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22. 角田慎一:サイトカインとタンパク質工学による粘膜アジュバント開発,次世代アジュバント研究会,大阪,2011 年 1 月.
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24. Arita S., Kayamuro H., Abe Y., Yoshioka Y., Kamada H., Furuya T., Itoh N., Yoshikawa T., Nagano K., Tsutsumi Y., Tsunoda S., THE POTENTIAL OF IL-1 FAMILY CYTOKINES AS MUCOSAL ADJUVANTS FOR NASAL INFLUENZA VACCINE, Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR), Chicago, October, 2010.
25. 吉岡靖雄, 森重智弘, 角田慎一, 向 洋平, 岡田直貴, 堤 康央, 中川晋作:非晶質ナノシリカの粒子特性と自然免疫応答の連関評価とそのメカニズム解析., 第 37 回日本トキシコロジー学会学術年会, 那覇, 2010 年 6 月.

H. 知的財産権の出願・登録状況

H-1 特許

角田慎一、堤 康央、吉岡靖雄、粘膜ワクチン用アジュバント、特願 2009-055953.

H-2 実用新案

該当なし

I. 研究協力者

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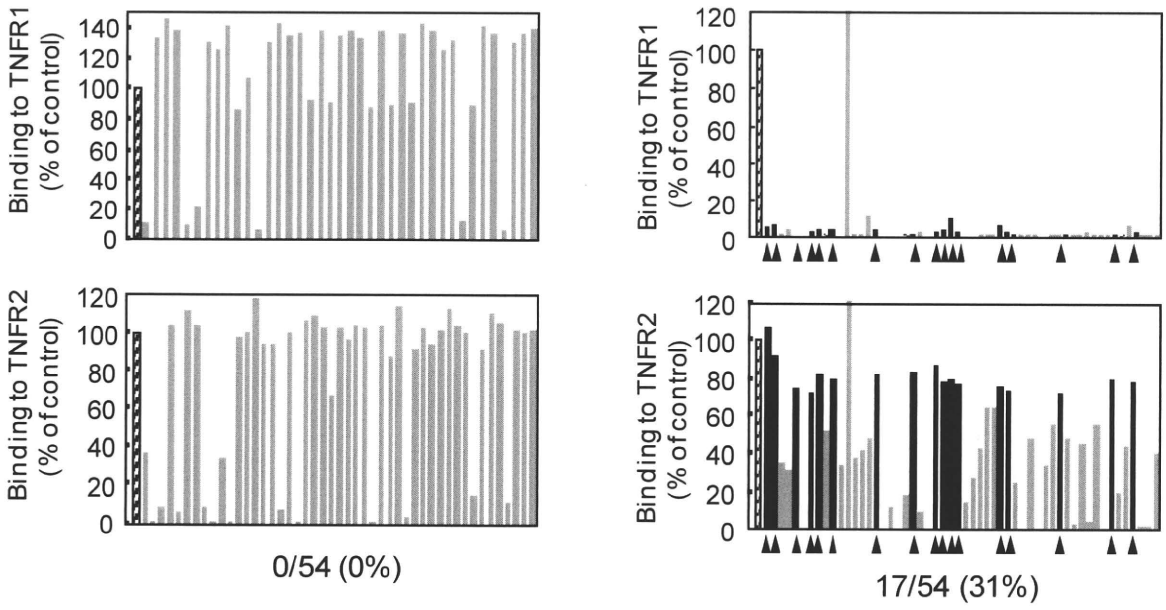

図1　ELISA による TNFR2 指向性 TNF 変異体のスクリーニング.

表1 TNFR2指向性変異体候補のアミノ酸配列と各TNFレセプターに対する結合特性の評価.

(A) TNFR1に対する結合特性

	Residue Position						k_{on} [1]	k_{off} [2]	K_D [3]	Relative [4]
	29	31	32	145	146	147	($\times 10^6 M^{-1}s^{-1}$)	($\times 10^{-4} s^{-1}$)	($\times 10^{-10}$ M)	affinity (%)
wtTNF	L	R	R	A	E	S	0.45	1.3	2.9	100.0
R2-6	L	R	R	H	E	D	0.79	54.5	68.8	4.2
R2-7	L	R	R	T	S	D	1.19	50.1	42.3	6.9
R2-8	L	R	R	N	D	D	1.22	50.3	41.1	7.1
R2-9	V	R	R	D	D	D	0.44	116.0	262.0	1.1
R2-10	L	R	R	Q	D	D	0.67	43.9	63.7	4.6
R2-11	L	R	R	T	D	D	0.81	87.5	108.0	2.7
R2-12	L	R	R	D	G	D	1.36	98.8	72.6	4.0
R2-13	L	R	R	D	E	D	0.97	104.0	107.0	2.7

(B) TNFR2に対する結合特性

	Residue Position						k_{on} [1]	k_{off} [2]	K_D [3]	Relative [4]
	29	31	32	145	146	147	($\times 10^6 M^{-1}s^{-1}$)	($\times 10^{-4} s^{-1}$)	($\times 10^{-10}$ M)	affinity (%)
wtTNF	L	R	R	A	E	S	1.98	12.1	6.11	100.0
R2-6	L	R	R	H	E	D	3.24	7.9	2.43	251.4
R2-7	L	R	R	T	S	D	3.83	12.6	3.30	185.2
R2-8	L	R	R	N	D	D	3.13	6.6	2.10	291.0
R2-9	V	R	R	D	D	D	2.06	7.4	3.60	169.7
R2-10	L	R	R	Q	D	D	2.22	5.3	2.41	253.5
R2-11	L	R	R	T	D	D	2.33	5.4	2.31	264.5
R2-12	L	R	R	D	G	D	4.06	10.6	2.60	235.0
R2-13	L	R	R	D	E	D	2.85	8.2	2.88	212.2

[1] k_{on} ; 結合速度定数 [2] k_{off} ; 解離速度定数 [3] K_D ; 解離平衡速度定数 ($K_D = k_{off} / k_{on}$)
[4] Relative affinity = K_D (wtTNF) / K_D (TNF variants) × 100

表2 TNFR1およびTNFR2を介した生物活性の評価.

