 expression levels.
DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (**A**) or the indicated MOI (**B**). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h (**A**) or the indicated period (**B**) in GM-CSF-free medium. Total RNA was isolated from these DCs, and then mouse CCR7 mRNA expression was assessed by RT-PCR analysis. Relative CCR7 mRNA expression was calculated as ratio of the densitometric units of PCR-products derived from CCR7 transcripts to the densitometric units of PCR-products derived from β-actin transcripts. Data are presented as mean ± SD of results from three independent experiments.

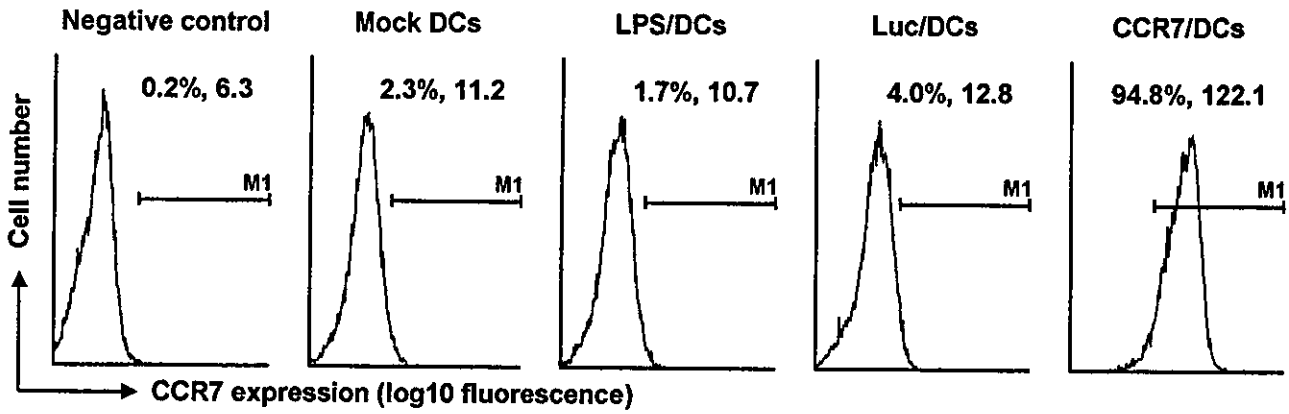


Fig.18 Flow cytometric analysis for mouse CCR7 expression levels.
DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI. These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h in GM-CSF-free medium. Flow cytometric analysis was performed by using anti-mouse CCR7 antibody. Negative control represents mock DCs stained by second antibody alone. The data are representative of two independent experiments, and the % value and the numerical value indicated in the upper part of each panel express % of M1-gated cells and mean fluorescence intensity (MFI), respectively.

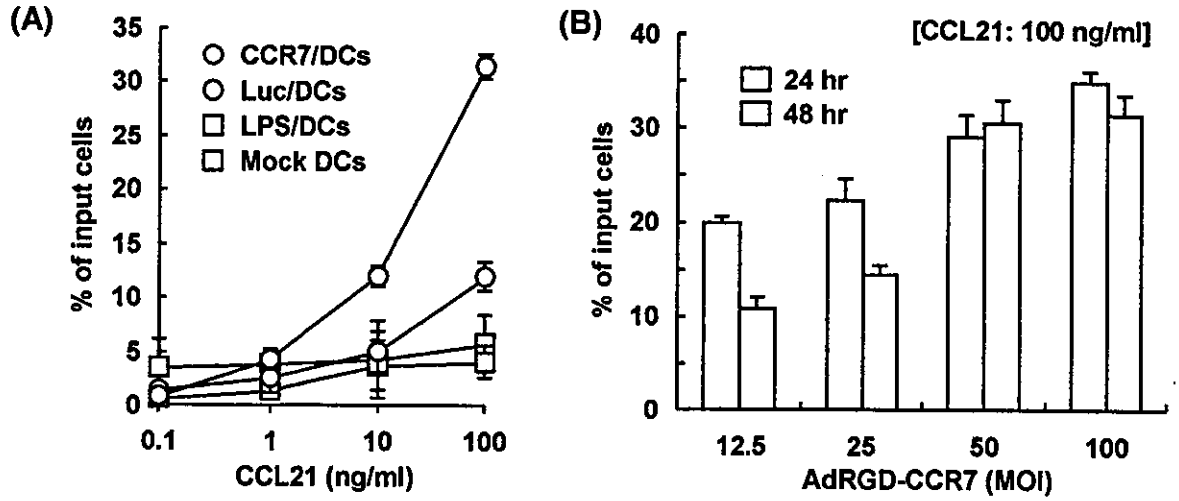


Fig. 19 Chemotactic activity of CCR7/DCs in response to CCL21.
DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI (**A**) or the indicated MOI (**B**). These transduced cells, LPS-stimulated DCs, and mock DCs were cultured for 24 h (**A** and **B**) or 48 h (**B**) in GM-CSF-free medium. In vitro chemotaxis assay was performed by a Chemotaxicell-24 installed on 24-well culture plate. CCL21 solution was added in the lower compartment at the indicated concentration, and DCs were placed in the upper chamber at 10^6 cells. After 4 h-incubation, the number of cells that migrated to the lower compartment was counted on a NucleoCounter. Data are presented as mean ± SD of four independent cultures.

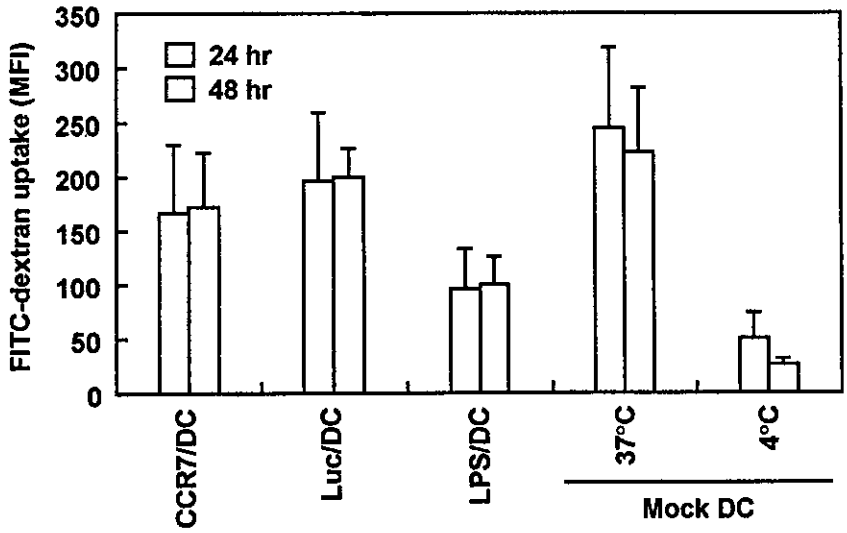


Fig. 20 Uptake of FITC-dextran by CCR7/DCs.
DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 or 48 h. These transduced cells, LPS-stimulated DCs, and mock DCs were incubated with PBS containing 1 mg/ml FITC-dextran at 4 or 37°C. One hour later, cells were washed five times with ice-cold PBS and uptake of FITC-dextran was assessed by flow cytometry. MFI of flow cytometric analysis is presented in bar chart. Data are presented as mean ± SD of four independent cultures.

