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Polyfunctional T Cell and Neutralizing Antibody Responses to ACAM2000™ Smallpox Vaccine Immunization in Primary-Vaccinated Individuals

Suchada Sukhumvittaya[1*], Silawun Ampol[1*], Kovit Pattanapanyasat[2], Wannee Kantakamalakul[1#]

[1]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[2]Center of Excellence for Flow Cytometry, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
Email: [#]wannee.kan@mahidol.ac.th

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Abstract

Smallpox eradication was successful via prophylactic administration of live attenuated vaccinia virus. As a result of the discontinuation of the smallpox immunization program, many individuals are now susceptible to smallpox virus infection should it be used as a biological weapon. Presently, only individuals at high risk for exposure are required to receive smallpox vaccine, such as laboratory personnel that handle variola/vaccinia virus. This study endeavored to investigate a one-year period of vaccinia virus-specific T cell responses using polychromatic flow cytometry and neutralizing (Nt) antibody responses using plaque reduction neutralization test (PRNT) in individuals receiving primary immunization (n = 5) with ACAM2000™ smallpox vaccine. Functional and phenotypic profiles of vaccinia virus-specific T cell responses were characterized. Each single functional measurement {CD107a/b expression, production of interferon γ (IFN-γ), macrophage inflammatory protein 1β (MIP-1β), interleukin 2 (IL-2), and tumor necrosis factor α (TNF-α)} demonstrated that vaccinia virus-specific CD8$^+$ T cells were functional at least one time point after vaccination ($p \leq 0.05$). However, vaccinia virus-specific CD4$^+$ T cells were functional only for MIP-1β production ($p \leq 0.05$). Vaccinia virus-specific CD8$^+$ T cells induced in these individuals showed increased polyfunctionality in at least 2 phenotypes relative to pre-vaccination ($p \leq 0.05$). Although only three of five individuals (60%) showed positive Nt antibody (titer ≥ 20) at first month after vaccination, all five individuals (100%) demonstrated Nt antibody at 2 months, post-immunization. Interestingly, all vaccinees could retain the Nt antibody for 6 months after primary vacci-

[*]S.S. and S.A. contributed equally to this article.
[#]Corresponding author.

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nation. In conclusion, ACAM2000™ smallpox vaccine induced both polyfunctional T cell-and Nt antibody-responses in primary immunized individuals.

Keywords

Smallpox Vaccine, Primary Immunization, T Cell, Neutralizing Antibody

1. Introduction

The smallpox vaccine is considered the gold standard of all vaccines, since its use led to the complete eradication of the disease from the human population. Although routine vaccination against smallpox ended globally in around 1972, smallpox vaccine has recently been given to some scientists and medical professionals who work with variola and related viruses. Less virulent replication-competent vaccinia viruses and attenuated replication-defective vaccinia viruses, such as Dryvax, Modified Vaccinia Ankara (MVA), and NYVAC have been used for smallpox vaccination [1]. ACAM2000™ is a single plaque-purified vaccinia virus derivative of Dryvax, with similar induction of immunity [2]. Vaccinia virus-containing smallpox vaccines elicit strong humoral and cellular immune responses that confer protective immunity against variola virus for decades after immunization [3] [4]. However, a detailed understanding of human immune responses to poxviruses is still incomplete. Smallpox vaccination provides a high level of T cell immune responses for up to 5 years [5] and neutralizing (Nt) antibodies at protective levels for up to 80 years [6]. The breadth of the vaccinia-specific effector T-cell repertoire at the single cell level has been demonstrated using polychromatic flow cytometry and intracellular cytokine staining (ICS). Subjects who received MVA and Dryvax vaccines showed polyfunctionality of the "5^{+}" $CD8^{+}$ T cell population; specifically, CD107a/b, IFN-γ, MIP-1β, IL-2, and TNF-α [7]. In this study, we used this same advanced technique to investigate virus-specific T cell response profiles and PRNT to examine Nt antibody induced by ACAM2000™ smallpox vaccine in primary-immunized Thai individuals.