	HEp-2 Assay		TNFR2/Fas Assay		
	EC50 [1] (ng/ml)	Relative activity [2]	EC50 [1] (ng/ml)	Relative activity [2]	TNFR2/TNFR1 [3]
wtTNF	0.6	1.0	0.56	1.00	1.0
R2-6	8.1×10^3	7.3×10^{-5}	0.39	1.44	2.0×10^4
R2-7	2.1×10^3	2.8×10^{-4}	0.21	2.67	9.5×10^3
R2-8	4.6×10^3	1.2×10^{-4}	0.67	0.84	7.0×10^3
R2-9	$>1.0 \times 10^5$	$< 6.0 \times 10^{-6}$	0.51	1.10	1.8×10^5
R2-10	1.1×10^4	5.4×10^{-5}	0.72	0.78	1.4×10^4
R2-11	6.7×10^4	8.9×10^{-6}	0.95	0.59	6.6×10^4
R2-12	2.6×10^4	2.2×10^{-5}	0.23	2.43	1.1×10^5
R2-13	$>1.0 \times 10^5$	$< 6.0 \times 10^{-6}$	0.63	0.89	1.5×10^5

[1] 50%効果濃度(EC50)をロジスティック回帰法により算出した。
[2] Relative activities = EC50 (wtTNF) / EC50 (TNF variants).
[3] TNFR2-selectivities = Relative activity (via TNFR1) / Relative activity (via TNFR2).

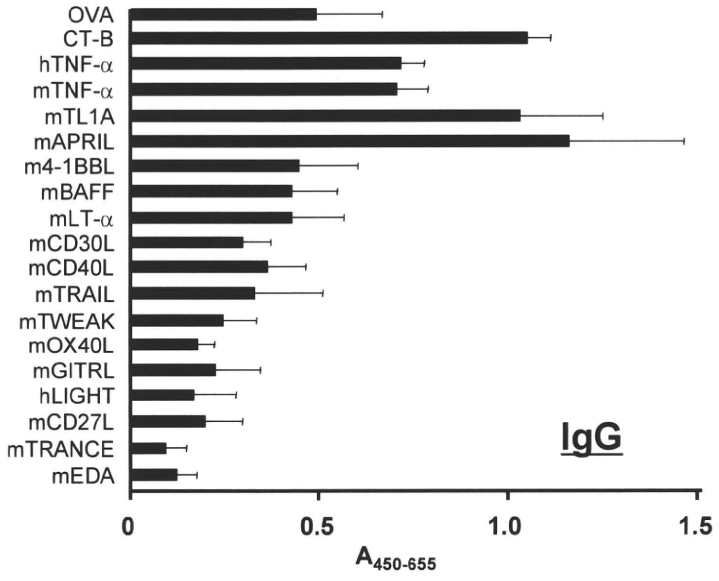

図2. Comparison of OVA-specific serum IgG Abs responses in mice immunized with TNFsfs. BALB/c mice were immunized intranasally with 100 μg OVA alone or in combination with 1 μg TNFsfs once per week for three consecutive weeks. Serum was collected on day 21 and analyzed by ELISA for OVA specific IgG Abs responses. Values represent the mean $OD_{450-655}$ ± S.E. at a 1:50 dillution of seven mice for each groups.

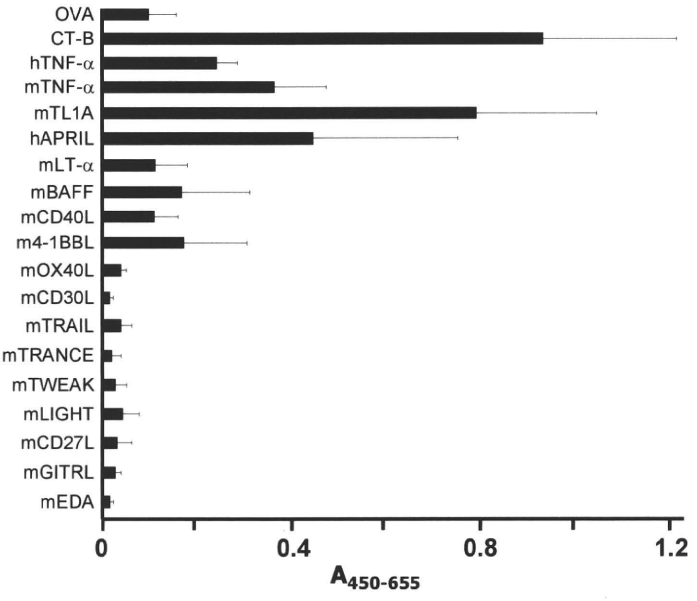

図3. Comparison of OVA-specific IgA Abs responses in nasal washes of mice immunized with TNFsfs.
BALB/c mice were immunized intranasally with 100 μg OVA alone or in combination with 1 μg TNFsfs once per week for three consecutive weeks. Nasal washes were collected on day 21 and analyzed by ELISA for OVA specific IgA Abs responses. The data represent the mean reciprocal titers and each points represents the mean ± SE of seven mice for each groups.

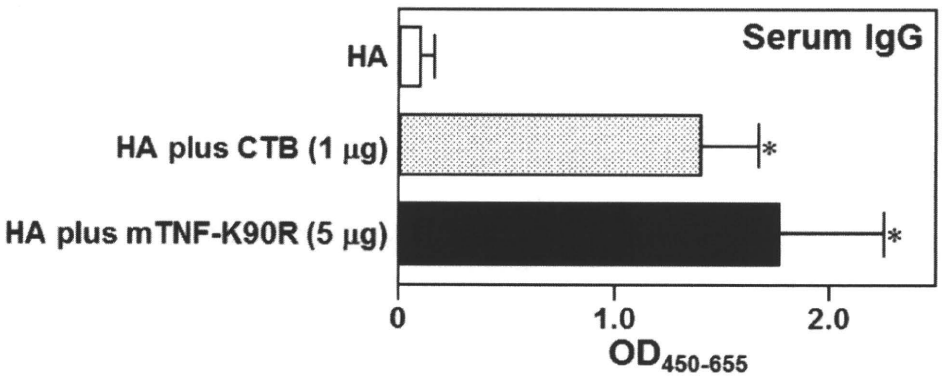

図4. mTNF-K90R induced serum IgG responses against influenza virus HA in mice.
BALB/c mice were immunized intranasally with HA together with 1 μg CTB or 5 μg mTNF-K90R. One week after the last immunization, the levels of HA-specific IgG in serum at a 1:500 dilution was assessed by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). Data are presented as means ± SEM (n = 4-6; * $P < 0.05$ *versus* value for HA alone treated group by ANOVA).

