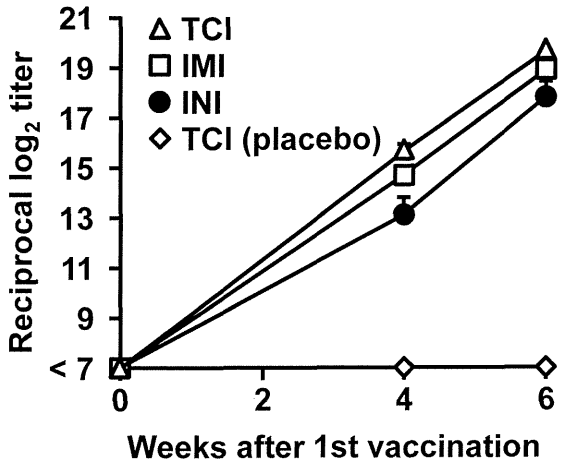
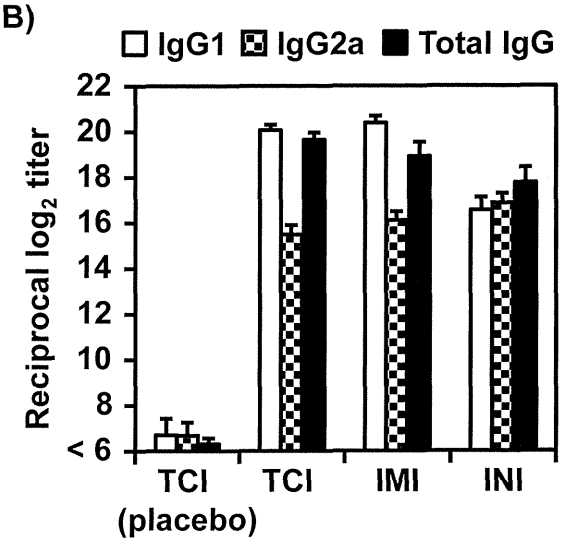
(A)



(B)



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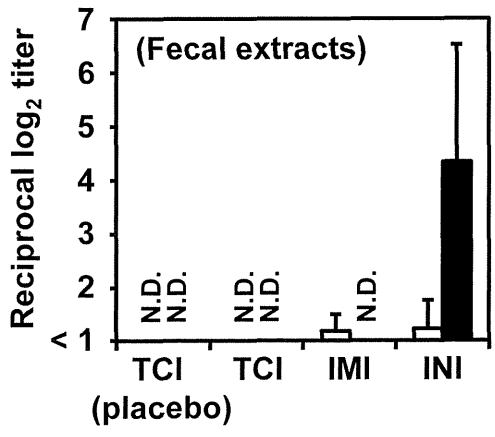

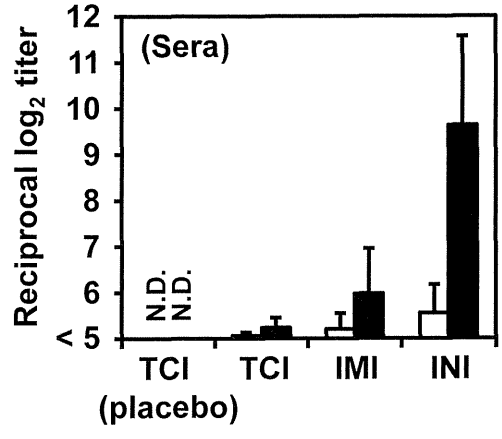

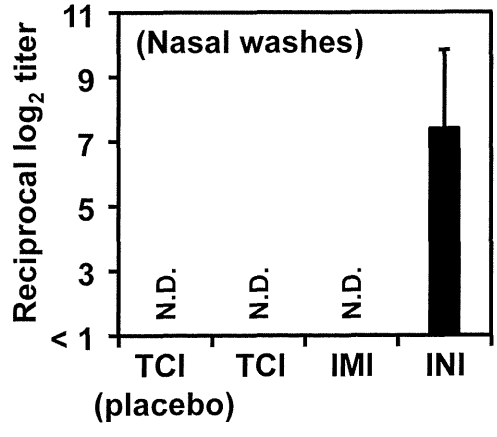

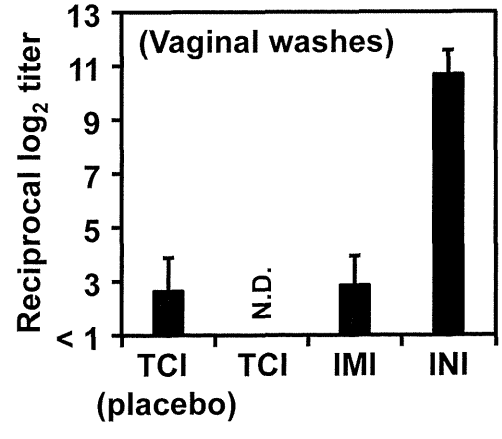

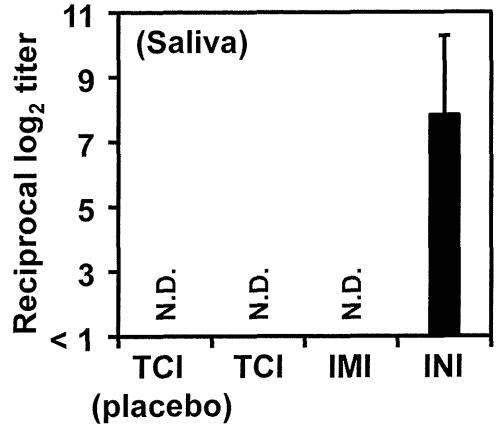

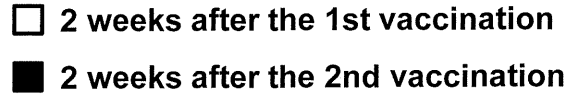

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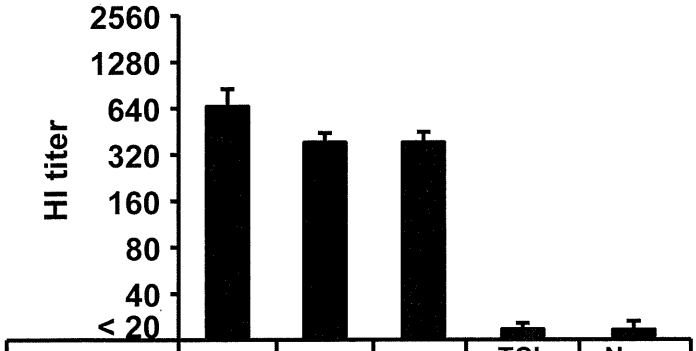
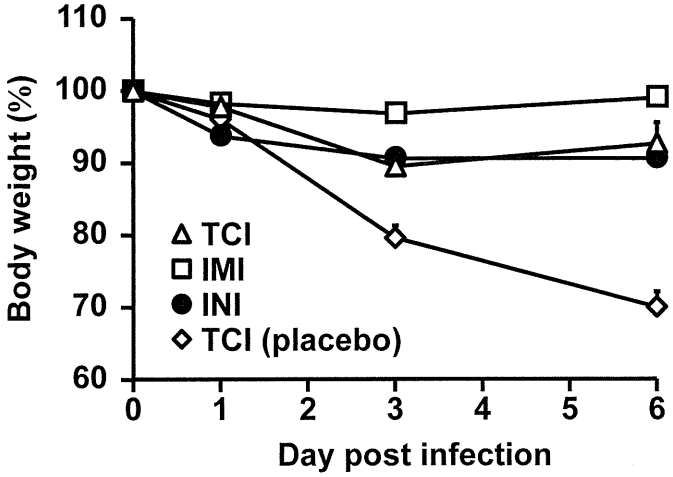
(D)



Route	TCI	IMI	INI	TCI (placebo)	Non-treated
Adjuvant	—	—	CT	—	—

(E)



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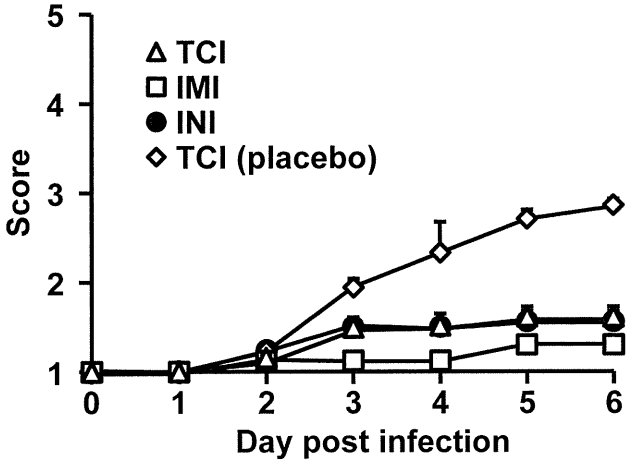