2. Materials and Methods

2.1. Subjects

This study was conducted from May 2009 to December 2010 and received approval from the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University. Five healthy vaccinia-naïve adults aged 30 - 47 years provided written informed consent. Volunteer participants received ACAM2000™ smallpox vaccine as a primary immunization. Vaccines were administered percutaneously (15 punctures) using a bifurcated needle at a dose of 1×10^{8} PFU/ml. All participants had a primary skin reaction indicating a successful vaccination. Blood samples were collected in acid citrate dextrose (ACD) anticoagulant tubes for PBMCs preparation and as clotted blood for serum preparation at pre-vaccination, 0.5, 1, 3, 6, and 12 months, post-vaccination.

2.2. Peripheral Blood Mononuclear Cells (PBMCs) and *in Vitro* Stimulation (IVS)

PBMCs were prepared by standard Ficoll-Hypaque method and cryopreserved in liquid nitrogen. Cells were thawed, resuspended at 2×10^{6} cells/ml with RPMI growth medium (RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate), and left overnight at 37˚C. Unattached cells were washed and stimulated with vaccinia virus vP1170, strain WR (Virogenetics Corp., Troy, NY, USA) at 5 pfu/cell at infected:uninfected ratio of 1:5 and incubated for 1.5 hours at 37˚C/5% CO_2. Recombinant human interleukin 7 (rhIL-7) (Roche Diagnostics, Indianapolis, IN, USA) was added to a final concentration of 300 U/ml. On day 7, interleukin-2 (IL-2) (Roche Diagnostics) was added to a final concentration of 200 U/ml. Effector cells were then tested for vaccinia virus-specific responses on days 14 - 21, following culture initiation.

2.3. Transformed B Lymphocytes (TBLs)

Transformed B lymphocytes (TBLs) were established from autologous PBMC of each volunteer, as previously

described [8]. Sixteen hours prior to assay, TBLs were infected with vP1170 at 5 pfu/cell, then adjusted to 5 × 10^4 cells/ml and used as target cells.

2.4. Antibody Panels for Polychromatic Flow Cytometry

The following antibody panel was used to independently quantify five individual T cell functions: CD3-Cy7 allophycocyanin (APC), CD8-Cy5.5PerCP, CD107a/b-FITC, IFN-γ-APC, MIP-1β-PE, IL-2-Cy7PE, TNF-α-Alexa700 (all from Becton Dickinson Biosciences (BDB), San Jose, CA, USA), and CD4-ECD (Beckman Coulter, Inc. Pasadena, CA, USA).

2.5. Intracellular Cytokine Staining (ICS)

Effector and target cells were resuspended in complete RPMI medium and adjusted to an effector:target (E:T) ratio of 50:1. Co-stimulatory antibodies (anti-CD28/CD49d, 1 µg/ml each; BDB), monensin (Golgistop, 0.7 µl/ml; BDB), brefeldin A (10 µg/ml; BDB), and anti-CD107a/b (BDB) were then added to all tubes. Cell suspensions were then incubated at 37˚C/5% CO_2 for 5 hours. Following stimulation, cells were washed with PBS and then permeabilized according to manufacturer's instructions (Cytofix/Cytoperm Kit; BDB). Cells were intracellularly stained with pretitered antibodies against CD3, CD4, CD8, cytokines, and chemokines. CD107a/b on cell surfaces was stained with specific antibody. Cells were subsequently washed in flow wash buffer (BDB) and fixed in PBS containing 1% paraformaldehyde. Cells were collected for 350,000 - 1,000,000 events per sample on an LSRII flow cytometer (BDB) configured to detect 8 fluorochromes. Analysis was performed using FlowJo software version 9.6.2 (TreeStar, Inc., Ashland, OR, USA).

2.6. Serum

All serum samples were heat-inactivated at 56˚C for 30 minutes, aliquoted, and stored at −20˚C until use. The negative-control serum sample was a non-vaccinated human pool serum. The positive-control serum sample was a post-vaccination serum sample.