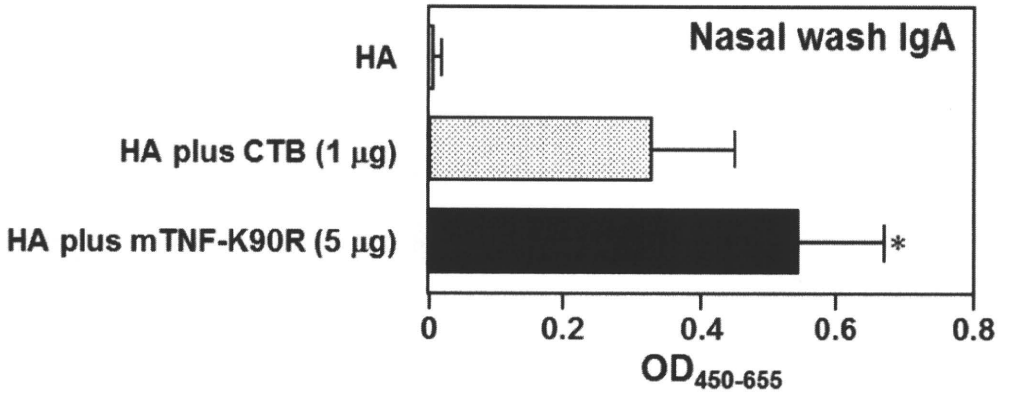

図5. mTNF-K90R induced nasal IgA against influenza virus HA in mice.
BALB/c mice were immunized intranasally with HA together with 1 μg CTB or 5 μg mTNF-K90R. One week after the last immunization, the levels of HA-specific IgA in nasal at a 1:8 dilution were assessed by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). N.D; not detected. Data are presented as means ± SEM (n = 4-6; * $P < 0.05$ *versus* value for HA alone treated group by ANOVA).

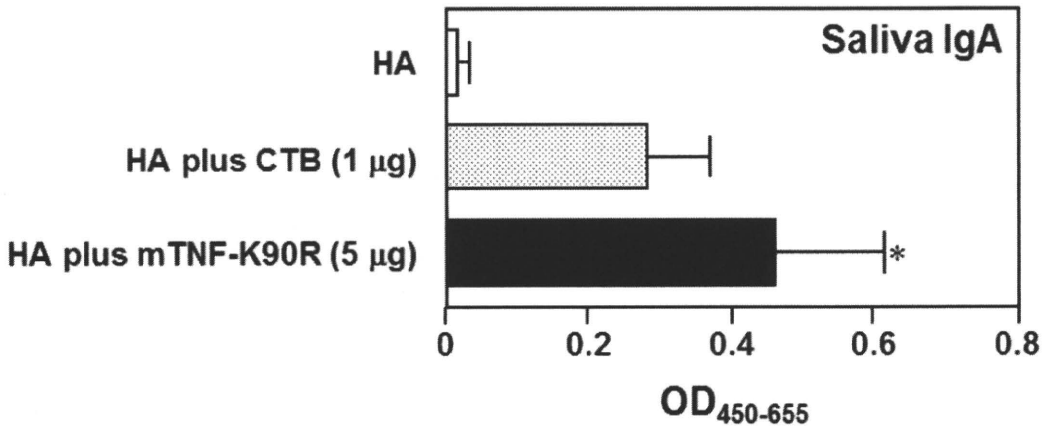

図6. mTNF-K90R induced saliva IgA against influenza virus HA in mice.
BALB/c mice were immunized intranasally with HA together with 1 μg CTB or 5 μg mTNF-K90R. One week after the last immunization, the levels of HA-specific IgA in saliva at a 1:8 dilution were assessed by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). N.D; not detected. Data are presented as means ± SEM (n = 4-6; * $P < 0.05$ *versus* value for HA alone treated group by ANOVA).

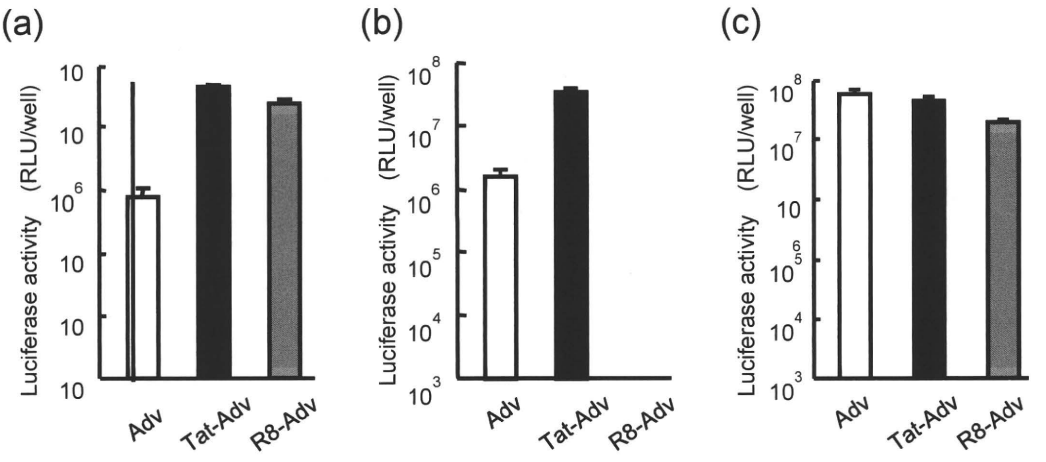

図7. Transduction efficiency of CPP-Advs with various modification ratios into (a) B16BL6, (b) CT26, and (c) A549 cells.
Cells (1×10^4) were transduced with 1×10^4 vp/cell of Tat-Adv, Pro-Adv, or R8-Adv encoding the luciferase gene. After 24 h of cultivation, luciferase expression was measured. Each bar represents the mean ± S.D. (n = 4). Each CPP-Adv was used at molar ratios of 1:25 (Adv lysine residue: CPP-NHS).