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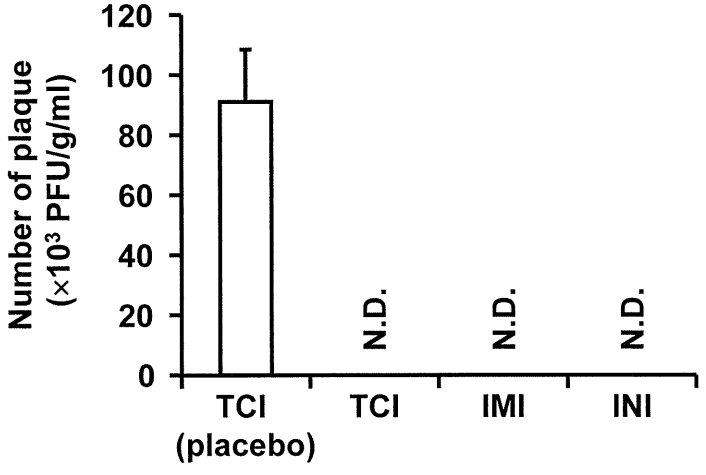
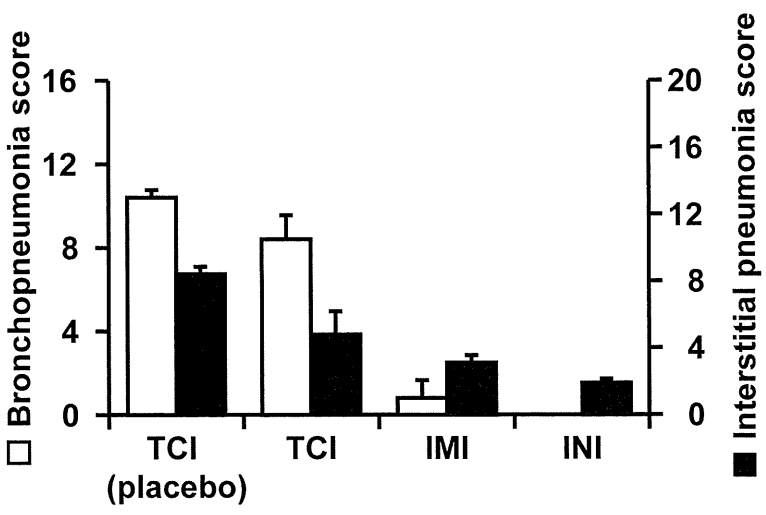


(H)



(continued)

Fig. 34 Protection of vaccinated mice against influenza virus challenge. BALB/c mice were transcutaneously vaccinated with HA from [A/PR/8/34 (H1N1)] (0.4 μg) for 6 h twice at 4-week interval. Control groups were treated with transcutaneous application without HA, intramuscular injection of HA (0.4 μg), or intranasal application of HA (0.4 μg) combined with CT (10 μg) twice at 4-week interval. These mice were each infected intranasally with 6×10^5 PFU of the A/PR/8/34(H1N1) virus. (A) At the indicated points, sera collected from these mice were assayed for the HA-specific IgG titer by ELISA. (B, D) Two weeks after the final treatment, sera collected from these mice were assayed for (B) anti-HA IgG subclass (IgG1 and IgG2a) and (D) the HI titer. HI activity is expressed as the highest dilution that resulted in complete inhibition of hemagglutination. (C) Two weeks after the 1st or 2nd vaccination, fecal extracts, sera, nasal washes, vaginal washes, and saliva collected from these mice were assayed for the HA-specific IgA titer by ELISA. (A-D) Data are expressed as mean ± SE of results from 13 mice. (E) Body weight was measured each day and is presented as a percentage of the original weight before infection (day 0). (F) The performance status of the mice was scored every day. (G) Six days after infection, the lungs were collected from these mice and number of viruses in the lung homogenate was determined using a plaque assay system. (H) The degree of inflammation of the lung was scored as follows: 0, none; 1, very slight; 2, mild; 3,moderate; and 4, severe. Pathologic findings were classified into bronchopneumonia and interstitial pneumonia. (E-H) Data are expressed as mean ± SE of results from 10 mice. TCI; transcutaneous immunization, IMI; intramuscular immunization, INI; intranasal immunization, N.D.; not detectable.

Table 14 Lung weight

Vaccination	Lung weight (g; Mean ± SE)	
	Left lobe	Right lobe
TCI (placebo)	0.154 ± 0.007	0.243 ± 0.022
TCI	0.068 ± 0.007	0.142 ± 0.013
IMI	0.059 ± 0.003	0.123 ± 0.005
INI	0.057 ± 0.002	0.117 ± 0.003

Table 15 Degree of lung consolidation

Vaccination	Consolidation (/10 tested mice)				Score (Mean ± SE) [a]	
	Left lobe	Right lobe	Right and left lobe	No	Left lobe	Right lobe
TCI (placebo)	1	0	9	0	4.2 ± 0.2	3.4 ± 0.5
TCI	0	1	0	9	1.0 ± 0.0	1.1 ± 0.1
IMI	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0
INI	0	0	0	10	1.0 ± 0.0	1.0 ± 0.0

[a] Degree of consolidation: 1; no consolidation, 2; ≤1/3 lobe, 3; >1/3 to ≤1/2 lobe, 4; >1/2 to ≤ 2/3 lobe, 5; >2/3 lobe.

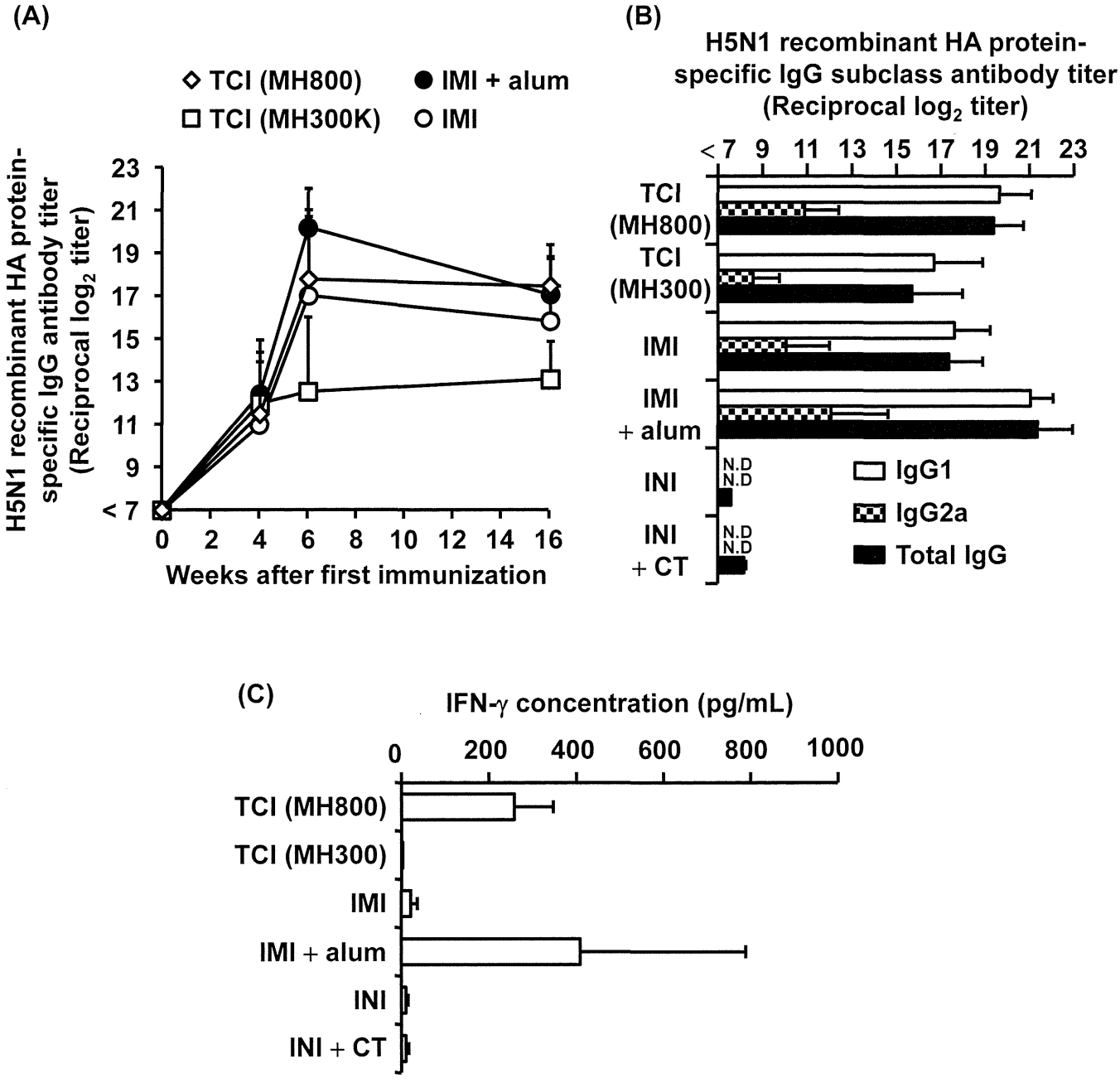


Fig. 35 Anti-H5N1 HA immune responses in BALB/c mice after TCI. BALB/c mice were transcutaneously vaccinated with 0.05 μg H5N1 recombinant HA protein using by MH800 or MH300K for 6 h twice at 4-week interval. Control group was intramuscularly vaccinated with 0.05 μg H5N1 recombinant HA protein with or without 10 μg alum twice at 4-week interval. Another control group was intranasally vaccinated with 0.05 μg H5N1 recombinant HA protein with or without 10 μg CT twice at 4-week interval. (A) At the indicated points, sera collected from these mice were assayed to determine the HA-specific IgG titer by ELISA. (B) Sera collected 2 weeks after the last treatment were assayed for HA-specific IgG subclass (IgG1 and IgG2a) by ELISA. (C) Two weeks after final vaccination, single-cell suspensions of splenocytes were prepared and IFN-γ concentration was assayed by ELISA after stimulating the cells with 1 μg/mL H5N1 recombinant HA protein for 24 h. Data are expressed as mean ± SE of results from 3 mice. TCI; transcutaneous immunization, IMI; intramuscular immunization, INI; intranasal immunization, CT; cholera toxin, N.D.; not detectable.