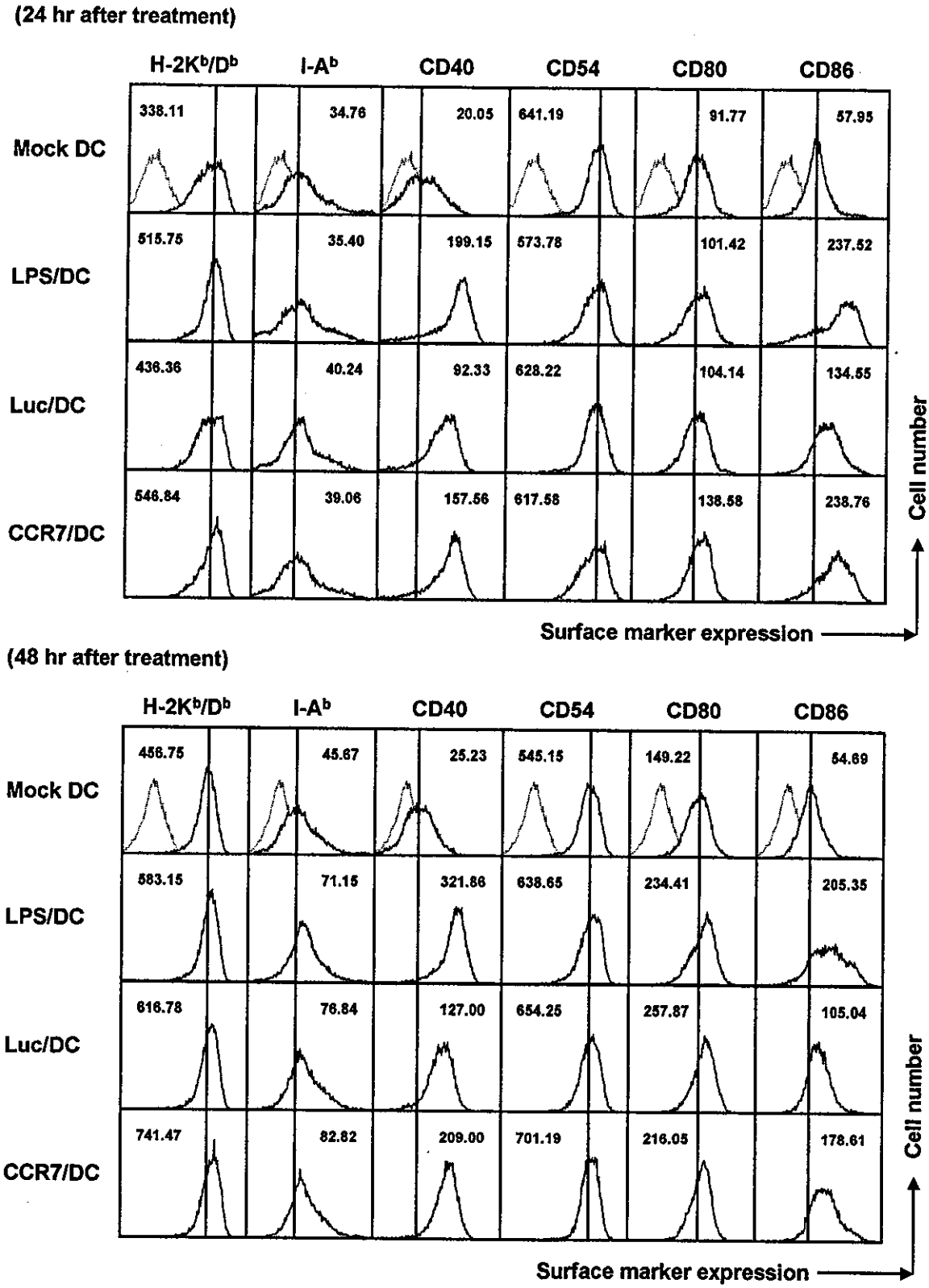


Fig.21 Flow cytometric analysis of surface markers in CCR7/DCs.
DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 or 48 h. These transduced cells, LPS-stimulated DCs, and mock DCs were stained by indirect immunofluorescence using monoclonal antibodies of the indicated specificities (solid histogram). Dotted histograms represent cells stained by phycoerythrin-conjugated streptavidin alone. Values indicated in the upper part of each panel represent MFI of flow cytometric analysis. The data are representative of three independent experiments.

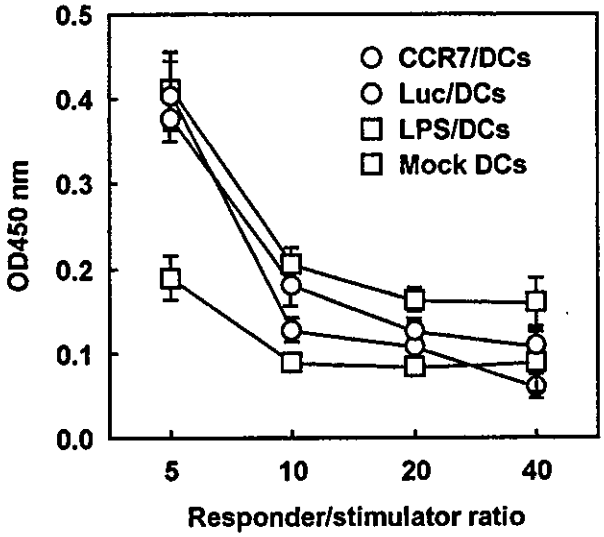


Fig. 22 Allogenic T cell proliferation-stimulating ability of CCR7/DCs.
C57BL/6 DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. Naive BALB/c T lymphocytes were co-cultured with CCR7/DCs, Luc/DCs, LPS/DCs, or mock DCs at the indicated responder/stimulator ratio for 3 days. Cell cultures were pulsed with BrdU during the last 18 h, and then T cell proliferation was assessed by BrdU-ELISA. Results are expressed as mean ± SE of three independent cultures using T cells prepared from three individual mice.

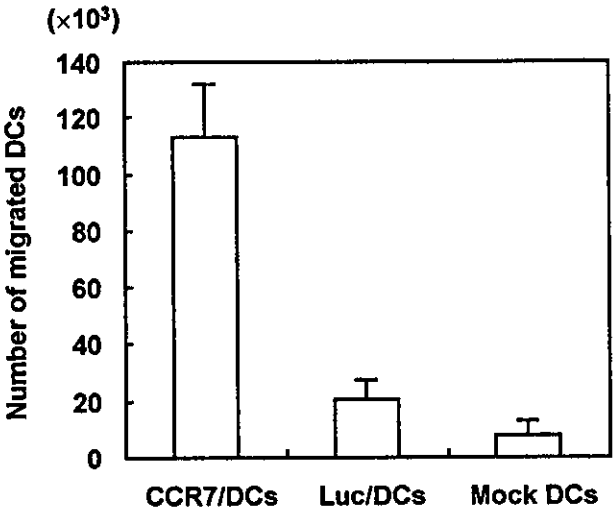


Fig. 23 Migration of CCR7/DCs from administration site to draining lymph node.
DCs derived from EGFP-transgenic mouse were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were cultured for 24 h. These transduced cells and mock DCs were intradermally injected into the left flank of C57BL/6 mice at 2×10^6 cells/50 μl. Two days later, the draining inguinal lymph nodes were collected from these mice, and a single cell suspension was prepared and stained by indirect immunofluorescence using anti-CD11c monoclonal antibody. The abundance of $EGFP^+CD11c^+$ DCs was assessed by flow cytometric analysis acquiring 500,000 events. The number of DCs that had migrated into draining lymph nodes was calculated by multiplying the $EGFP^+CD11c^+$ DC-frequency by the total number of isolated lymph node cells. Data are presented as mean ± SE of results from four mice.