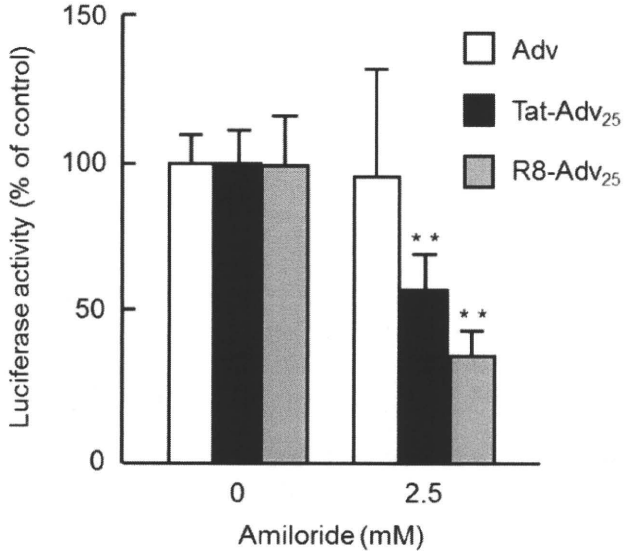

図8. The cellular uptake of Tat-Adv and R8-Adv depends on macropinocytosis.
A549 cells (1×10^4 cells) were transduced with 1×10^4 vp/cell for each vector in the absence or presence of 2.5 mM amiloride. After 24 h of cultivation, luciferase expression was measured. Each bar represents the mean ± S.D. (n = 5; ** $P < 0.01$ versus value for the absence of amiloride by Student's *t*-test)

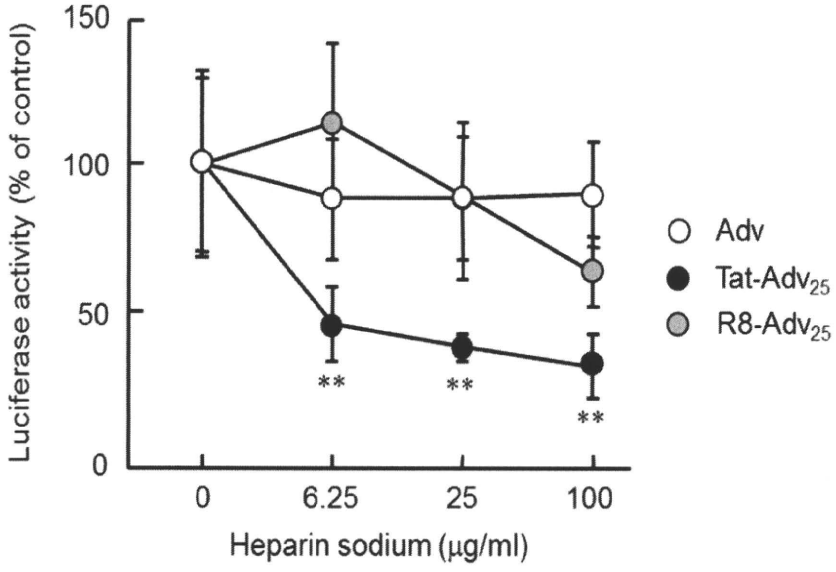

図9. The cellular uptake of Tat-Adv and R8-Adv depends on heparan sulfate proteoglycans.
A549 cells (1×10^4 cells) were transduced with 1×10^4 vp/cell of each vector in the absence or presence of 6.25, 25, or 100 μg/ml of heparin sodium. After 24 h of cultivation, luciferase expression was measured. Each bar represents the mean ± S.D. (n = 5; ** $P < 0.01$ versus value for absence of heparin sodium by ANOVA)

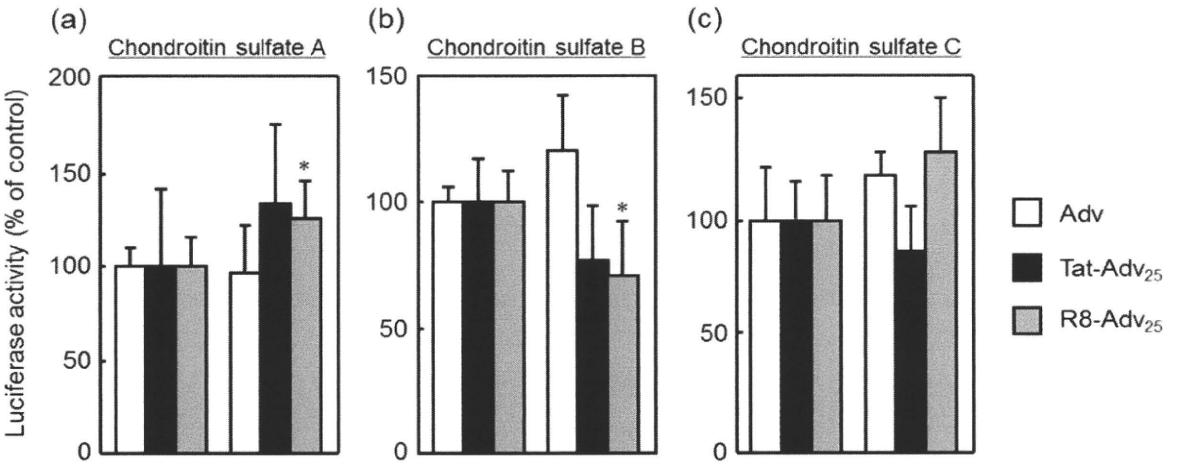

図10. Cellular uptake of Tat-Adv and R8-Adv depends on chondroitin sulfate.
A549 cells (1×10^4) were transduced with 1×10^4 vp/cell of each vector in the absence or presence of 90 μg/ml of (a) chondroitin sulfate-A, (b) chondroitin sulfate-B, or (c) chondroitin sulfate-C. After 2 h incubation, the cells were washed twice by PBS, and the virus solution was replaced with fresh medium. After 24 h cultivation, luciferase expression was measured. Each point represents the mean ± S.D. (n = 5; * $P < 0.05$ versus value for the absence of chondroitin sulfate by Student's *t*-test)