2.7. Cell Line

Thymidine kinase (TK) cell line was used as susceptible cell to vaccinia virus. Cells were cultured at 37˚C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate.

2.8. Reference Virus

Vaccinia virus, vP1170 used in the assay was propagated in TK cells, frozen, and thawed for 3 freeze-thaw cycles. Vaccinia virus titer was then determined in term of plaque forming units (PFU)/ml. The virus was then stored at −80˚C until use.

2.9. Plaque Reduction Neutralization Test (PRNT)

Serial two-fold dilutions of sample sera and control sera were prepared in serum-free DMEM with antibiotics. Each dilution of sera was mixed with an equal volume of vaccinia virus, containing approximately 100 PFU. Serum-virus mixtures were incubated at 37˚C/5% CO_2 for 15 hours [9]. Back titration of viral control for each assay was prepared by mixing vaccinia virus diluted to 50, 100, and 200 PFU with equal volumes of DMEM. Overnight-prepared monolayer of TK cells in 24-well tissue culture plates were washed once with 2% DMEM. Serum-virus mixtures were then inoculated to the cell monolayer in duplicate and allowed to adsorb at 37˚C/5% CO_2 for 1 hour. Then, 2% DMEM was added and the plates were returned to the incubator for 48 hours. Tissue culture plates were stained with 1% crystal violet for 5 minutes and air-dried. Plaques in each well were manually counted by microscope. Total plaque count for each serum dilution was defined as mean plaque count of duplicate well. The endpoint for serum Nt antibody titer was defined as the reciprocal of the highest dilution of the serum with mean plaque count less than or equal to the 50% plaque reduction cutoff value. Sera that had Nt antibody titer > 160 were further diluted to ratios of 1:320, 1:640, 1:1280, and 1:2560 before re-testing.

2.10. Statistical Analysis

SPSS version 13.0 was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). Comparison of pre-vaccination and post-vaccination T cell responses by different time points was performed using paired-samples t-test. Descriptive statistics were used to analyze and present results from PRNT. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. ACAM2000™ Smallpox Vaccine Induces T Cell Functions Independently and Simultaneously in Primary-Vaccinated Individuals

Settings for polychromatic flow cytometry to measure five T cell functions (expression of surface CD107a/b and production of IFN-γ, MIP-1β, IL-2, and TNF-α) independently and simultaneously were performed using PBMCs from vaccinia-naïve individuals stimulated with staphylococcal enterotoxin B vs. unstimulated PBMCs (data not shown). Induction of vaccinia virus-specific $CD8^+$ and $CD4^+$ T cells from primary-vaccinated individuals (n = 5) at different time points following immunization was demonstrated. Each of five functions of $CD8^+$ T cells at least one time point (1, 3, 6, and 12 months, post-vaccination) showed significantly higher responses, compared to pre-vaccination ($p \leq 0.05$). For example, CD107a/b expression of $CD8^+$ T cells demonstrated significantly higher at 3 time points (1, 3, and 6 months post-vaccination) at p-value of 0.025, 0.015, 0.019, respectively. Results of $CD8^+$ T cells showed a predominance of CD107a/b expression, with a hierarchy of CD107a > MIP-1β > IFN-γ > TNF-α > IL-2 (**Table 1**, **Figure 1(a)**). For the $CD4^+$ T cell subset, only production of MIP-1β was significantly higher as shown at 3 time points (1, 3, and 6 months post-vaccination) at p-value of 0.006, 0.006, 0.030, respectively, when compared to that of pre-vaccination (**Table 1**, **Figure 1(a)**).