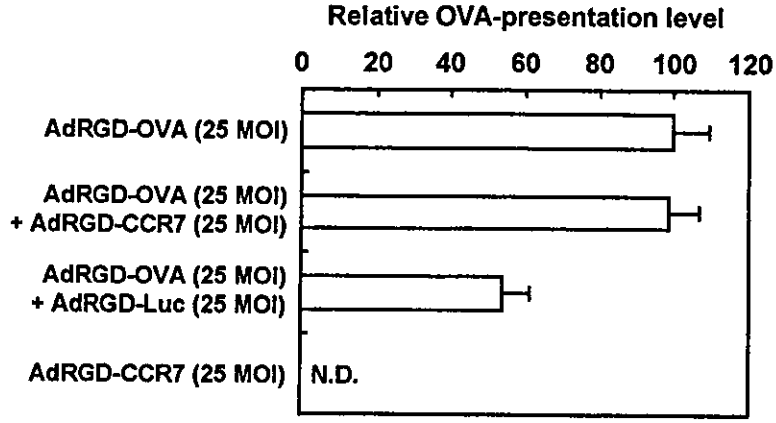


Fig. 24 Antigen presentation on MHC class I molecules by CCR7/DCs.
DCs were transduced with the indicated combination of AdRGD-OVA, AdRGD-CCR7 and AdRGD-Luc. These cells were co-cultured with CD8-OVA 1.3 cells for 20 h. IL-2 levels released from stimulated CD8-OVA 1.3 cells into culture supernatants were determined by ELISA, and relative OVA-presentation level via MHC class I molecules in each transduced DC was expressed as a percentage of the group using DCs transduced with AdRGD-OVA alone. Data represent the mean ± SD of three independent cultures. N.D.: IL-2 secreted from CD8-OVA 1.3 cells was not detectable.

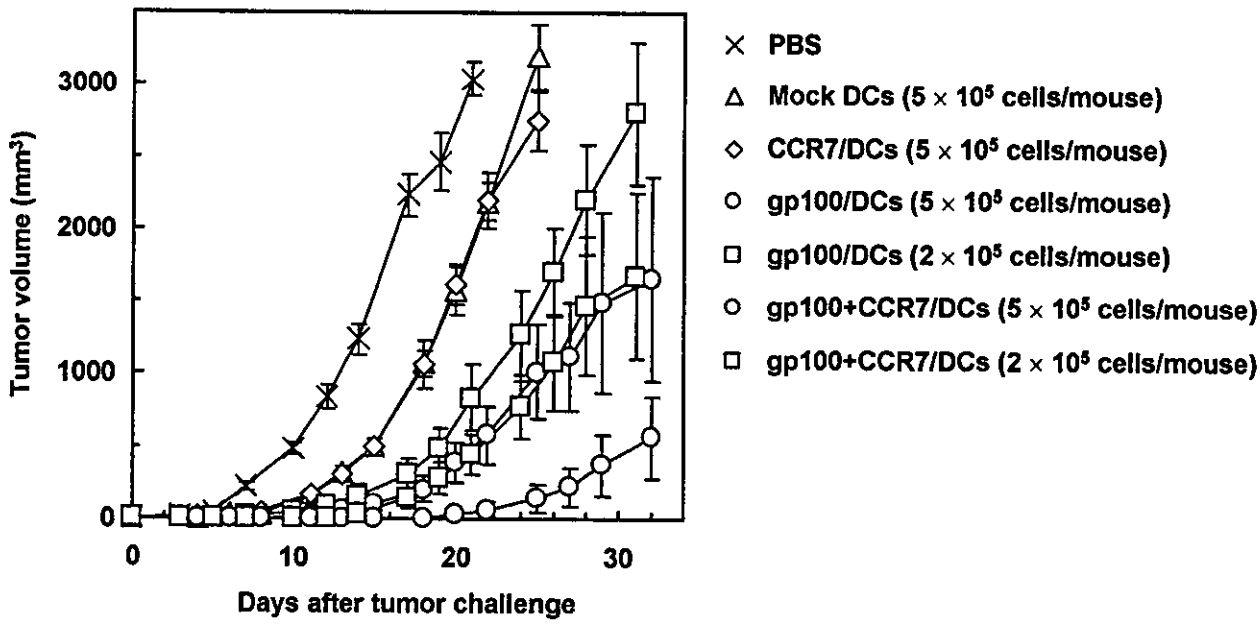


Fig. 25 Vaccine efficacy of DCs co-transduced with CCR7 and gp100 gene against B16BL6 melanoma challenge.
CCR7/DCs, gp100/DCs, and gp100+CCR7/DCs were prepared using corresponding vectors at 25 MOI, and then cultured for 24 h. C57BL/6 mice were immunized by intradermal injection of transduced DCs into the left flank at the indicated cell dosage, and then 4 × 10^5 B16BL6 melanoma cells were inoculated into the right flank of the mice at 1 week post-vaccination. The tumor sizes were assessed using microcalipers three times per week. Each point represents the mean ± SE of 5-10 mice.