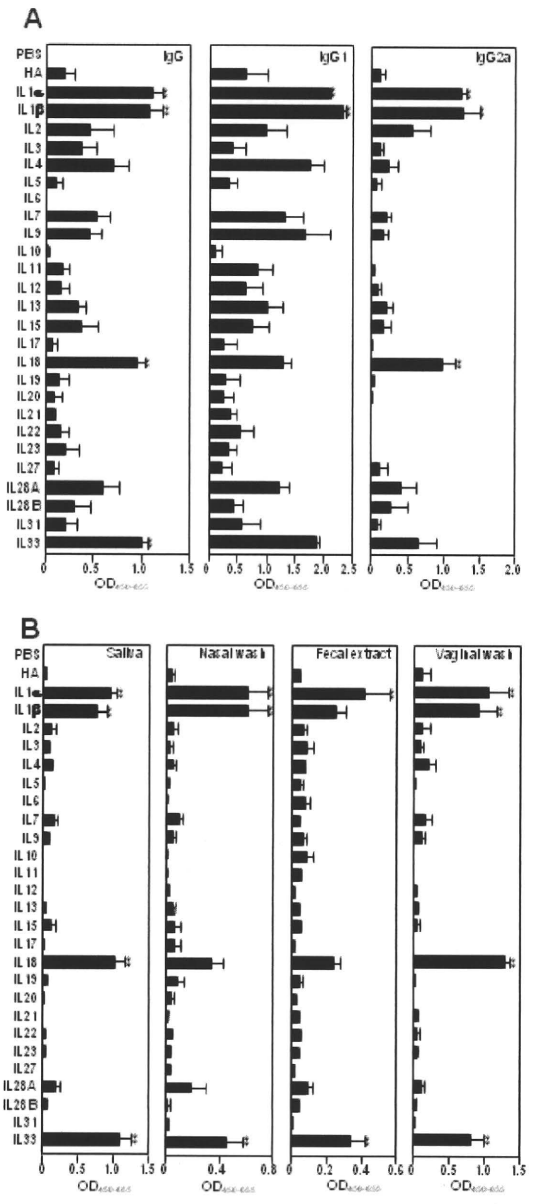

図 11 Serum rHA-specific IgG responses by nasal immunization with rHA plus interleukins.

BALB/c mice were intranasally immunized with rHA alone or rHA plus each interleukins two times at 4 weekly intervals. Serum was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific (A) IgG, IgG1, and IgG2a responses. rHA-specific IgA responses in (B) saliva, nasal wash, fecal extract and vaginal wash were determined by ELISA. Data are presented as means ± SEM (n = 5;)

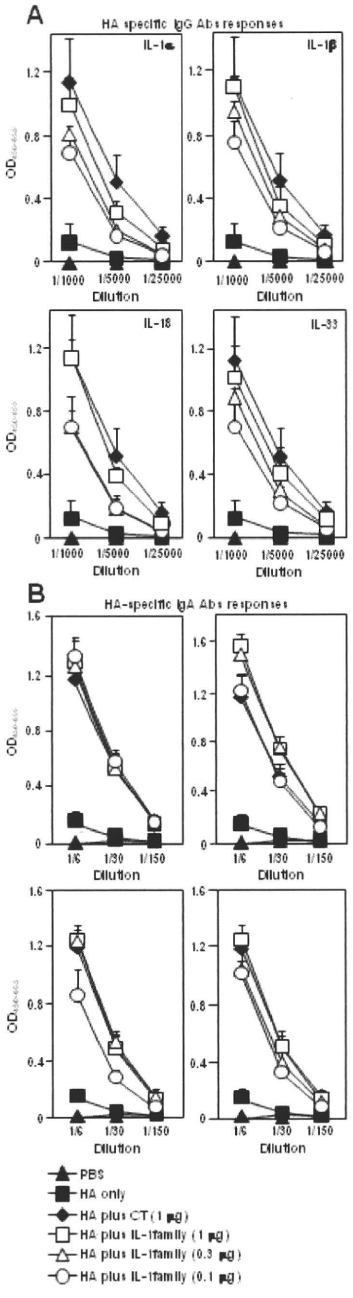

図 12 The dose response for the induction of rHA-specific Ab responses by nasal immunization with rHA plus IL-1 family cytokines.

BALB/c mice were intranasally immunized with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus IL-1 family cytokines (0.1, 0.3, or 1 μg/mouse) two times at 4 weekly intervals. Serum was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific (A) IgG responses at a dilution of 1/1000, 1/5000, and 1/250000. (B) The nasal wash was examined for the presence of rHA-specific IgA responses at dilutions of 1/6, 1/30, and 1/150. Data are presented as means ± SEM (n = 5;)