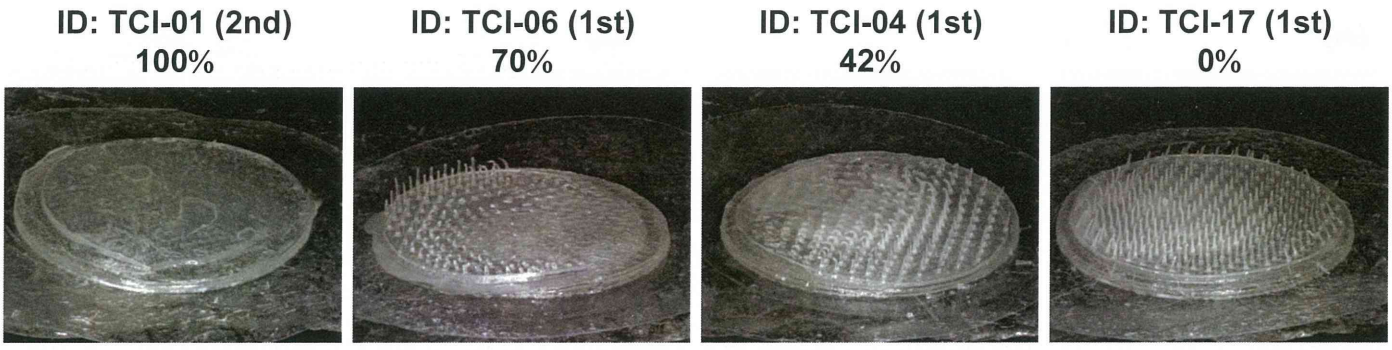


Fig. 36 Digital microscopic images of MH800 containing influenza HA antigens after application to human skin. The MH800 was applied to the skin of the left lateral upper arm of healthy volunteers for 6 h, and then the MH800 patches were observed under digital microscope. Percentage of MN dissolution in each subjects was calculated by counting remained MNs.

Table 16 MN dissolution in each subjects

		Type A	Type A	Type B
Handheld applicator used in application				
Study	**ID**	**% of MN dissolution**		
Pilot	***TCI-01***	86	100	
	TCI-02	100	57	
	TCI-03	100	96	
Prospective	TCI-04		42	100
	TCI-05		16	100
	TCI-06		70	100
	TCI-07		94	100
	TCI-08		13	100
	TCI-09		3	100
	TCI-10		26	100
	TCI-11		51	100
	TCI-12		0	100
	TCI-13		41	100
	TCI-14		29	100
	TCI-15		0	100
	TCI-17		0	100
	TCI-18		27	100
	TCI-19		59	100
	TCI-20		16	100

The IDs of 7 subjects (Per Protocol Set; PPS) whom more than 50% of MNs dissolved on both 1st and 2nd vaccination are written in italic and bold.

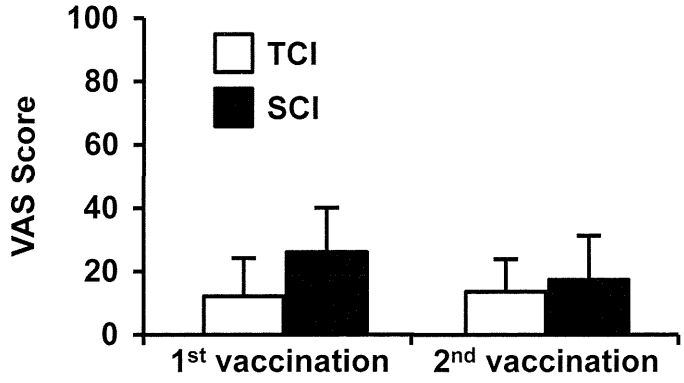


Fig. 37 Pain scale assessment on TCI using MH800 loaded with influenza HA or SCI using injectable HA vaccine. Subjects were asked to grade the pain experienced using a VAS from 0 (no pain) to 100 (unbearable pain). Data are expressed as mean ± SE of results from 19 (TCI) or 20 (SCI) subjects.

Table 17 Local adverse events by application of MH800 loaded with influenza HA

		Erythema				Purpura		Pigmentation	
		Diameter (mm)		No. of positive subjects		No. of positive subjects		No. of positive subjects	
		TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)
1st	Day 2	14.0 ± 4.8	12.2 ± 24.3	19 (100%)	5 (25%)	13 (68%)	1 (5%)	0 (0%)	0 (0%)
	Day 7	10.4 ± 4.9	1.0 ± 1.3	16 (84%)	8 (40%)	12 (63%)	1 (5%)	3 (16%)	0 (0%)
	Day 21	3.3 ± 6.0	1.0 ± 1.3	5 (26%)	8 (40%)	0 (0%)	1 (5%)	10 (53%)	0 (0%)
2nd	Day 23	14.8 ± 6.8	1.7 ± 1.5	19 (100%)	13 (65%)	14 (74%)	13 (65%)	0 (0%)	0 (0%)
	Day 28	11.6 ± 3.5	1.3 ± 1.2	19 (100%)	12 (60%)	6 (32%)	1 (5%)	3 (16%)	1 (5%)
	Day 42	9.3 ± 3.6	1.1 ± 1.0	17 (89%)	13 (65%)	0 (0%)	0 (0%)	10 (53%)	0 (0%)

		Induration		Pressure pain		Feeling of feverishness		A water blister	
		No. of positive subjects		No. of positive subjects		No. of positive subjects		No. of positive subjects	
		TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)	TCI (n=19)	SCI (n=20)
1st	Day 2	8 (42%)	1 (5%)	1 (5%)	5 (25%)	0 (0%)	5 (25%)	0 (0%)	0 (0%)
	Day 7	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Day 21	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2nd	Day 23	5 (26%)	1 (5%)	0 (0%)	1 (5%)	3 (16%)	1 (5%)	0 (0%)	0 (0%)
	Day 28	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)
	Day 42	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