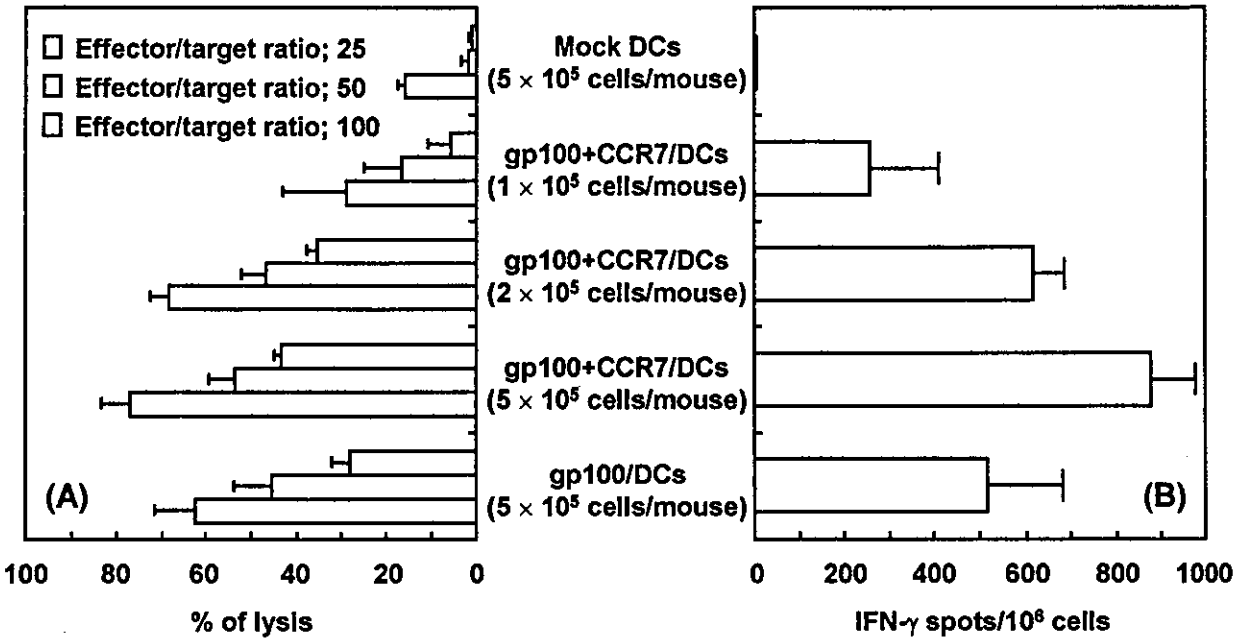


Fig. 26 CTL activity and the frequency of IFN-γ-producing cells in splenocytes from mice immunized with DCs cotransduced with CCR7 and gp100 gene by AdRGD.

gp100/DCs and gp100+CCR7/DCs were prepared using corresponding vectors at 25 MOI, and then cultured for 24 h. These transduced cells and mock DCs were vaccinated once intradermally into C57BL/6 mice at the indicated cell dosage. At 1 week after immunization, non-adherent splenocytes were prepared from these mice, and then were re-stimulated in vitro for 5 days with IFN-γ-stimulated and MMC-inactivated B16BL6 cells. (**A**); a cytolytic assay using the re-stimulated splenocytes (effector cells) was performed against IFN-γ-stimulated B16BL6 cells (target cells). The data represent the mean ± SE of four independent cultures from four individual mice. (**B**); IFN-γ-producing cells in re-stimulated splenocytes were evaluated by mouse IFN-γ ELISPOT assay. The data represent the mean ± SD of results from four mice.

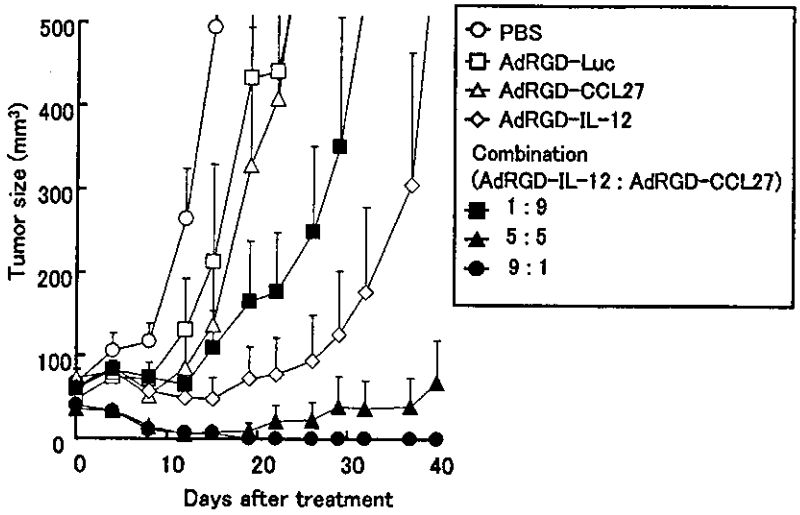


Fig. 27 Combination of AdRGD-IL-12 and AdRGD-CCL27 induced synergistic anti-tumor activity. B6C3F1 mice 1×10^6 OV-HM cells were inoculated intradermally into B6C3F1 mice. After the tumor diameter reached 7-8mm, indicated adenovirus vectors in total of 2×10^7 PFU or PBS were injected intratumorally. Tumor size was measured twice a week.

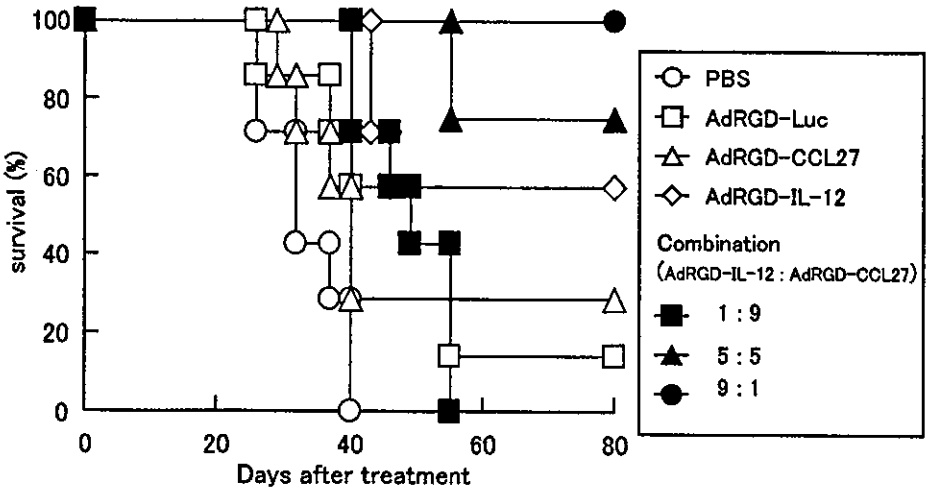


Fig. 28 The survival percent of mice with the treatment of indicated adenovirus vectors

Table 4 Induction of specific long-term immunity against OV-HM cells in tumor rejected mice

Groups	Challenging cells	Tumor rejected mice/ challenged mice		
		After 3 months		After 6 months
		1st Exp.	2nd Exp.	3rd Exp.
Intact	OV-HM	0/6	0/6	0/6
Combination AdRGD-IL-12 (9) AdRGD-CCL27 (1)	OV-HM	4/5	5/5	5/5
	B16BL6	0/3	0/5	0/5
Combination AdRGD-IL-12 (5) AdRGD-CCL27 (5)	OV-HM	4/4		
	B16BL6	0/2		

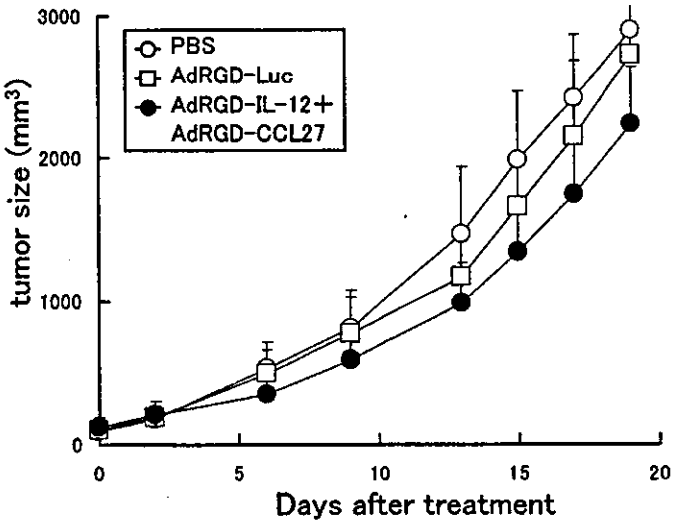


Fig. 29 Anti-tumor effect induced by the combination of AdRGD-IL-12 and AdRGD-CCL27 is T cell-dependent

Balb/c nude mice were inoculated intradermally with OV-HM cells (1×10^6 cells/mouse). After one week, 50 μl of PBS, 2×10^7 PFU of AdRGD-Luc, or AdRGD-IL-12 plus AdRGD-CCL27, in total of 2×10^7 PFU at the ratio of 9:1, were intratumorally injected. Tumor size was measured twice a week.