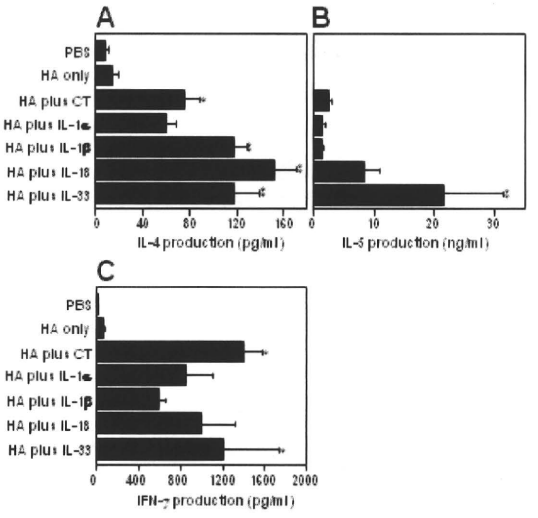

図 13 Cytokine responses induced by nasal immunization with rHA plus IL-1 family cytokines. BALB/c mice were intranasally immunized with rHA alone, rHA plus CT, or rHA plus IL-1 family cytokines two times at 4 weekly intervals. 14 days after the final immunization, splenocytes from each group were cultured with 10 μg/mL rHA. Culture supernatants were harvested after 3 days of incubation, and then rHA-specific (A) IL-4, (B) IL-5, and (C) IFN-γ productions in culture supernatant were analyzed using the Bio-Plex Multiplex Cytokine Assay. Data are presented as means ± SEM (n = 5;)

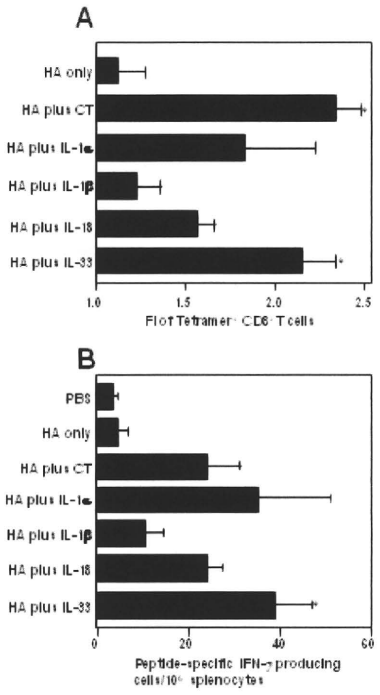

図 14 The frequency of H-2Kd/HA240-248 tetramer+ CD8+ T cells and H-2Kd/HA240-248 specific IFN-γ secreting cells in spleen after nasally immunization with rHA plus IL-1 family cytokines.

BALB/c mice were intranasally immunized with rHA alone, rHA plus CT, or rHA plus IL-1 family cytokines two times at 4 weekly intervals. 14 days after the final immunization, splenocytes from immunized mice were harvested and stimulated in the presence of H-2Kd- restricted class I HA peptide at a final concentration of 10 μg/ml total peptide. (A) For detection of H-2Kd/HA240-248 tetramer+ CD8+ T cells, splenocytes from immunized mice were cultured in media containing CTL epitope peptide (HA240-248; IYSTVASSL) plus 10 U/mL human IL-2 for 7 days, and then stained for the presence of CD8 and tetramer-binding cells using flow cytometry. (B) After 24 h incubation, the IFN-γ-producing cells were measured by an ELISPOT assay. Data are presented as means ± SEM (n = 5;)

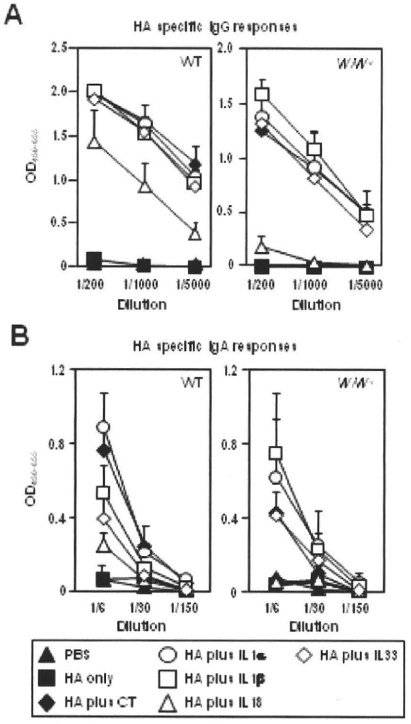

図 15 The dependency of MCs for the induction of rHA-specific immune responses by nasal immunization with rHA plus IL-1 family cytokines.

WBB6F1 W/Wv and WT mice were intranasally immunized with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus IL-1 family cytokines (1 μg/mouse) two times at 4 weekly intervals. Plasma and fecal extracts were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG responses in (A) plasma and rHA-specific IgA responses in (B) fecal extract. Data are presented as means ± SEM (n = 5;)

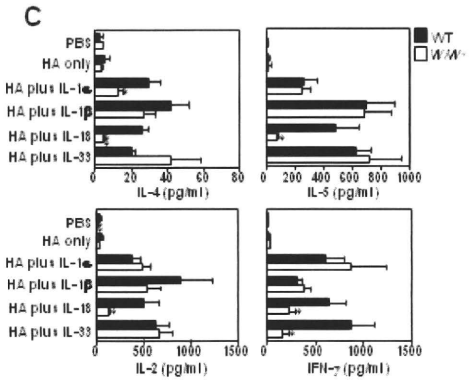

図 16 The dependency of MCs for the induction of rHA-specific immune responses by nasal immunization with rHA plus IL-1 family cytokines.

WBB6F1 W/Wv and WT mice were intranasally immunized with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus IL-1 family cytokines (1 μg/mouse) two times at 4 weekly intervals. Splenocytes from each group of WBB6F1 W/Wv and WT mice were cultured with 10 μg/mL rHA. Culture supernatants were harvested after 3 days of incubation, and then rHA-specific cytokine productions in culture supernatant (IL-4, IL-5, IL-2, and IFN-γ) were analyzed using the Bio-Plex Multiplex Cytokine Assay. Data are presented as means ± SEM (n = 5;)