Quality of T cell responses was assessed by simultaneous measurement of all five functions. $CD4^+$ T cells showed higher responses for only 2 phenotypes (p-value of 0.024) at one time point, 6 months post-vaccination (**Table 2**, **Figure 1(b)**). Polyfunctional $CD8^+$ T cells, however, showed significantly higher responses in at least 2 phenotypes (p-value of 0.027, 0.042, 0.040) at three time points (1-, 3- and 6-month post-vaccination) as compared to pre-vaccination (**Table 2**, **Figure 1(c)**). Moreover, these $CD8^+$ T cells also demonstrated the higher responses for 3 phenotypes (p-value of 0.022 and 0.047) and 5 phenotypes (p-value of 0.037) at two time points (6- and 12-month) and one time points (12 months) post-vaccination, respectively.

3.2. ACAM2000™ Smallpox Vaccine Induces Neutralizing Antibody in Primary-Vaccinated Individuals

Using PRNT to measure Nt antibody responses of primary-vaccinated individuals to vaccinia virus, three of five (60%) individuals showed Nt antibody (at titers ≥ 20) at 1-month after vaccination (**Table 3**). However, other two individuals demonstrated Nt antibody at 2 months, post-immunization. One individual showed peak Nt antibody at 2 months, while the other four participants demonstrated peak Nt antibody at 3 months, post-vaccination. All 5 participants were able to sustain Nt antibody responses for 6 months. Interestingly, three vaccinees (60%) showed Nt antibody responses until 12 months after primary vaccination.

4. Discussion

Variola virus, the causative agent of smallpox, was declared eradicated in 1977 after a global immunization program using live vaccinia virus [10]. Thus, a live vaccinia vaccine approach was chosen for the vaccine production for the US Strategic National Stockpile. Up to now, ACAM2000™ has been replaced Dryvax for all smallpox vaccination in US since February 2008 [11]. The main advantage of live attenuated virus vaccines is that they mimic the natural infection and could induce a more balanced of both arms of immune responses than other vaccine types [12]. Moreover, the induced immunity is longer lasting. Immunologic correlates of protection against smallpox following primary vaccination are not clearly defined but are likely to involve a combination of humoral and cell-mediated immune responses [13].

Thailand has been certified to be free of smallpox since December 1978 [14]. Currently, only specialized laboratory workers receive the smallpox vaccine. Thus, only 5 vaccinia-naïve volunteers have been involved in this study. They received ACAM2000™, a single clonal derivative of Dryvax vaccine as a primary immunization.

Table 1. Mean values of net percent positive of each functional response of $CD4^+$ and $CD8^+$ T cells, demonstrated as markers: CD107a/b, IFN-γ, MIP-1β, IL-2, and TNF-α at single-cell level of primary vaccinated-individuals (n = 5). Statistical differences between mean values at pre-vaccination, 1, 3, 6, and 12 months after immunization were assessed by paired-samples t-test.