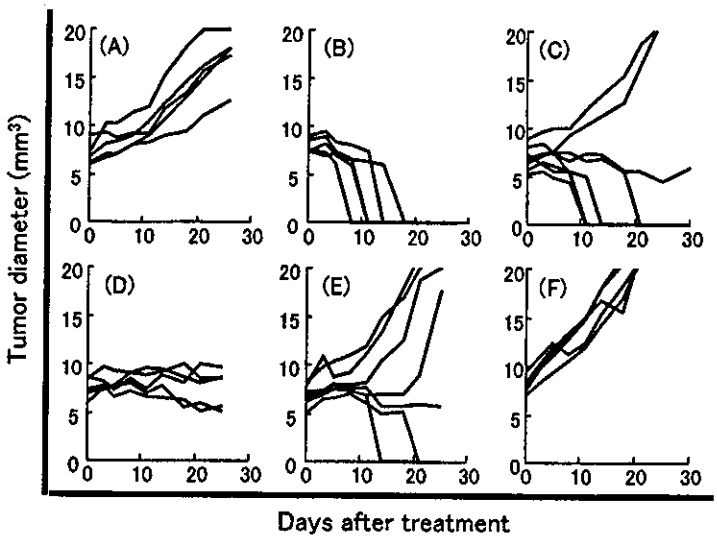


Fig. 30 Both CD4 or CD8 T cells contributed to the anti-tumor activity induced by combination. CD4 positive T, CD8 positive T or NK cell-depleted naive mice were inoculated intradermally with OV-HM cells (1×10^6 cells/mouse). (A) Tumor-bearing mice treated with PBS, (B) intact mice, (C) NK cell-depleted mice, (D) CD4 positive T cell-depleted mice, (E) CD8 positive T cell-depleted mice, (F) CD4 positive T cell and CD8 positive T cell-depleted mice treated with AdRGD-IL-12 and AdRGD-CCL27 at a ratio of 9:1 (in total 2×10^7 PFU/mouse).

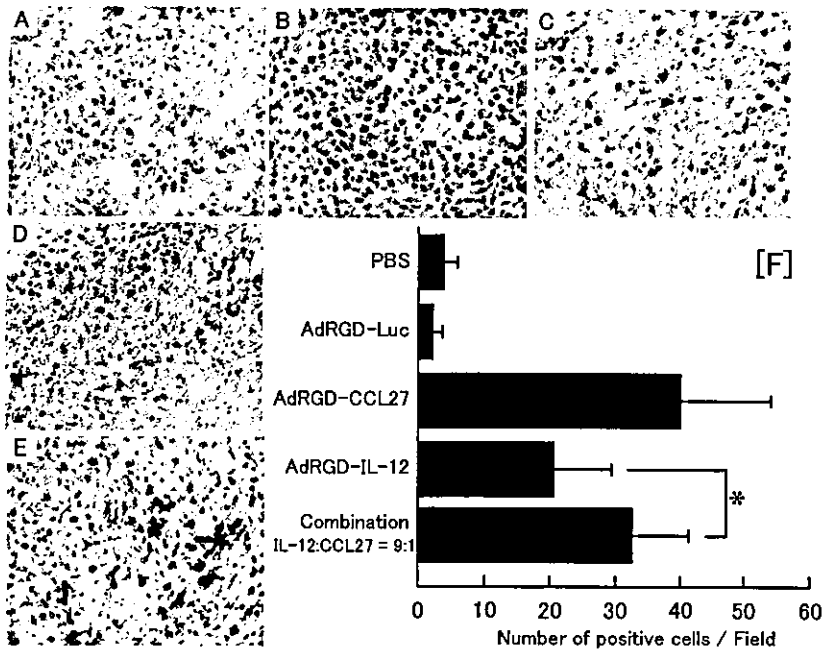


Fig.31 CD3 positive lymphocyte infiltrate into OV-HM tumor.
Immunohistochemical analysis was utilized to determine lymphocytes infiltrated into tumors. When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administration of (A) PBS, (B) AdRGD-Luc, (C) AdRGD-CCL27, (D) AdRGD-IL-12 or (E) combination (AdRGD-IL-12:AdRGD-CCL27=9:1). The tumor nodules were harvested, embedded in the O.C.T. compound, and stored at -80°C. Frozen thin sections of the nodules were fixed and stained for CD3 positive T cells using the method described above. (F) The number of immunostained cells were counted under light microscope with ×400 magnihication. For countinf positive cell number infiltrated into tumor tissue, six fields were randomly selected. Statistical analysis was carried out by Student's t-test. *; < 0.05

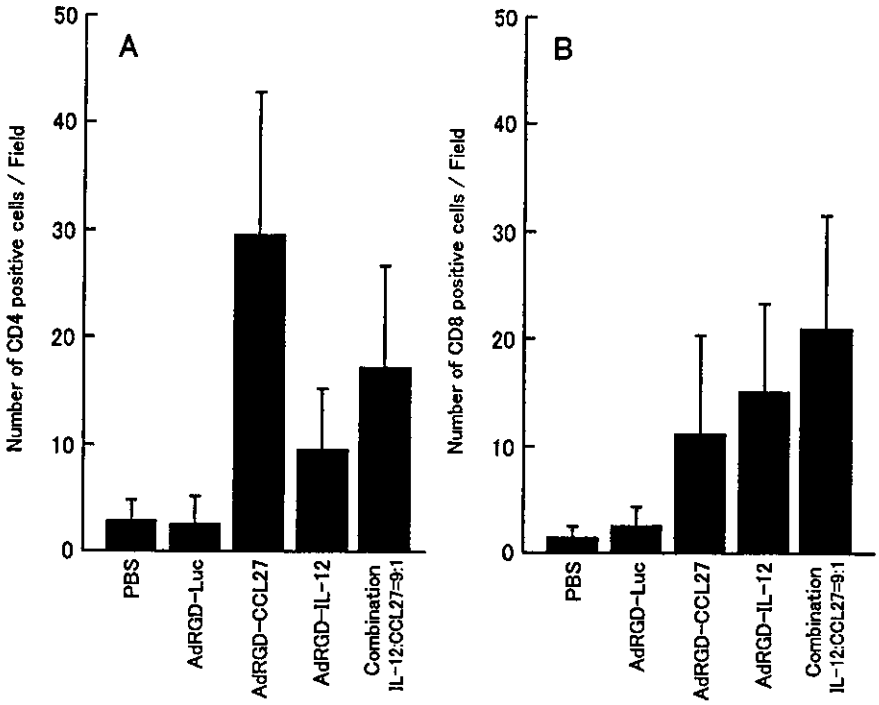


Fig. 32 CD4 or CD8 positive lymphocyte infiltrate into OV-HM tumor.
When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administration of AdRGD-CCL27, AdRGD-IL-12 or combination. The tumor nodules were harvested, embedded in the O.C.T. compound., stored at -80°C. Frozen thin sections of the nodules were fixed and stained for CD4 (A) or CD8 (B)-positive cells using the method described above. The number of immunostained cells were counted under light microscope with ×400 magnification. For counting the positive cell number infiltrated into tumor tissue, six fields were randomly selected.