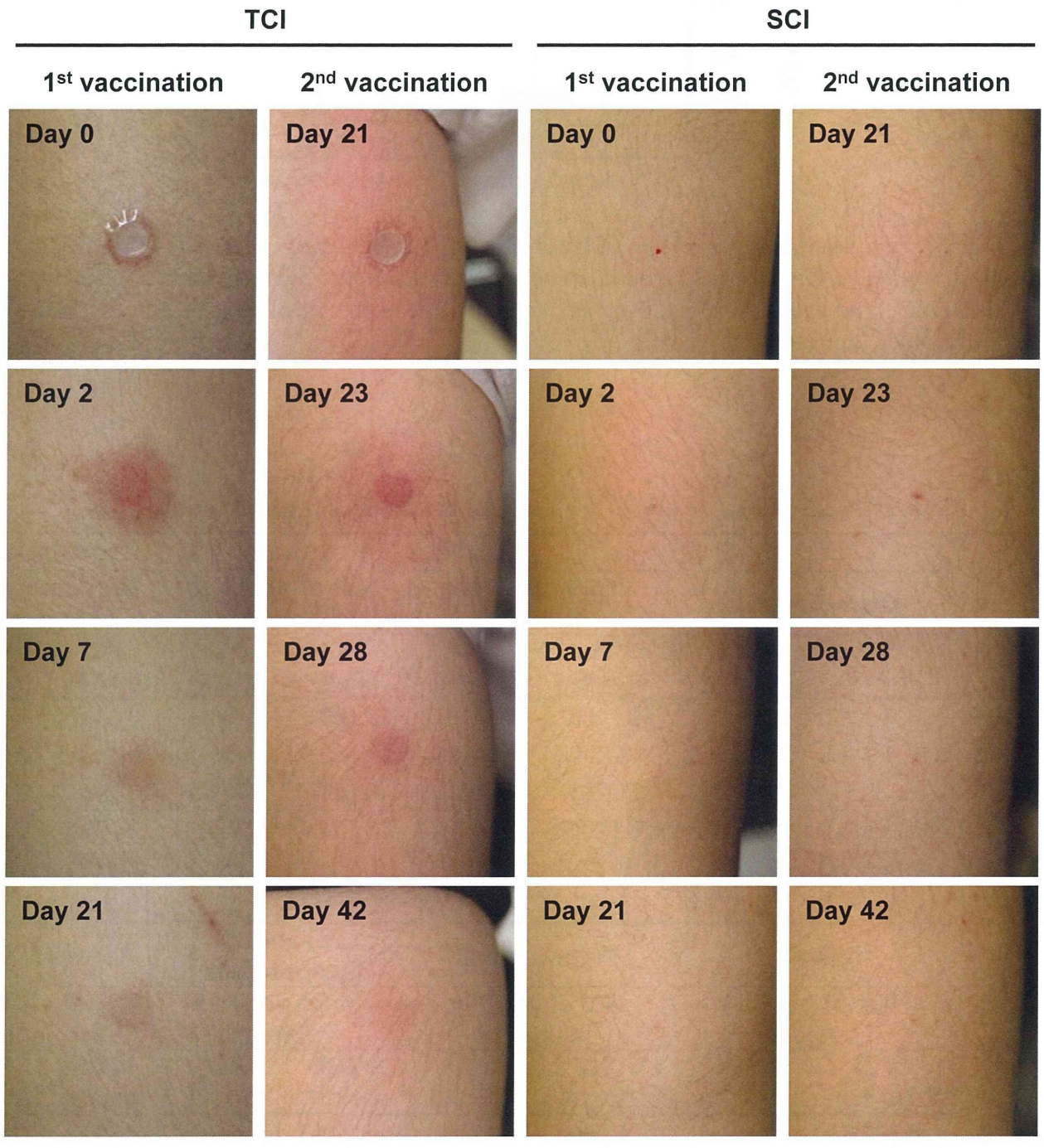


Fig. 38 Photographs of human skin after TCI using MH800 loaded with influenza HA or SCI using injectable HA vaccine.

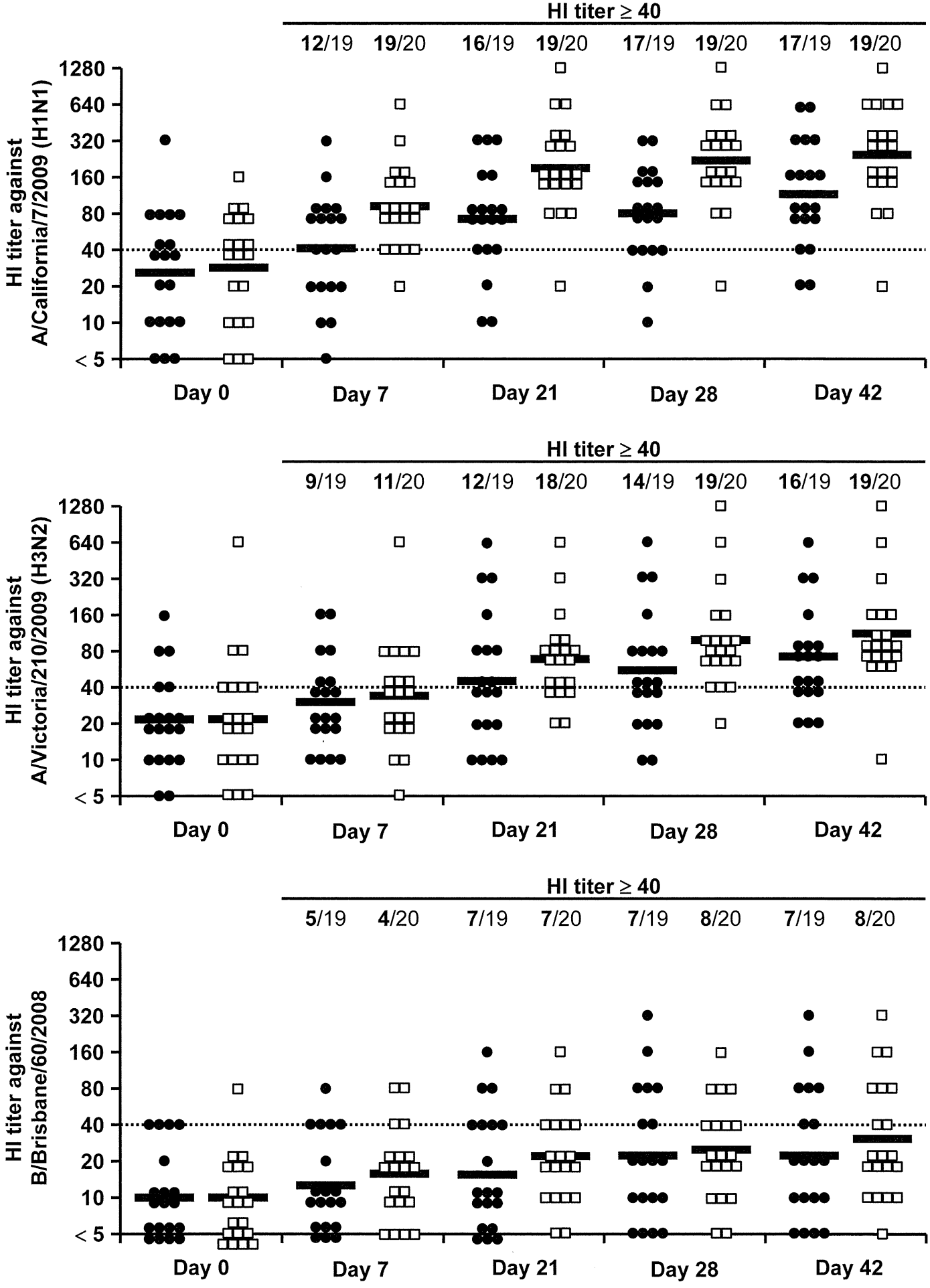


Fig. 39 HI titer against each influenza HA antigens in sera. Each symbols mean HI titer of each subjects (●, TCI and □, SCI) and geometric titer (GMT) is shown as a bar.

Table 18 EMA influenza vaccine criteria

Parameters	EMA criteria	Calculation method
Seroconversion	**> 40%**	Percentage of subjects who have a pre-vaccination titer <10 and a post-vaccination titer >40, or have a pre-vaccination titer ≥10 and at least a fourfold increase in post-vaccination titer.
Seroprotection	**> 70%**	Percentage of subjects with a post-vaccination titer of at least 40.
GMT fold increase value	**> 2.5**	Geometric means of the ratio of the post-vaccination titer to the pre-vaccination titer.

Table 19 Determination of the efficacy based on HI titer in sera

Day	Parameters	A/California/7/2009 (H1N1)		A/Victoria/210/2009 (H3N2)		B/Brisbane/60/2008	
		TCI	SCI	TCI	SCI	TCI	SCI
7	**Seroconversion**	16%	35%	11%	15%	5%	10%
	Seroprotection	63%	**95%**	47%	55%	26%	20%
	GMT fold increase value	1.6	**3.2**	1.4	1.5	1.2	1.6
21	**Seroconversion**	32%	**60%**	11%	40%	16%	25%
	Seroprotection	**84%**	**95%**	63%	**90%**	37%	35%
	GMT fold increase value	**2.8**	**7.0**	2.2	**3.1**	1.6	2.3
28	**Seroconversion**	37%	**65%**	21%	**50%**	16%	30%
	Seroprotection	**89%**	**95%**	**74%**	**95%**	37%	40%
	GMT fold increase value	**3.1**	**7.7**	**2.7**	**4.4**	1.8	2.5
42	**Seroconversion**	**53%**	**65%**	**42%**	**60%**	16%	35%
	Seroprotection	**89%**	**95%**	**84%**	**95%**	37%	40%
	GMT fold increase value	**4.5**	**8.6**	**3.5**	**5.1**	2.2	**3.1**

The numerical values written in bold and underline satisfy EMA criteria.

Table 20 Determination of the efficacy based on HI titer in sera in PPS

Day	Parameters	A/California/7/2009 (H1N1)		A/Victoria/210/2009 (H3N2)		B/Brisbane/60/2008	
		TCI	SCI	TCI	SCI	TCI	SCI
7	Seroconversion	29%	35%	29%	15%	14%	10%
	Seroprotection	57%	**95%**	57%	55%	57%	20%
	GMT fold increase value	2.0	**3.2**	1.5	1.5	1.5	1.6
21	Seroconversion	**57%**	**60%**	29%	40%	**43%**	25%
	Seroprotection	**100%**	**95%**	**71%**	**90%**	**86%**	35%
	GMT fold increase value	**4.4**	**7.0**	**3.6**	**3.1**	**3.0**	2.3
28	Seroconversion	**57%**	**65%**	29%	**50%**	**43%**	30%
	Seroprotection	**100%**	**95%**	**71%**	**95%**	**86%**	40%
	GMT fold increase value	**4.4**	**7.7**	**4.0**	**4.4**	**3.0**	2.5
42	Seroconversion	**71%**	**65%**	**43%**	**60%**	**43%**	35%
	Seroprotection	**100%**	**95%**	**71%**	**95%**	**86%**	40%
	GMT fold increase value	**6.6**	**8.6**	**4.9**	**5.1**	**4.4**	**3.1**

The numerical values written in bold and underline satisfy EMA criteria.