Visit	Statistic	Net % positive of each functional response of $CD4^+$ T cells					Net % positive of each functional response of $CD8^+$ T cells				
		CD107a/b	IFN-γ	MIP-1β	IL-2	TNF-α	CD107a/b	IFN-γ	MIP-1β	IL-2	TNF-α
Pre-vac	Mean ± SD	0.002 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.006 ± 0.013	0.000 ± 0.000	0.010 ± 0.022	0.000 ± 0.000	0.002 ± 0.004	0.000 ± 0.000
	Median (min - max)	0.00 (0.00 - 0.01)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.03)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.05)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.01)	0.00 (0.00 - 0.00)
1 month	Mean ± SD	1.028 ± 1.171	0.342 ± 0.759	2.954 ± 1.218	0.560 ± 1.230	2.250 ± 3.363	30.620 ± 19.545	5.502 ± 7.423	24.128 ± 20.056	0.576 ± 0.860	3.286 ± 4.052
	Median (min - max)	0.33 (0.06 - 2.59)	0.00 (0.00 - 1.70)	3.38 (1.27 - 4.28)	0.00 (0.00 - 2.76)	1.13 (0.00 - 8.19)	24.15 (11.41 - 57.07)	2.26 (0.90 - 18.49)	33.24 (1.27 - 42.91)	0.15 (0.02 - 2.08)	1.40 (0.58 - 10.40)
	p-value	0.122	0.371	0.006	0.366	0.211	0.025	0.174	0.055	0.21	0.144
3 months	Mean ± SD	0.606 ± 0.579	0.268 ± 0.588	2.670 ± 1.144	0.138 ± 0.303	1.160 ± 1.926	28.576 ± 15.683	5.022 ± 4.295	27.624 ± 22.110	0.168 ± 0.263	2.644 ± 1.996
	Median (min - max)	0.53 (0.12 - 1.54)	0.01 (0.00 - 1.32)	2.38 (1.68 - 4.65)	0.00 (0.00 - 0.68)	0.42 (0.00 - 4.57)	23.80 (8.67 - 48.88)	3.45 (0.73 - 10.77)	36.60 (1.06 - 48.37)	0.02 (0.01 - 0.62)	1.77 (0.84 - 5.61)
	p-value	0.081	0.366	0.006	0.366	0.252	0.015	0.06	0.049	0.231	0.041
6 months	Mean ± SD	0.364 ± 0.385	0.238 ± 0.450	2.906 ± 1.964	0.070 ± 0.105	1.408 ± 1.349	23.886 ± 13.982	3.872 ± 3.132	22.642 ± 18.693	0.096 ± 0.116	2.118 ± 1.467
	Median (min - max)	0.33 (0.06 - 1.01)	0.05 (0.00 - 1.04)	2.11 (1.31 - 5.85)	0.03 (0.00 - 0.25)	1.03 (0.00 - 3.44)	27.91 (5.76 - 42.37)	4.19 (0.32 - 8.44)	32.08 (1.32 - 43.14)	0.03 (0.00 - 0.28)	2.08 (0.66 - 3.75)
	p-value	0.103	0.302	0.03	0.209	0.081	0.019	0.051	0.054	0.134	0.032
12 months	Mean ± SD	0.502 ± 0.632	0.098 ± 0.120	2.598 ± 3.197	0.020 ± 0.045	0.668 ± 0.954	17.032 ± 14.319	1.364 ± 0.990	20.742 ± 19.239	0.084 ± 0.061	1.316 ± 0.869
	Median (min - max)	0.20 (0.01 - 1.51)	0.07 (0.00 - 0.29)	1.17 (0.54 - 8.16)	0.00 (0.00 - 0.10)	0.38 (0.00 - 2.30)	20.45 (0.23 - 37.09)	1.06 (0.16 - 2.79)	27.76 (0.14 - 43.81)	0.09 (0.00 - 0.15)	1.31 (0.14 - 2.55)
	p-value	0.152	0.143	0.143	0.374	0.191	0.056	0.038	0.073	0.034	0.028

We investigated whether the use of ACAM2000™ could induce T cell functions independently and/or simultaneously and also specific neutralizing antibody in these individuals. Despite our small sample size, $CD8^+$ T cells from these vaccinated individuals showed significantly higher virus-specific responses in both single function and polyfunctionality in phenotypes. These results were similar to those of Precopio, *et al*. [7]. They suggested that Dryvax offers the protection against smallpox infection because of its inherent ability to induce highly polyfunctional T cell responses. Our results emphasized their idea since ACAM2000™ is a derivative of Dryvax. However, Precopio, *et al*. reported higher frequency responses in individuals who were pre-immunized with two or three doses of MVA and challenged with Dryvax. The kinetics of each single function of $CD8^+$ T cells in our study peaked mostly at 1 month after immunization and then gradually declined, as previously reported [15] [16]. However, the persistence of virus-specific $CD8^+$ T cell functions was demonstrated up to at least 12 months after immunization.