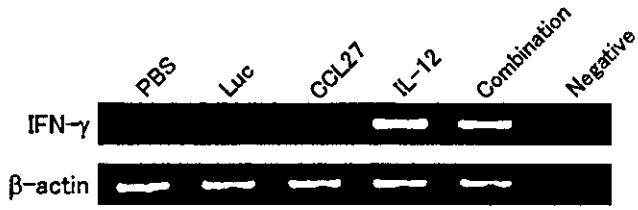


Fig. 33 RT-PCR analysis of murine IFN-γ in indicated adenovirus vectors injected OV-HM tumor nodules.
Total RNA was extracted from OV-HM tumor nodules, and then RT-PCR was performed to amplify the mRNA levels of mouse IFN-γ (379bp) and β-actin (514bp). PCR products were visualized by ethidium bromide staining after electrophoresis on an agarose gel. Negative was performed PCR using water as template.

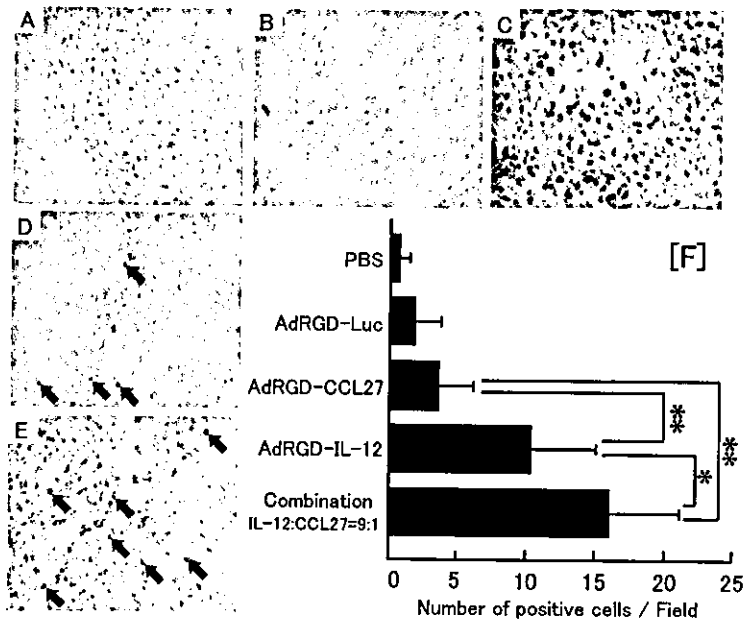


Fig. 34 Perforin positive cells infiltrate into OV-HM tumor.
When the length of tumor reached about 7-8 mm, intratumoral administrations of indicated adenovirus vectors were carried out. Tumor-bearing mice were sacrificed in six days after the intratumoral administrations of (A) PBS, (B) AdRGD-Luc, (C) AdRGD-CCL27, (D) AdRGD-IL-12 and (E) combination (AdRGD-IL-12:AdRGD-CCL27=9:1). The tumor nodules were harvested, embedded in the O.C.T. compound, and stored at -80℃. Frozen thin sections of the nodules were fixed and stained for perforin-positive cells using the method described above. The number of immunostained cells were counted under light microscope with ×400 magnification. For counting the positive cell number infiltrated into tumor tissue, 6 fields were randomly selected. (F) Quantitation of perforin-positive cells in treated tumors. Statistical analysis was carried out by Student's t-test. *; < 0.05, **; < 0.01

Table 5 Adenovirus vectors used in the present study

Ad vectors	The RGD motif of the penton base	Type of the fiber shaft	The fiber knob			
			AB loop	FG loop	HI loop	C-terminus
Ad-L2	intact	5	intact	intact	——	——
Ad/ΔF(FG)ΔP-S35-L2	mutation	35	intact	mutation	——	——
Ad/ΔF(AB)ΔP-S35-L2	mutation	35	mutation	intact	——	——
Ad/ΔF(FG)ΔP-S35-RGD(HI)-L2	mutation	35	intact	mutation	RGD motif	——
Ad/ΔF(AB)ΔP-S35-RGD(HI)-L2	mutation	35	mutation	intact	RGD motif	——

Table 6 Adenovirus vectors used in the present study

Ad vector	Penton base	Ad type of tail	Ad type of shaft	Amino acid sequence of knob domain			
				AB loop	FG loop	HI loop	C-terminus
Conventional Ad							
Ad-L2	MND-HAIRGDTFAT-RAE	5	5 (22 β-repeats)	- NCRLNAEKDA-	- TEGTAYTNAV-	-DTTPSA-	-QE stop
Mutant Ad							
Ad/ΔF(FG) ΔP-S35-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCRLNAEKDA-	- TEG - - - - NAV- Δ a.a. 489-492	-DTTSNPSA-	-QEID stop
Ad/ΔF(AB) ΔP-S35-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCSLNGGGDA- 4 a.a. mutation	- TEGTAYTNAV-	-DTTSNPSA-	-QEID stop
Ad/ΔF(FG)ΔP -S35-RGD(HI)-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCRLNAEKDA-	- TEG - - - - NAV- Δ a.a. 489-492	-DTTSACDCR GDCFCANPSA	-QEID stop
Ad/ΔF(AB)ΔP -S35-RGD(HI)-L2	MND-TS----------RAE ΔRGD motif	5	35 (6 β-repeats)	- NCSLNGGGDA- 4 a.a. mutation	- TEGTAYTNAV-	-DTTSACDCR GDCFCANPSA	-QEID stop