PPS (Per Protocol Set) contain 7 subjects in TCI group whom more than 50% of microneedles dissolved and 20 subjects in SCI group.

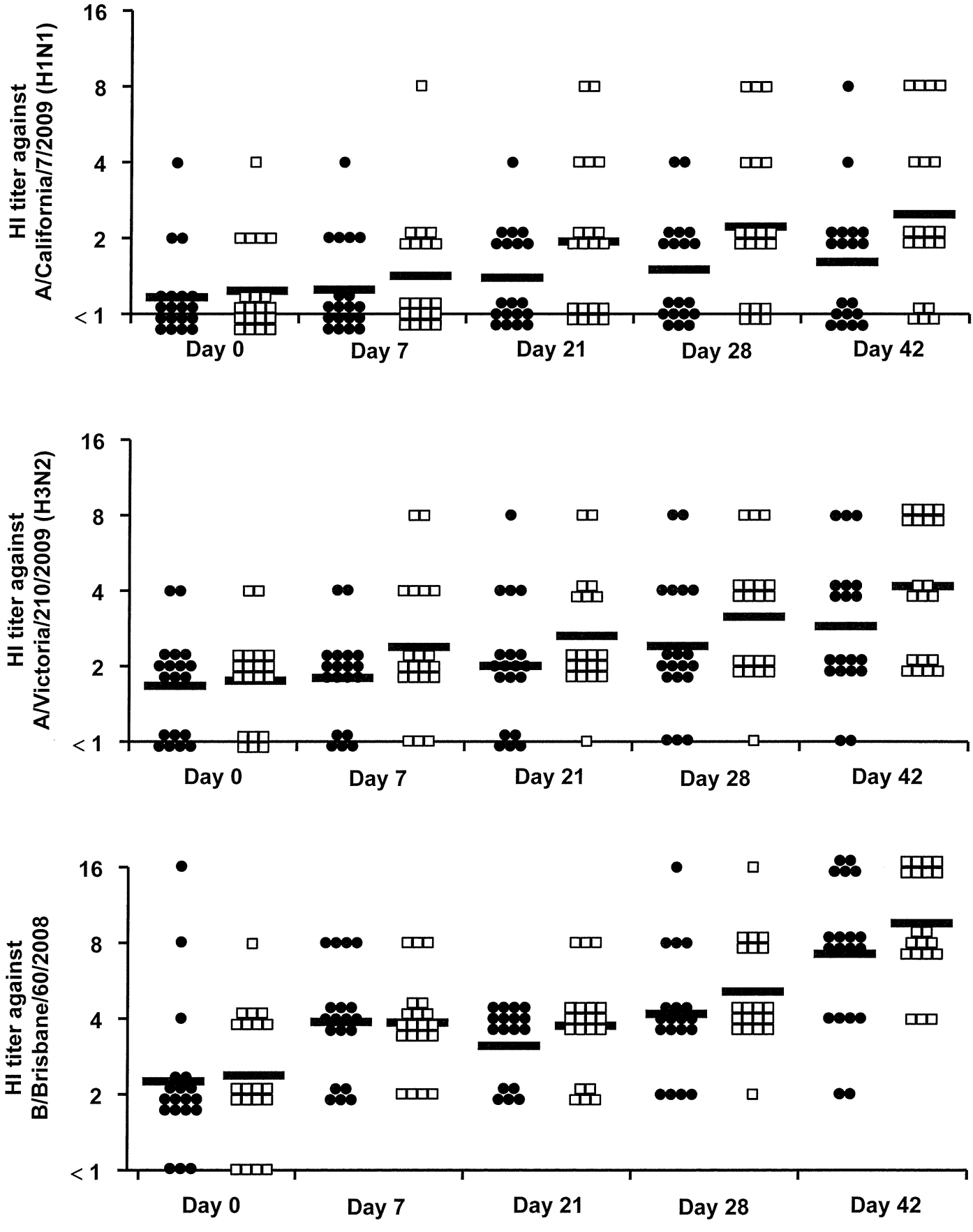


Fig. 40 HI titer against each influenza HA antigens in nasal washes. Each symbols mean HI titer of each subjects (●, TCI and □, SCI) and geometric titer (GMT) is shown as a bar.

Table 21 Determination of the efficacy based on HI titer in nasal washes

Day	Parameters	A/California/7/2009 (H1N1)		A/Victoria/210/2009 (H3N2)		B/Brisbane/60/2008	
		TCI	SCI	TCI	SCI	TCI	SCI
7	Seroconversion	0%	5%	0%	5%	11%	5%
	GMT fold increase value	1.1	1.1	1.1	1.4	1.7	1.6
21	Seroconversion	0%	10%	5%	10%	0%	5%
	GMT fold increase value	1.2	1.6	1.2	1.5	1.4	1.6
28	Seroconversion	0%	15%	11%	15%	16%	25%
	GMT fold increase value	1.3	1.8	1.4	1.8	1.9	2.1
42	Seroconversion	0%	20%	16%	30%	**58%**	**65%**
	GMT fold increase value	1.4	2.0	1.7	2.4	**3.2**	**4.0**

The numerical values written in bold and underline satisfy EMA criteria.

Table 22 Determination of the efficacy based on HI titer in nasal washes in PPS

Day	Parameters	A/California/7/2009 (H1N1)		A/Victoria/210/2009 (H3N2)		B/Brisbane/60/2008	
		TCI	SCI	TCI	SCI	TCI	SCI
7	Seroconversion	0%	5%	0%	5%	0%	5%
	GMT fold increase value	1.2	1.1	1.1	1.4	1.8	1.6
21	Seroconversion	0%	10%	14%	10%	0%	5%
	GMT fold increase value	1.3	1.6	1.3	1.5	1.8	1.6
28	Seroconversion	0%	15%	14%	15%	0%	25%
	GMT fold increase value	1.5	1.8	1.5	1.8	2.0	2.1
42	Seroconversion	0%	20%	14%	30%	**57%**	**65%**
	GMT fold increase value	1.6	2.0	1.5	2.4	**4.4**	**4.0**

The numerical values written in bold and underline satisfy EMA criteria.

PPS (Per Protocol Set) contain 7 subjects in TCI group whom more than 50% of microneedles dissolved and 20 subjects in SCI group.

Table 23 ELISpot assay for IFN-γ-producing cells specific for each HA antigen

Spots/10^5 cells	A/California/7/2009 (H1N1)					
	TCI			SCI		
	Day 0	Day 21	Day 42	Day 0	Day 21	Day 42
≤ 10	**14**	7	7	**9**	2	2
10 – 30	4	**12**	**9**	6	**9**	**14**
30 <	1	0	2	5	**9**	4
Spots/10^5 cells	**A/Victoria/210/2009 (H3N2)**					
	TCI			**SCI**		
	Day 0	**Day 21**	**Day 42**	**Day 0**	**Day 21**	**Day 42**
≤ 10	**13**	6	6	3	1	2
10 – 30	4	**10**	**10**	**10**	**13**	**14**
30 <	2	3	2	7	6	4
Spots/10^5 cells	**B/Brisbane/60/2008**					
	TCI			**SCI**		
	Day 0	**Day 21**	**Day 42**	**Day 0**	**Day 21**	**Day 42**
≤ 10	**13**	5	**7**	**8**	3	1
10 – 30	3	**9**	6	5	**9**	**13**
30 <	3	5	5	6	8	6

