

# Residual Immunity from Smallpox Vaccination and Possible Protection from Mpox, China

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Among persons born in China before 1980 and tested for vaccinia virus Tiantan strain (VVT), 28.7% (137/478) had neutralizing antibodies, 71.4% (25/35) had memory B-cell responses, and 65.7% (23/35) had memory T-cell responses to VVT. Because of cross-immunity between the viruses, these findings can help guide mpox vaccination strategies in China.

On July 23, 2022, the World Health Organization declared the global mpox outbreak to be a public health emergency of international concern (<https://www.who.int/europe/news/item/23-07-2022-who-director-general-declares-the-ongoing-monkeypox-outbreak-a-public-health-event-of-international-concern>). No specific treatment is currently approved for mpox. Vaccines such as JYNNEOS (Bavarian Nordic, <https://www.bavarian-nordic.com>) and ACAM2000 (Emergent BioSolutions Inc., <https://www.emergentbiosolutions.com>) are available for preexposure protection from mpox (1), and tecovirimat can be used for patients who are at risk for severe disease (2).

Vaccinia virus Tiantan strain (VTT) was historically used for vaccines in the smallpox virus eradication campaign in China. Given the high level of sequence homology among their surface proteins,

smallpox vaccination provided ≈85% protection against mpox (3). Because the World Health Organization declared that smallpox had been eradicated and routine use of vaccinia vaccine was terminated in most countries by 1980–1981, most persons born after 1980 do not have vaccinia virus-elicited immunity. Vaccinia-derived protection wanes in the vaccinated population over time, which may lead to an increase in susceptibility to monkeypox virus (MPXV) infection because of cross-immunity between the 2 viruses.

After the first mpox case imported from Europe to mainland China on September 14, 2022 (4), investigation of the level of residual VTT-specific immunity in the population of China became pressing, as researchers assessed susceptibility to mpox and guided development of appropriate protective strategies. Different patterns of residual immunity against vaccinia suggest different strategies in responding to mpox transmission. However, levels of residual immunity to poxviruses in the population in China are not well assessed. We measured VTT-specific humoral and cellular immune responses in a diverse population born during 1930–2008 in China.

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**Table 1.** Characteristics of 1,070 participants in a cross-sectional cohort study to determine IgG titers against vaccinia virus Tiantan strain, China\*

Characteristic	Decade of birth, no. (%)							Total
	1930–1939	1940–1949	1950–1959	1960–1969	1970–1979	1980–1989	1990–2008	
Overall	106 (9.91)	104 (9.72)	76 (7.10)	95 (8.88)	97 (9.07)	84 (7.85)	508 (47.48)	1,070 (100)
Sex								
M	63 (5.89)	61 (5.70)	39 (3.64)	53 (4.95)	40 (3.74)	42 (3.93)	285 (26.64)	583 (54.49)
F	43 (4.02)	43 (4.02)	37 (3.46)	42 (3.93)	57 (5.33)	42 (3.93)	223 (20.84)	487 (45.51)

\*Participants lived in Beijing, Shanxi Province, Heilongjiang Province, Hubei Province, or Shenzhen.

## The Study

In this cross-sectional cohort study, we collected blood specimens from 1,070 healthy donors who lived in Beijing, Shanxi Province, Heilongjiang Province, Hubei Province, or Shenzhen during regular health check-ups. Among the participants (Table 1), 478 were born during 1930–1979 and 592 were born during 1980–2008; ages ranged from 1 month to 90 years. The study was approved by the institutional review boards of the Chinese Academy of Medical Sciences' Institute of Pathogen Biology (approval no. 2013-IPB-03, IPB-2021-15).