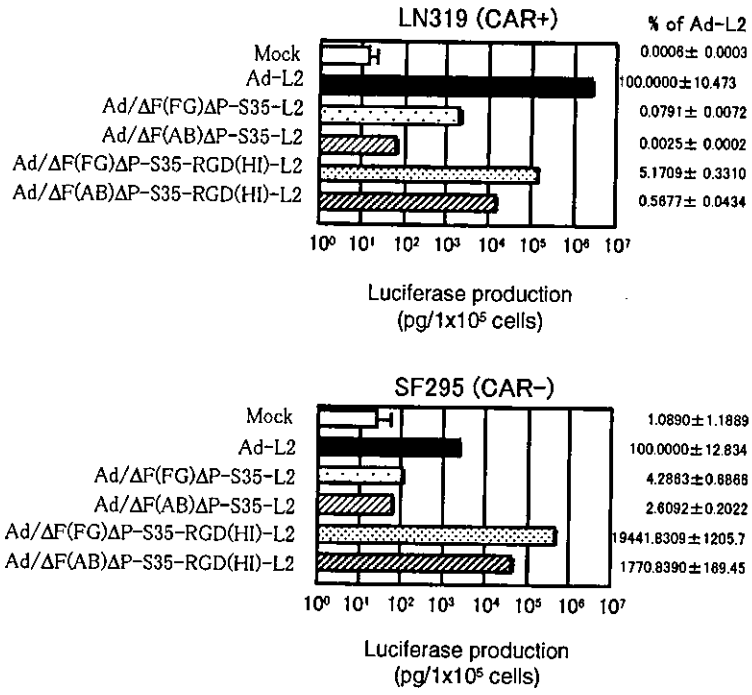


Fig. 35 Comparison of luciferase production in cultured cells transduced with modified Ad vectors.
Cells were transduced with 3000 VP/cell of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2, Ad/ΔF(FG)ΔP-S35-RGD(HI)-L2, or Ad/ΔF(AB)ΔP-S35-RGD(HI)-L2 for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean ± S.D. (n = 4).

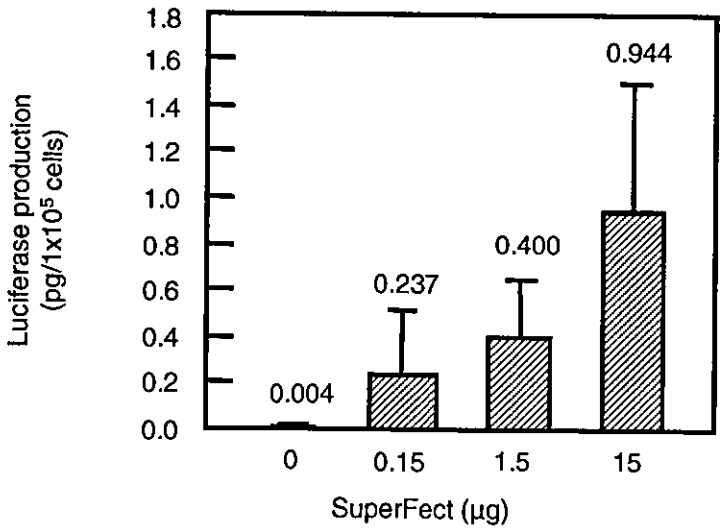


Fig. 36 Luciferase production in SF295 cells trsnsduced by Ad/ΔF(AB)ΔP-S35-L2 in the presence of Superfect Transduction Reagent.
Cells were transduced with 30000 VP/cell of Ad/ΔF(AB)ΔP-S35-L2 in the presence of polyamidoamine dendrimer reagent SuperFect for 1.5 h. After culture for 48 h, luciferase production was measured by a luminescent assay. The data are expressed as mean ± S.D. (n = 6).

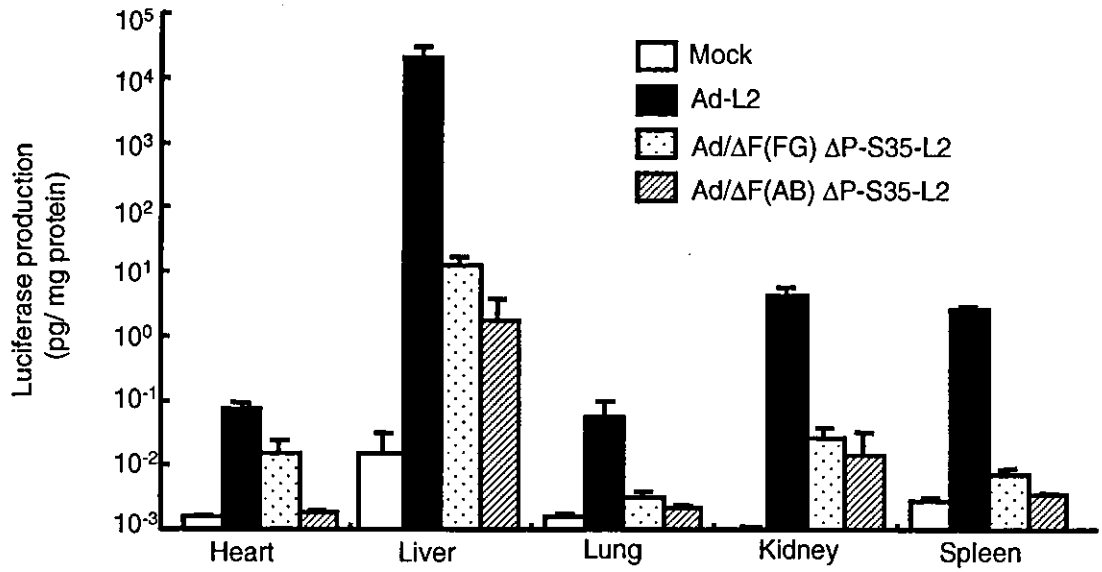


Fig. 37 Luciferase production in mice after the intravenous administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2.

Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2 (3.0x1010 VP) were intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean ± S.E. of six mice.

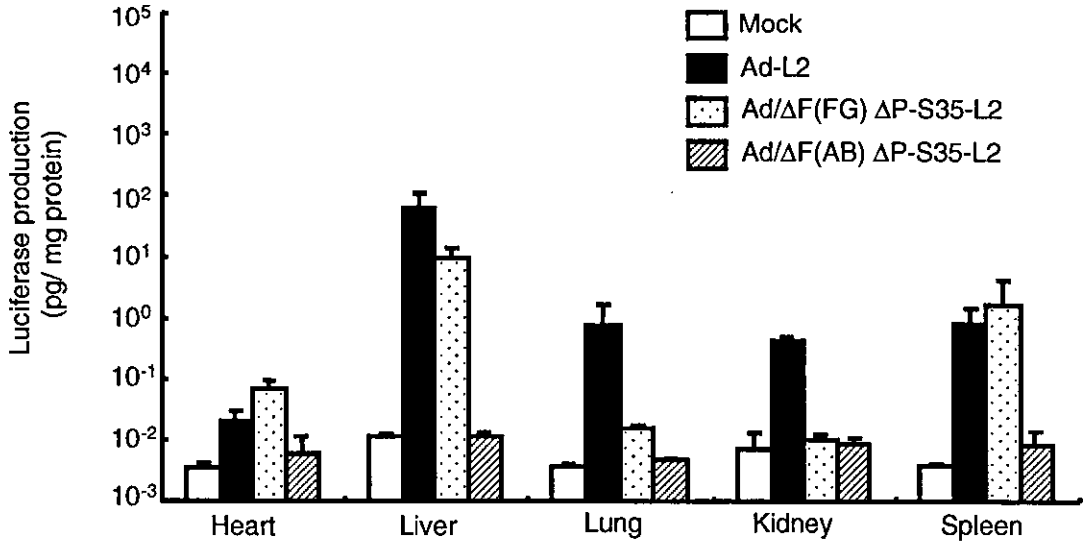


Fig. 38 Luciferase production in mice after the intraperitoneal administration of Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2.