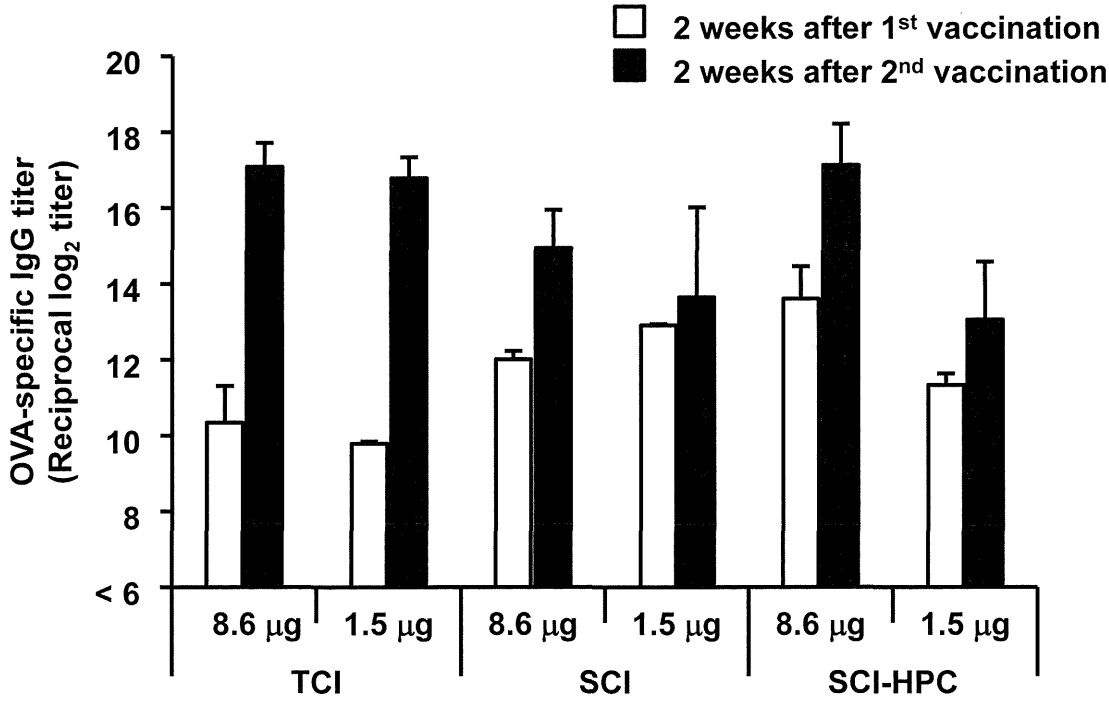


Fig. 41 OVA-specific antibody titers in mice immunized transcutaneously with OVA-coated nMN. OVA-coated nMNs were applied to back skin of C57BL/6 mice twice at 2-week interval (TCI). As a control, OVA/PBS solution (SCI) or OVA/HPC solution (SCI-HPC) were administrated subcutaneously at the same schedule. Sera collected from these mice were assayed for the IgG titer specific against OVA by ELISA. Data are expressed as mean ± SD of results from 3 mice.

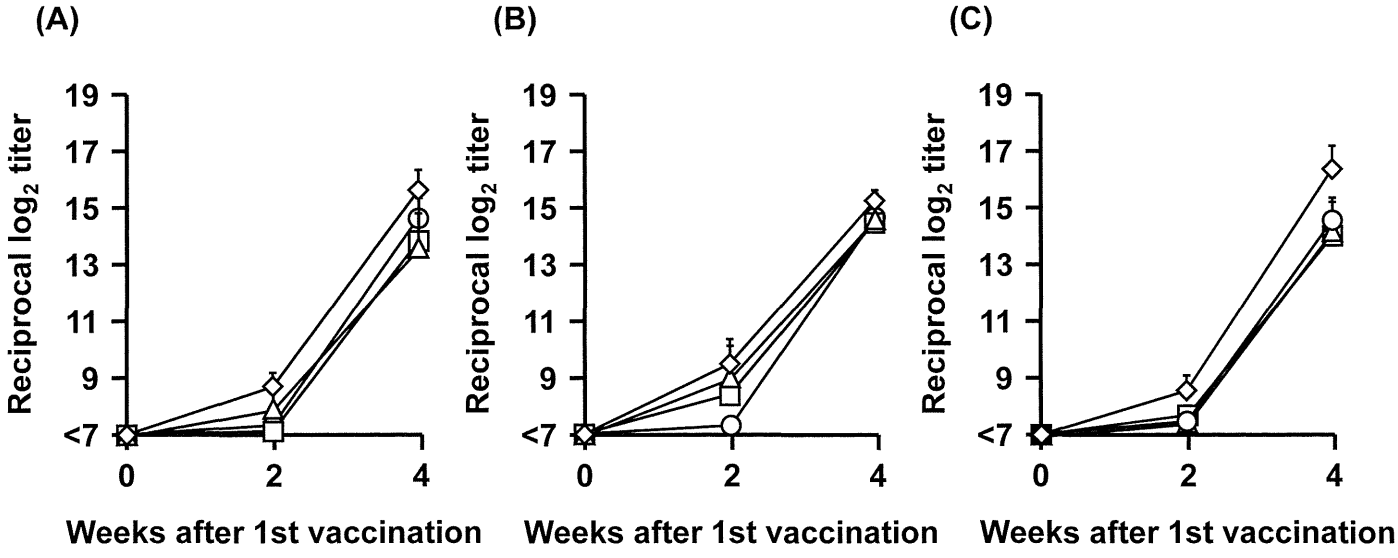


Fig. 42 OVA-specific antibody response in BALB/c mice after intradermal immunization with OVA and components of MH. OVA (2 μg) without (○) or with 0.01 mg (□), 0.1 mg (△) or 1 mg (◇) hyaluronic acid (clinic grade) (A), polyvinylpyrrolidone (B), or dextran (C) twice at 2-week interval. Sera collected from these mice were assayed to determine the OVA-specific IgG titer by ELISA. Data are expressed as mean ± SE of results from 4 animals.

Table 24 OVA-specific IgG titer by transcutaneous vaccination with OVA (50 μg) plus adjuvant candidates

Candidates	Dose (μg)	ID	IgG titer	
			Day 13	Day 27
Non	–	1	< 5	12.5
		2	6.2	13.8
		3	13.9	17.9
		4	11.5	15.5
		5	< 5	15.1
		6	< 5	16.6
		7	< 5	12.5
		8	< 5	15.7
		Ave.	–	**15.0**
Pam3CSK4	10	1	< 5	12.9
		2	< 5	15.3
		3	11.6	17.5
		4	< 5	14.7
		5	< 5	15.1
		Ave.	–	**15.1**
Poly (I:C)	10	1	< 5	18.5
		2	< 5	16
		3	< 5	16.3
		4	< 5	14.7
		5	10.8	16.3
		Ave.	–	**16.4**
ODN 1826	10	1	5	18.3
		2	13.5	20.1
		3	18.2	19.9
		4	14.6	18.2
		5	14.3	22.2
		Ave.	**13.1**	**19.8**
LTA-SA	10	1	< 5	15.7
		2	< 5	17.4
		3	< 5	14.2
		4	< 5	17.9
		5	< 5	13.6
		Ave.	–	**15.8**
Imiquimod	10	1	< 5	17.5
		2	< 5	16.8
		3	< 5	17.9
		4	< 5	13.6
		5	5.4	11.5
		Ave.	–	**15.5**
R848	10	1	5	13.2
		2	< 5	16.8
		3	< 5	14.5
		4	< 5	16.1
		5	< 5	16
		Ave.	–	**15.4**

Candidates	Dose (μg)	ID	IgG titer	
			Day 13	Day 27
Non (10% DMSO)	-	1	< 5	12.1
		2	< 5	13.4
		3	5.6	13.8
		4	< 5	11.9
		5	< 5	12
		6	< 5	15.6
		Ave.	–	**13.1**
MPLA (10% DMSO)	5	1	6	17.5
		2	< 5	16.8
		3	10	17.2
		4	< 5	14.7
		5	12.3	19.1
		Ave.	–	**17.1**
ATRA (10% DMSO)	5	1	< 5	15.9
		2	< 5	17.8
		3	5.7	15.5
		4	5.1	15.8
		5	< 5	15.7
		Ave.	–	**16.1**

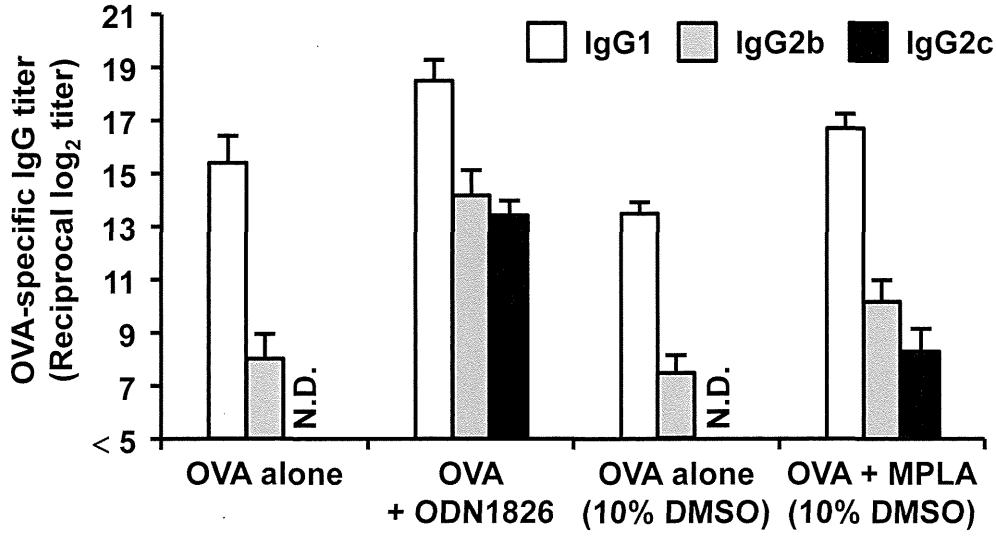


Fig. 43 OVA-specific IgG subclass analysis in mice immunized transcutaneously with OVA plus adjuvant candidate. C57BL/6 mice were transcutaneously immunized with 50 μg OVA alone or combined with either ODN1826 or MPLA by using puncturing method. Two weeks later, mice were immunized again with 50 μg OVA alone by using puncturing method. Two weeks after 2nd immunization, OVA-specific IgG subclass (IgG1, IgG2b, IgG2c) titer in sera collected from these mice were determined by ELISA. Data are expressed as mean ± SE of results from 5 mice. N.D.; not detected.