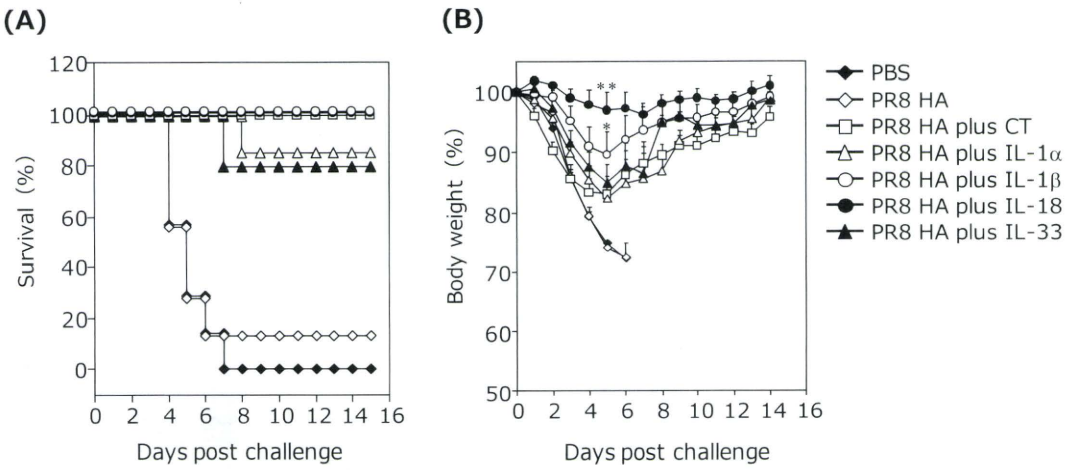

図 17. Protection of BALB/c mice against lethal influenza virus infection by IL-1 family cytokine adjuvants. BALB/c mice were immunized intranasally at 0 and 28 days with PR8 HA alone, PR8 HA plus CT, or PR8 HA plus an IL-1 family cytokine. 14 days after the final immunization, mice were intranasally infected with 256 HAU of influenza virus A/PR/8/34. Mice were monitored for survival (A) and weight loss (B) for 14 days after infection. The results are expressed as percent survival (A) and percent initial body weight (B). Data are presented as means ± SEM (n=4 to 7). Differences were compared using Dunnett analysis of variance (ANOVA). *, $P < 0.05$; **, $P < 0.01$ compared to the value for the PR8 HA treated group.

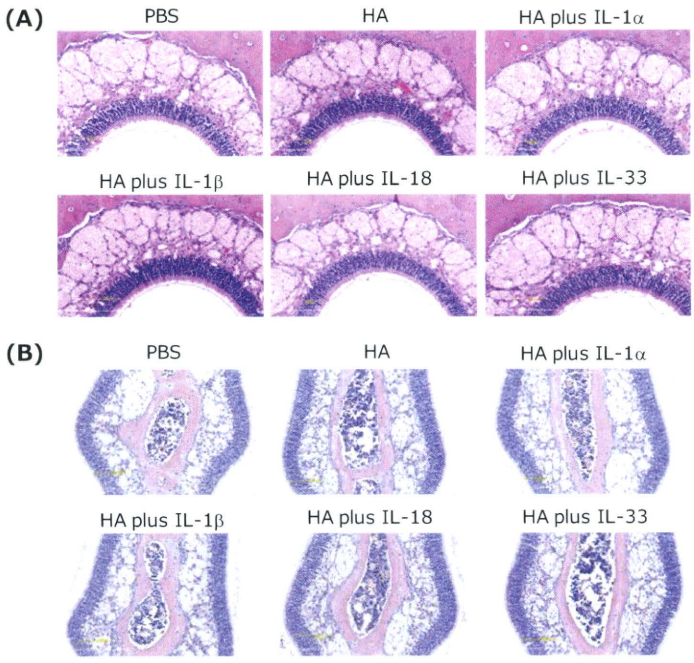

図 18. Histopathological analysis of the nasal cavities of mice immunized intranasally with rHA plus an IL-1 family cytokine. Frontal cross sections of nasal cavities of mice were taken after two administrations of PBS, rHA alone, or rHA plus an IL-1 family cytokine. Sections were prepared and stained with H&E (A) or Luna' s stain (B) to assess pathological changes. Overall views of the nasal epithelium (A) and of Luna' s-stained eosinophils in the nasal septum (B) are shown.

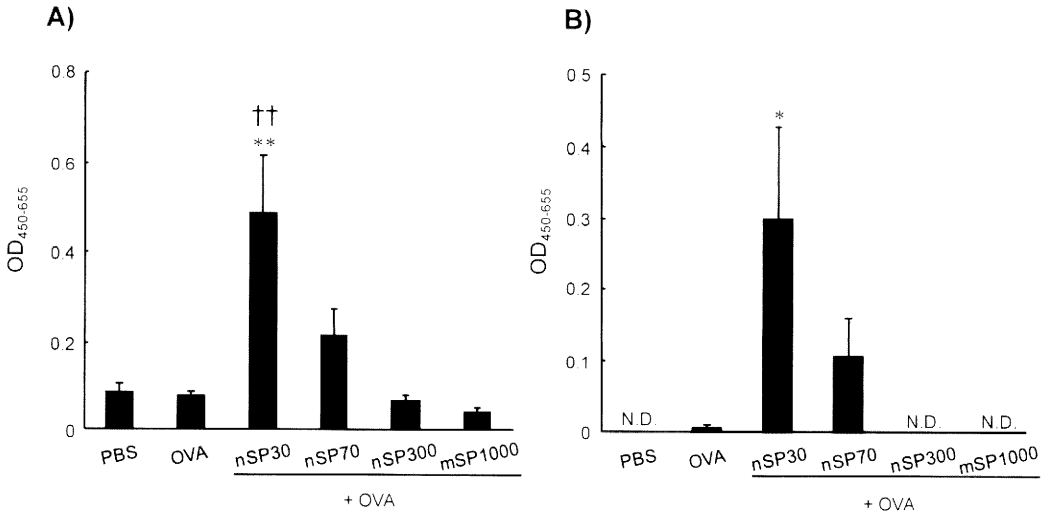

図 19. Plasma OVA-specific IgG and subclass IgG1 Ab response after intranasal exposure to OVA plus silica particles.