For Nt antibody responses, serum with Nt antibody titer ≥ 20 was considered Nt antibody positive. This was based upon study reports from the smallpox pre-eradication period, which found that anti-vaccinia Nt antibody titer of ≥20 or ≥32 or a 4-fold increase was protective [17] [18]. In our study, sera from only three of five participants (60%) showed positive Nt antibody at one month post-immunization. Artenstein, *et al*. reported 94% of vaccinia naïve adults who received primary ACAM2000™ vaccination developed Nt antibody at one month post-vaccination [19]. Interestingly, Frey, *et al*. also demonstrated the induction of Nt antibody in 96.7% of naïve adults but they measured at 45 days after vaccination [20]. However, sera from the other 2 participants in our study revealed positive Nt antibody at 2 months, post-vaccination. Unfortunately, their sera at 1.5 months (45 days) post-vaccination were not available. These 2 volunteers had a major cutaneous reaction as the other 3

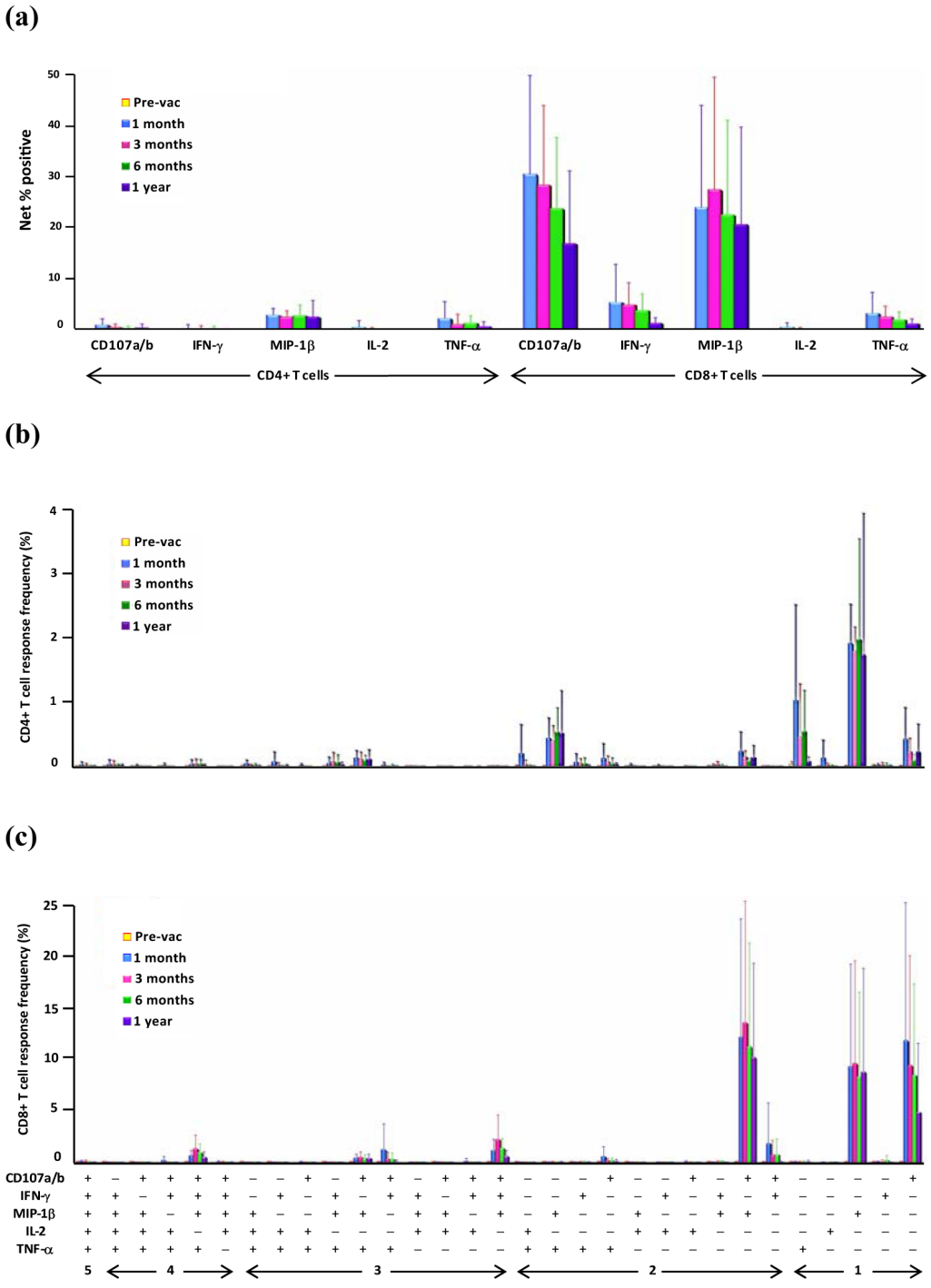

Figure 1. Functional and phenotypic profiles of vaccinia virus-specific T cell responses of primary vaccinated individuals (n = 5) at pre-vaccination, 1, 3, 6, and 12 month post-immunization. (a) Net % positive responses of each function of $CD4^+$ T cells (left panel) and $CD8^+$ T cells (right panel). (b), (c) Response frequency (%) of functional composition of $CD4^+$ T cells and $CD8^+$ T cells, respectively. Every possible combination of response is shown on the x-axis. Graph data is presented as mean values ± SD.

volunteers who had positive Nt antibody at one month post vaccination. This demonstrated that local viral replication in the skin and development of a pock may not accompany well by a systemic humoral immune response in some individuals. As such, the time needed to evaluate whether the ACAM2000™ smallpox vaccine was

Table 2. Mean values of frequency (%) of polyfunctional response of $CD4^+$ and $CD8^+$ T cells demonstrated by simultaneous response of markers: CD107a/b, IFN-γ, MIP-1β, IL-2, and TNF-α at single-cell level of primary vaccinated-individuals (n = 5). Statistical differences between mean values at pre-vaccination, 1, 3, 6, and 12 months after immunization were assessed by paired-samples t-test.