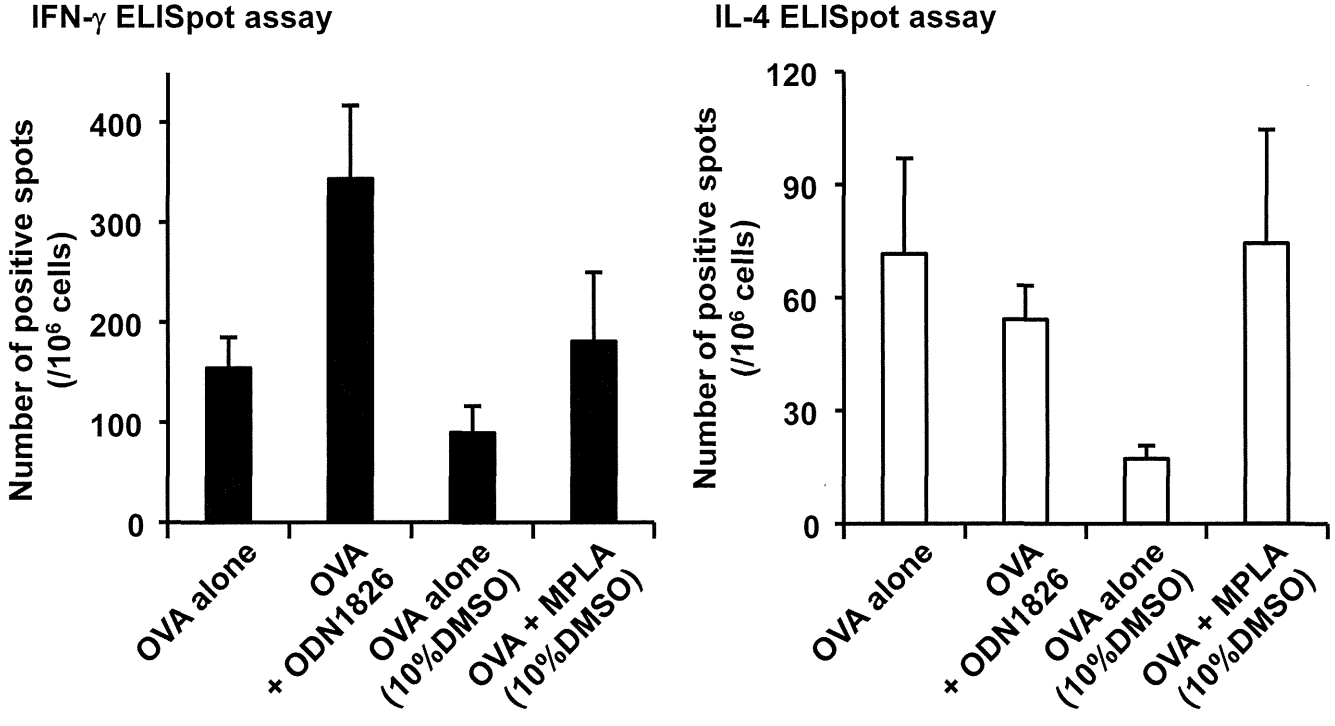


Fig. 44 ELISpot assay in splenocytes from mice immunized transcutaneously with OVA plus adjuvant candidate. C57BL/6 mice were transcutaneously immunized with 50 μg OVA alone or combined with either ODN1826 or MPLA by using puncturing method. Two weeks later, mice were immunized again with 50 μg OVA alone by using puncturing method. Two weeks after 2nd immunization, splenocytes prepared from these mice were cultured in media containing 1 mg/mL OVA for 24 h, and then ELISpot assay was performed in re-stimulated splenocytes to measure the number of OVA-specific IFN-γ- or IL-4-producing cells. Data are expressed as mean ± SE of results from 5 mice.

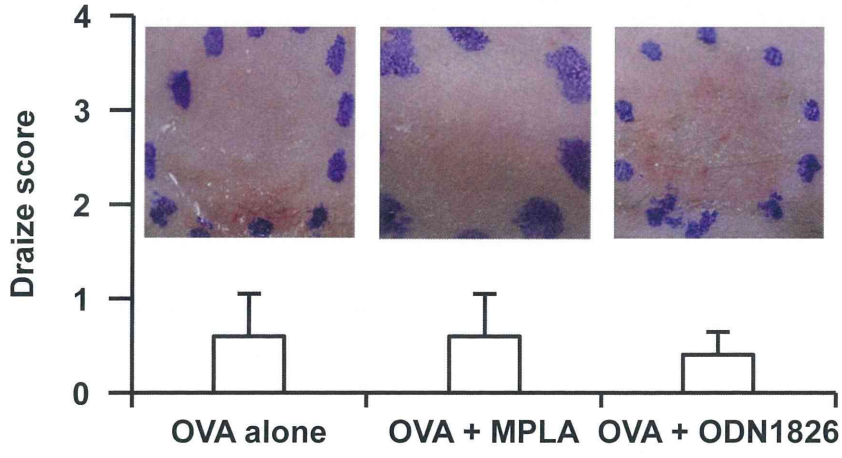


Fig. 45 Assessment of skin irritation in mice immunized transcutaneously with OVA plus adjuvant candidate. C57BL/6 mice were transcutaneously immunized with 50 μg OVA alone or combined with either ODN1826 or MPLA by using puncturing method. Immediately after removal of the hydrogel patch, the application site was observed and the degree of erythema on the skin of mice was scored using the Draize scoring system. Data are expressed as mean ± SE of results from 5 mice.

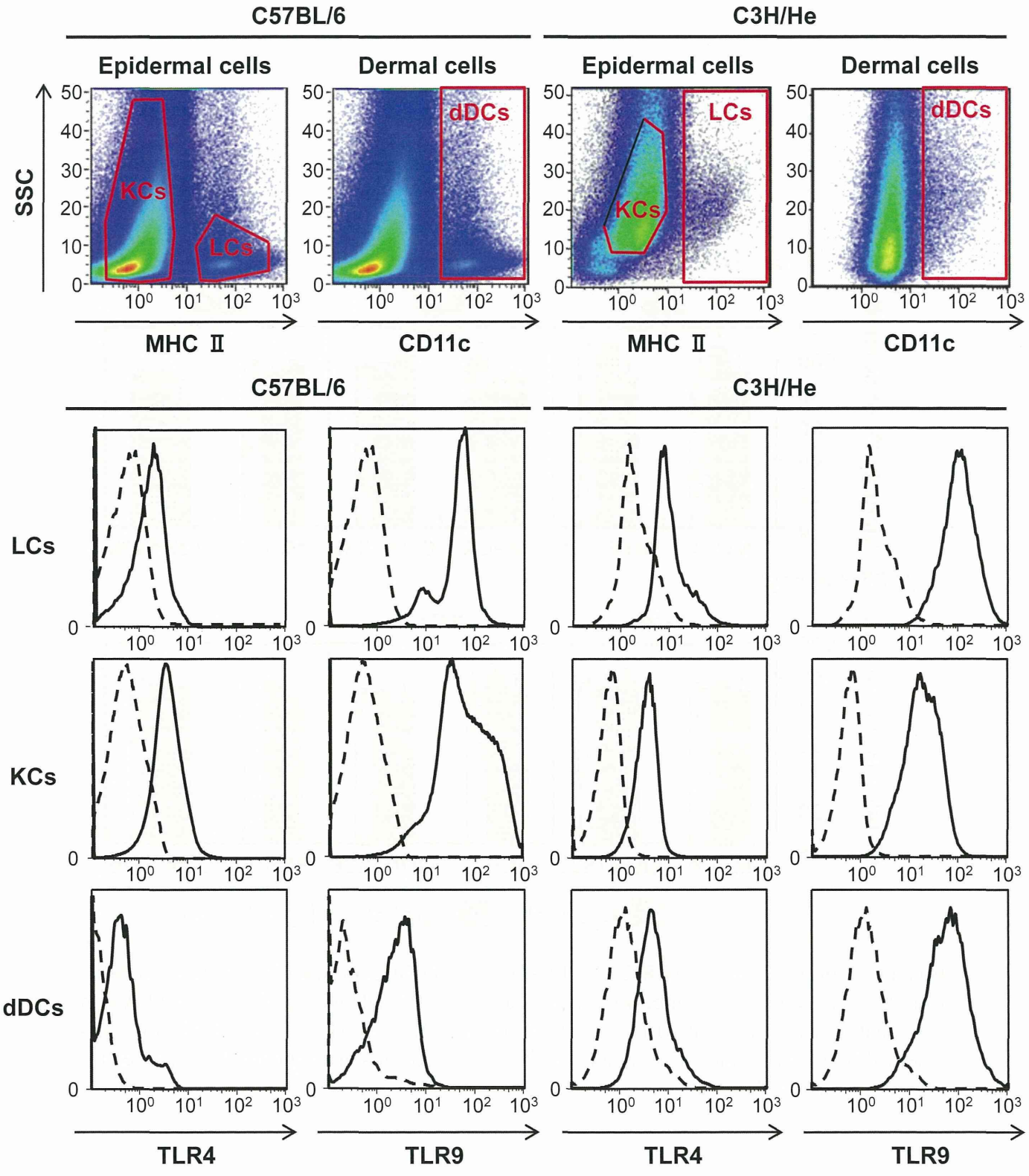


Fig. 46 Expression of TLR4 and TLR9 on LCs, KCs, and dDCs. Epidermal cells and dermal cells isolated from the skin of C57BL/6 or C3H/He mice were stained with anti-MHC class II, anti-CD11c, anti-TLR4, and anti-TLR9 antibodies. For intracellular detection of TLRs, cell fixation-permeabilization was performed before staining with antibodies. LCs, KCs, and dDCs were gated by MHC class II and CD11c expression, and were analyzed for the expression of TLR4 and TLR9 by flow cytometry. Dashed and solid histograms represent non-staining and staining groups, respectively.