Ad-L2, Ad/ΔF(FG)ΔP-S35-L2, Ad/ΔF(AB)ΔP-S35-L2 (3.0x1010 VP) were intraperitoneally injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean ± S.E. of six mice.

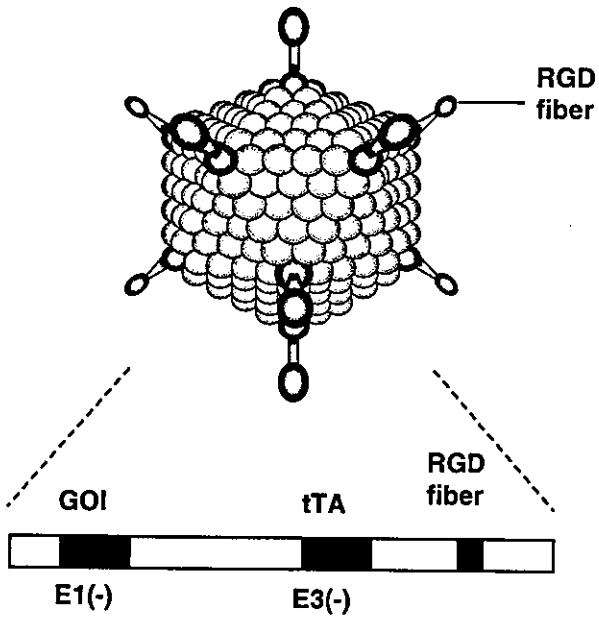


Fig.39 Capsid-modified single adenovirus vectors containing tet-off system

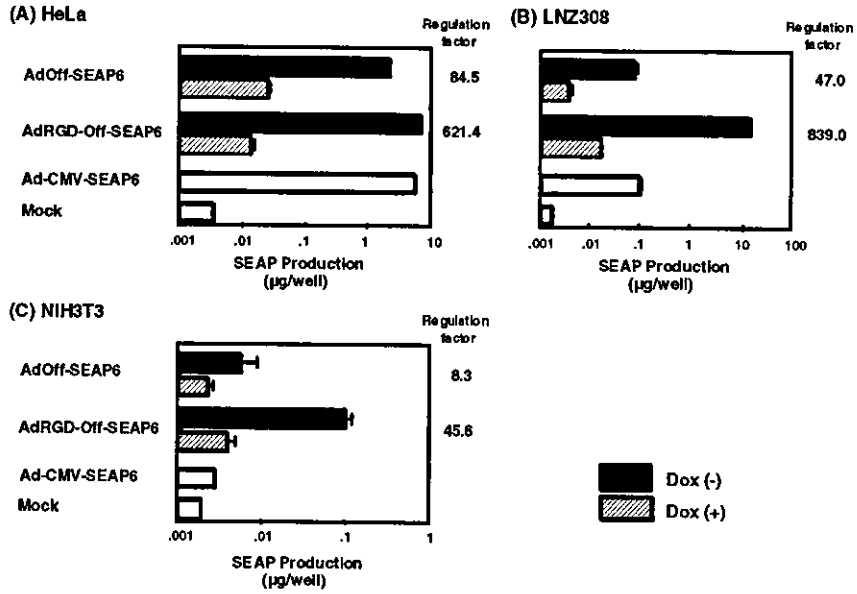
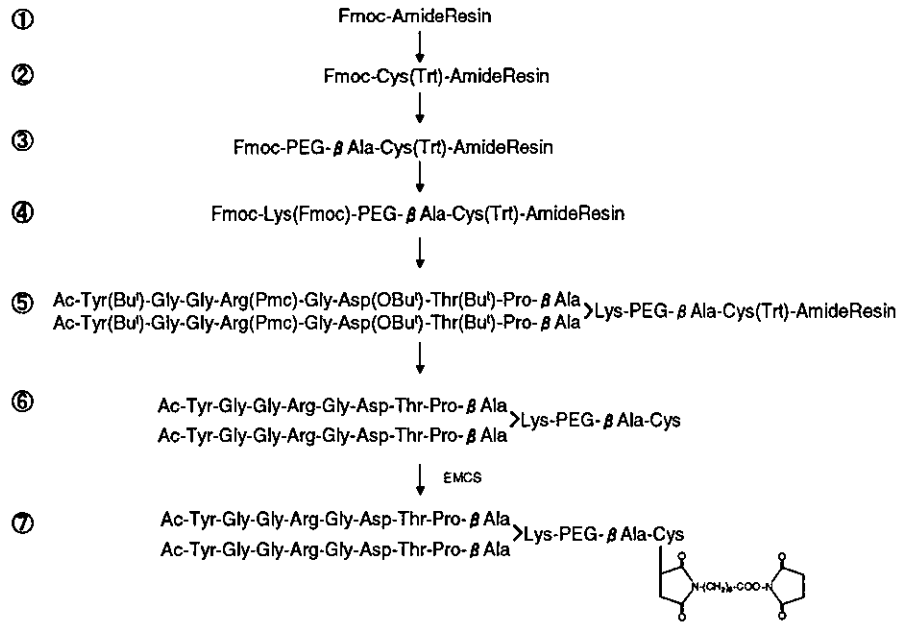


Fig.40 Regulated SEAP production in CAR-positive and -negative cells transduced by capsid-modified single adenovirus vectors containing tet-off system.
HeLa (A), LNZ308 (B), and NIH3T3 (C) cells were transduced with each Ad vector (300 VP/cell). The cells were cultured without (closed columns) and with (slashed columns) 10 ng/ml of doxycycline for 48 h. SEAP production in the medium was determined. Each data represents the mean S.D. of four experiments.



Scheme 1 Structure scheme for RGD-PEG

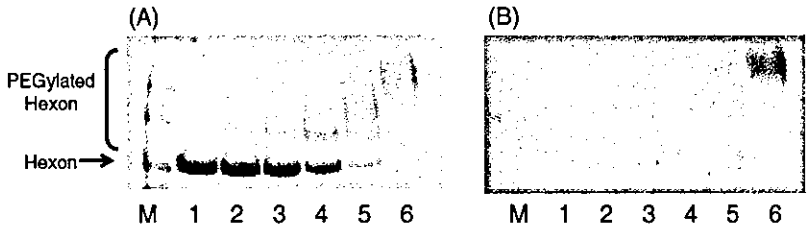


Fig. 41 SDS-PADE analysis of PEGylated adenovirus vectors.
Comparison of two SDS-gels (A, B) that were run under identical conditions and loaded as follows : lane M, protein markers ; lane 1, Ad : PEG=1:0 (unmodified-Ad) ; lane 2, 1:25 ; lane 3, 1:100 ; lane 4, 1:400 ; lane 5, 1:1600 ; lane 6, 1:6400. (A) The gel was stained with Coomassie blue. (B) The gel was stained for PEG using barium iodide.