BALB/c mice were intranasally exposed to PBS (vehicle control), OVA alone or OVA plus silica particles (250 μg/mouse) on days 0, 1, and 2. Plasma was collected on day 21 and analyzed by ELISA to detect the level of (A) OVA-specific IgG and (B) OVA-specific IgG1 Ab responses. Data represent mean absorbance at a wavelength of 450 nm (reference wavelength, 655 nm). N.D., not detected. Data are presented as mean ± SEM (n = 5 to 8); $*P < 0.05$, $**P < 0.01$ *vs* OVA alone; $^{††}P < 0.01$ *vs* PBS).

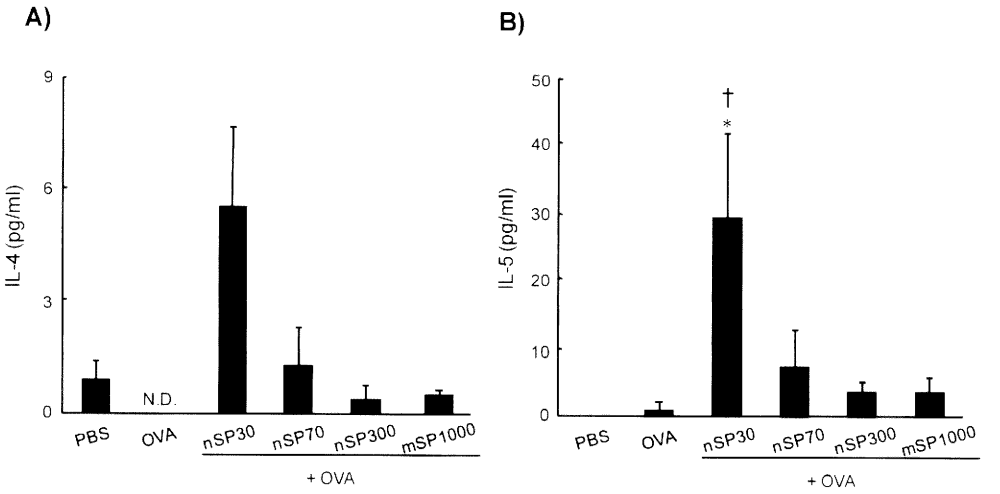

図 20. Cytokine responses induced after intranasal exposure to OVA plus silica particles.

BALB/c mice were intranasally exposed to PBS (vehicle control), OVA alone or OVA plus silica particles (250 μg/mouse) on days 0, 1, and 2. On day 21, splenocytes from each group were prepared and cultured with 1 mg/mL OVA. Culture supernatants were harvested after 3 days of incubation, and the level of OVA-induced IL-4 (A) and IL-5 (B) produced and released into the culture supernatant was analyzed using the Bio-Plex Multiplex Cytokine Assay. Data are presented as mean ± SEM (n = 3; $*P < 0.05$ *vs* OVA alone; $^{†}P < 0.05$ *vs* PBS).

研究成果の刊行に関する一覧表（H20 年度）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

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The therapeutic effect of TNFR1-selective antagonistic mutant TNF-α in murine hepatitis models

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is critically involved in a wide variety of inflammatory pathologies, such as hepatitis, via the TNF receptor-1 (TNFR1). To develop TNFR1-targeted anti-inflammatory drugs, we have already succeeded in creating a TNFR1-selective antagonistic mutant TNF-α (R1antTNF) and shown that R1antTNF efficiently inhibits TNF-α/TNFR1-mediated biological activity in vitro. In this study, we examined the therapeutic effect of R1antTNF in acute hepatitis using two independent experimental models, induced by carbon tetrachloride (CCl_4) or concanavalin A (ConA). In a CCl_4-induced model, treatment with R1antTNF significantly inhibited elevation in the serum level of ALT (alanine aminotransferase), a marker for liver damage. In a ConA-induced T-cell-mediated hepatitis model, R1antTNF also inhibited the production of serum immune activated markers such as IL-2 and IL-6. These R1antTNF-mediated therapeutic effects were as good as or better than those obtained using conventional anti-TNF-α antibody therapy. Our results suggest that R1antTNF may be a clinically useful TNF-α antagonist in hepatitis.

1. Introduction

Acute and chronic liver failure represents a worldwide health problem in humans for which there is no effective pharmacological treatment. For example, fulminant liver failure (FLF) is a devastating liver disease that is associated with significant mortality (40–80%) worldwide [1–3]. The incidence of FLF has increased in the last decade accounting for >2000 deaths annually in the United States alone [2]. Various etiologies result in acute and chronic liver failure. Immune-mediated mechanisms play a central role in autoimmune and viral hepatitis and thus determine disease progression.

Molecules belonging to the Tumor necrosis factor (TNF) superfamily, especially TNF-α, play an integral role in the regulation of innate and adaptive immunity, as well as contributing to inflammatory responses [4,5]. Overproduction of TNF-α has been implicated in the pathogenesis of various inflammatory conditions including autoimmune diseases [4]. Recent studies suggest that TNF-α may also play a crucial role in the progression of liver failure [6–8]. Elevated levels of TNF-α occur in various acute and chronic liver diseases, including viral and alcoholic hepatitis, FLF, and exposure to hepatotoxins [9,10]. Thus, TNF-α appears to be involved in mediating hepatic cell death in experimental models of hepatitis. Furthermore, the inhibition of TNF-α by means of antibody (Ab) or soluble decoy receptors has proven to have a clinical benefit [11,12]. However, these therapies can cause serious side effects, such as bacterial and virus infection, lymphoma development, and lupus inflammatory disease, because they also inhibit the TNF-α-dependent host defense function of the patients [13,14]. TNF-α binds to two receptor-subtypes, p55 TNF receptor (TNFR1) and p75 TNF receptor (TNFR2), to exert its biological activities [15]. The two receptors have distinct biological functions with different distribution patterns; TNFR1 is constitutively expressed in most tissues, whereas expression of TNFR2 is highly regulated and is typically found in cells of the immune system [16]. It is generally believed that most of the TNF-α activities, including inflammatory responses in hepatitis, are triggered by TNFR1, whereas TNFR2 plays a pivotal role in regulating the immune response [15,17,18]. Unfortunately, the therapies with Ab or soluble decoy

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