Visit	Statistic	$CD4^+$ T cell response frequency (%)					$CD8^+$ T cell response frequency (%)				
		5+	4+	3+	2+	1+	5+	4+	3+	2+	1+
Pre-vac	Mean ± SD	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.004	0.006 ± 0.009	0.038 ± 0.055	0.002 ± 0.004	0.000 ± 0.000	0.008 ± 0.018	0.006 ± 0.013	0.016 ± 0.036
	Median (min - max)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.01)	0.00 (0.00 - 0.02)	0.00 (0.00 - 0.12)	0.00 (0.00 - 0.01)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.04)	0.00 (0.00 - 0.03)	0.00 (0.00 - 0.08)
1 month	Mean ± SD	0.022 ± 0.049	0.092 ± 0.195	0.336 ± 0.514	1.120 ± 1.180	3.548 ± 2.222	0.090 ± 0.084	0.932 ± 0.773	3.004 ± 2.905	14.786 ± 9.650	21.334 ± 8.978
	Median (min - max)	0.00 (0.00 - 0.11)	0.01 (0.00 - 0.44)	0.12 (0.03 - 1.25)	0.70 (0.17 - 3.16)	2.95 (1.13 - 7.13)	0.06 (0.01 - 0.18)	0.79 (0.18 - 1.87)	2.00 (0.57 - 7.61)	12.83 (1.43 - 27.26)	21.08 (9.90 - 34.83)
	p-value	0.374	0.35	0.221	0.103	0.026	0.082	0.054	0.083	0.027	0.006
3 months	Mean ± SD	0.016 ± 0.036	0.072 ± 0.144	0.240 ± 0.353	0.730 ± 0.600	2.552 ± 1.319	0.094 ± 0.141	1.394 ± 1.416	3.146 ± 2.856	14.652 ± 11.128	19.236 ± 8.326
	Median (min - max)	0.00 (0.00 - 0.08)	0.01 (0.00 - 0.33)	0.08 (0.06 - 0.87)	0.45 (0.42 - 1.80)	1.88 (1.71 - 4.83)	0.01 (0.00 - 0.33)	1.20 (0.10 - 3.78)	2.56 (0.38 - 7.88)	16.42 (1.01 - 29.77)	21.73 (7.75 - 29.15)
	p-value	0.374	0.328	0.207	0.055	0.014	0.217	0.093	0.07	0.042	0.007
6 months	Mean ± SD	0.012 ± 0.016	0.066 ± 0.099	0.216 ± 0.237	0.758 ± 0.480	2.682 ± 1.379	0.054 ± 0.067	0.998 ± 0.863	2.124 ± 1.302	12.414 ± 9.226	17.184 ± 7.180
	Median (min - max)	0.01 (0.00 - 0.04)	0.04 (0.00 - 0.24)	0.21 (0.00 - 0.60)	1.05 (0.22 - 1.18)	2.6 (1.34 - 4.61)	0.01 (0.01 - 0.16)	0.84 (0.09 - 2.28)	2.73 (0.22 - 3.23)	12.54 (0.87 - 22.44)	18.85 (5.30 - 23.95)
	p-value	0.178	0.21	0.114	0.024	0.011	0.137	0.061	0.022	0.04	0.006
12 months	Mean ± SD	0.006 ± 0.009	0.032 ± 0.044	0.180 ± 0.170	0.714 ± 0.752	2.074 ± 2.313	0.042 ± 0.032	0.546 ± 0.515	1.130 ± 0.881	10.476 ± 9.062	13.818 ± 11.298
	Median (min - max)	0.00 (0.00 - 0.02)	0.00 (0.00 - 0.09)	0.14 (0.01 - 0.46)	0.45 (0.17 - 2.01)	0.75 (0.36 - 5.79)	0.05 (0.00 - 0.08)	0.45 (0.07 - 1.37)	1.08 (0.06 - 2.31)	15.51 (0.07 - 18.11)	13.26 (0.08 - 26.08)
	p-value	0.208	0.182	0.082	0.103	0.116	0.037	0.077	0.047	0.061	0.052