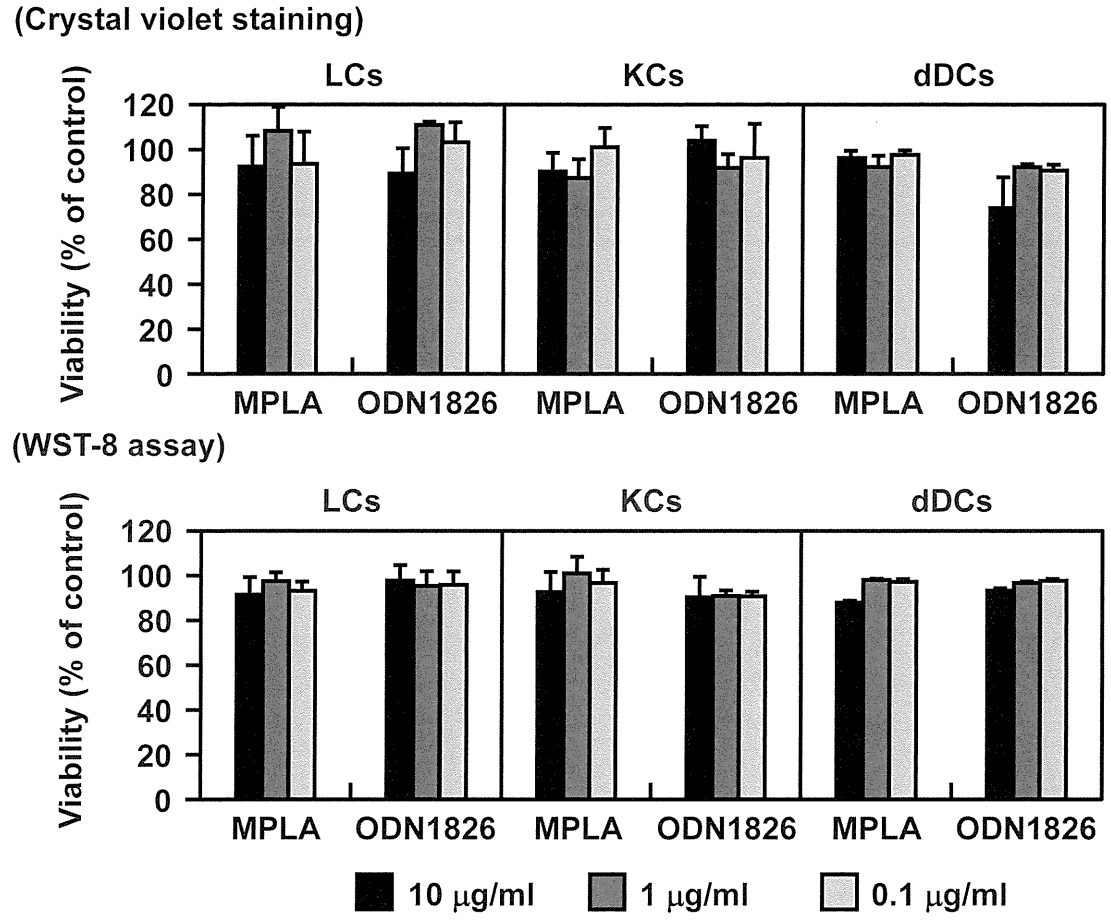


Fig. 47 Cytotoxic effect of TLR-Ls for LCs, KCs and dDCs. LCs, KCs, and dDCs isolated from the skin of C57BL/6 mice were cultured at 1×10^4 cells/well with 10 μg/ml OVA in combination without or with 0.1, 1, or 10 μg/ml each TLR-L. After 24 h, cell viability was assessed by crystal violet staining and WST-8 assay. Results are expressed as the mean ± S.E. of triplicate cultures.

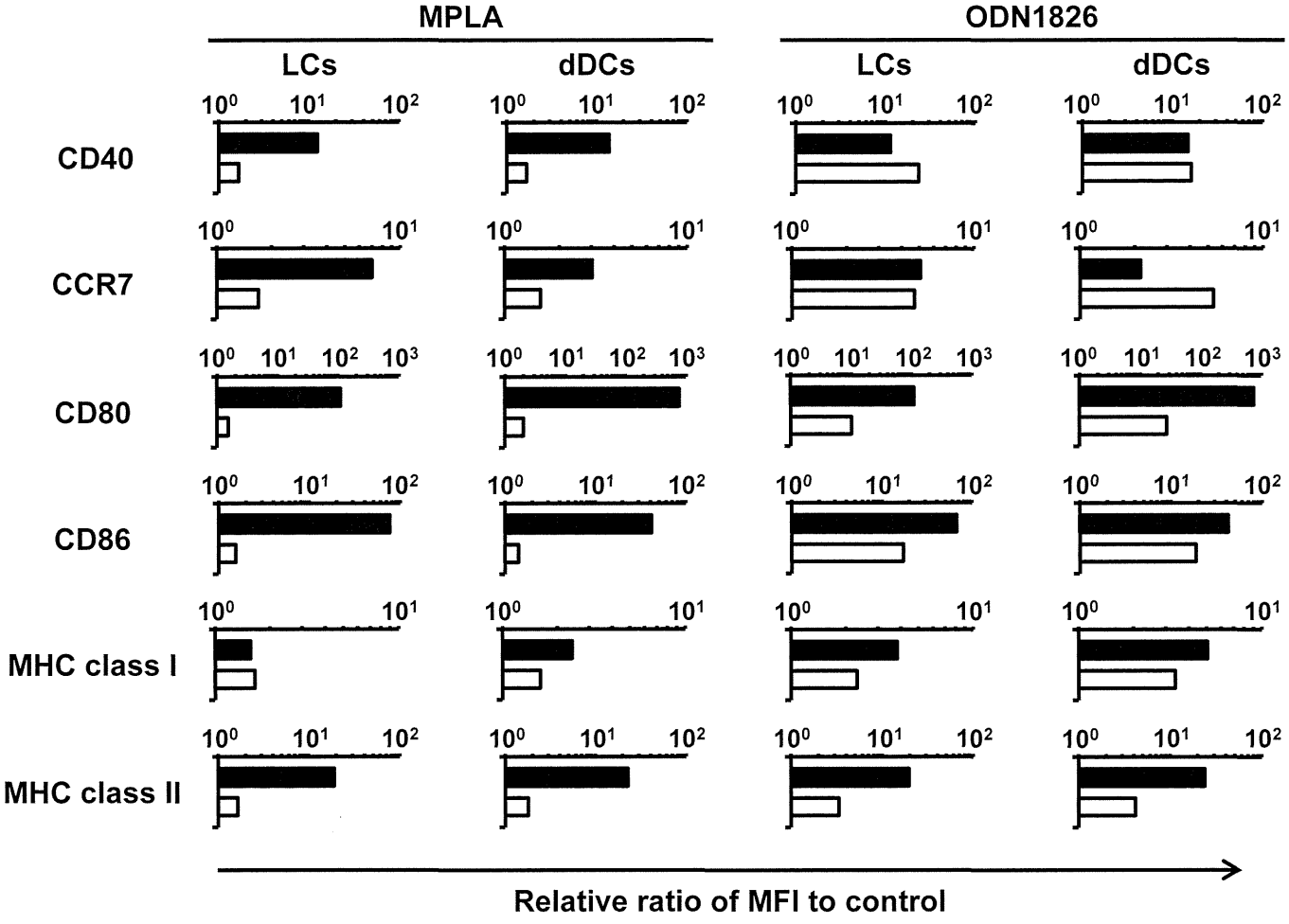


Fig. 48 Alteration of surface marker expression on LCs and dDCs by stimulation with TLR-Ls. LCs and dDCs isolated from C57BL/6 (solid column) or C3H/He (open column) mice were cultured at 5×10^5 cells/well with or without (control) 10 μg/ml MPLA or ODN1826. After 24-h cultivation, cells were stained with mAbs of the indicated specificities and analyzed by flow cytometry.