Table 3. Serum neutralizing antibody titers among primary vaccinated individuals over a one-year period following vaccination.

Subject	Gender	Pre-vac (day 0)	Post-vaccination at month					
			0.5^{th}	1^{st}	2^{nd}	3^{rd}	6^{th}	12^{th}
1	F	<10	10	80	160	1280	320	20
2	F	<10	<10	<10	80	160	40	<10
3	M	<10	10	160	320	160	160	160
4	F	<10	<10	40	40	320	80	<10
5	F	<10	ND	<10	40	160	160	160

effective in inducing protective humoral immunity in primary vaccination may need to be longer than the usual performed at one month time period. Nt antibody titers of most volunteers peaked at 3 months, post-vaccination. On the other hand, Walsh *et al*. reported that peak Nt antibody titers occurred on day 42 (14 days after second immunization) since they used 2 doses of MVA, a replication defective strain of vaccinia virus [21]. Persistence of protective Nt antibody was demonstrated for up to at least 6 months after primary vaccination in all participants. Interestingly, 3 of 5 vaccinees (60%) successfully maintained Nt antibody for up to 12 months, post-vaccination. Moreover, 2 of these 3 vaccinees were able to sustain Nt antibody for 18 months, post-vaccination (data not shown). In the case of a bioterrorism, one dose of primary vaccination should be enough to induce protective immunity among naïve individuals, unless a more than sufficient supply of vaccine is available.

Genetic factors may influence differences in vaccine response. Gender was significantly associated with vari-

ations in Nt antibody development after smallpox vaccination, with female shaving a significantly higher Nt antibody titer than males [22]. On the other hand, Troy *et al*, reported higher PRNT titers in men after vaccination [23]. In our study, Nt antibody titer between males and females were similar. However, the male participant showed faster Nt antibody peak (at month 2, post-vaccination) than the Nt antibody peak of the 4 female participants (at month 3, post-vaccination).

5. Conclusion

In conclusion, although the sample size of this study was extremely small for human study, our data demonstrated that primary immunization by ACAM2000™ smallpox vaccine in naïve individuals would be sufficient for induction of both single & polyfunctional $CD8^+$ T cells and Nt antibody responses. Persistence of both immune responses was at least 6 months post-immunization.

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Conflict of Interest

The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study.

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