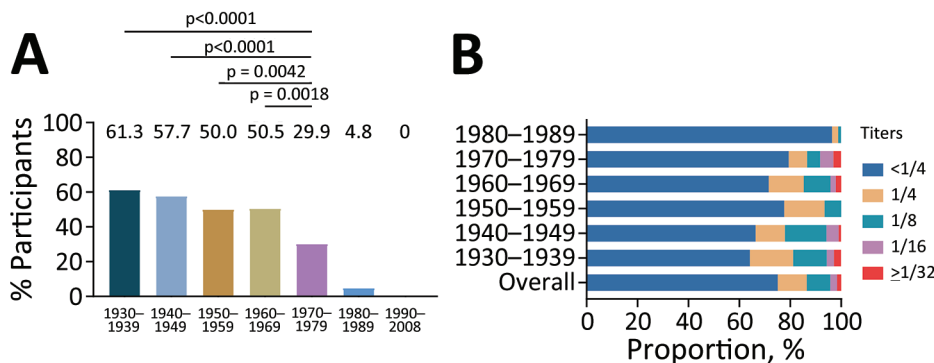
We tested serum samples from all participants to determine IgG titers against VTT by using ELISA. We performed a Gaussia luciferase-based vaccinia neutralization assay to determine the presence of neutralizing antibodies (NAbs). We performed memory B-cell and memory T-cell enzyme-linked immunospot (ELISpot) assays (Charles River Laboratories, <https://www.criver.com>) by using cryopreserved peripheral blood mononuclear cells (PBMCs). Because of insufficient PBMC samples, we evaluated memory B- and T-cell responses in a subgroup of the enrolled participants (Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/30/2/23-0542-App1.pdf>).

Overall VTT seropositivity was 50.2% (240/478) in participants born before 1980. Persons born during 1970–1979 had the lowest seropositivity, 29.9% (29/97), compared with 61.3% (65/106 [ $p < 0.0001$ ]) among persons born during 1930–1939, 57.7% (60/104 [ $p < 0.0001$ ]) among persons born during 1940–1949, 50.0% (38/76 [ $p = 0.0042$ ]) among persons

born during 1950–1959, 50.5% (48/95 [ $p = 0.0018$ ]) among persons born during 1960–1969. By comparison,  $\approx 4.8\%$  (4/84) participants born during 1980–1989 had VTT-specific IgG, and VTT-specific IgG was not detectable in persons born after 1990 (Figure 1, panel A). The VTT-specific IgG titers were not significantly different among participants born during 1930–1939, 1940–1949, 1950–1959, and 1960–1969 ( $p = 0.11$ ), but all were higher than in persons born during 1970–1979 (Appendix Figure 2, panel A).

We examined distribution of NAb levels in relation to year of birth (Table 2; Figure 1, panel B). Of the 478 serum samples from persons born before 1980, most (341 [71.3%]) had an NAb titer of  $< 1/4$ . Of the remaining samples, NAb titers were  $1/4$  for 62 (13.0%),  $1/8$  for 51 (10.7%),  $1/16$  for 15 (3.1%), and  $1/32$  for 9 (1.9%), suggesting the lack or low titers of NAb against VTT. VTT NAbs were detectable in 35.8% (38/106) of persons born during 1930–1939, 33.7% (35/104) born during 1940–1949, 22.4% (17/76) born during 1950–1959, 28.4% (27/95) born during 1960–1969, and 20.6% (20/97) born during 1970–1979 but were detectable in only 3.6% (3/84) born during 1980–1989 ( $\geq 1/4$ ) (Appendix Figure 2, panel B). We observed a significant correlation between NAb and IgG titers in persons born before 1990 (Spearman  $r = 0.54$ ;  $p < 0.0001$ ) (Appendix Figure 2, panel C).

We measured VTT-specific memory B-cell responses in 45 participants whose PBMCs were isolated successfully (Appendix Figure 3). Approximately 71.4% (25/35) of persons born before 1980 showed VTT-specific memory B-cell responses; positivity



**Figure 1.** Serum IgG and neutralizing antibody responses against vaccinia virus Tiantan strain (VTT) among 1,070 participants in a cross-sectional cohort study, China. A) Seropositivity of VTT-specific IgG by birth cohort in 1,070 persons born during 1930–2008, conducted with  $\chi^2$  or Fisher exact test as appropriate. B) Prevalence of neutralizing antibody by birth cohort in 562 persons born before 1990.

**Table 2.** Neutralizing antibody titers against vaccinia virus Tiantan strain in persons born during 1930–1979, by birth cohort, China\*

Decade of birth	Neutralizing antibody titers, no. (%)				
	<1/4	1/4	1/8	1/16	>1/32
1930–1939, n = 106	68 (64.2)	18 (17.0)	14 (13.2)	3 (2.8)	3 (2.8)
1940–1949, n = 104	69 (66.3)	12 (11.5)	17 (16.3)	5 (4.8)	1 (0.96)
1950–1959, n = 76	59 (77.6)	12 (15.8)	5 (6.6)	0 (0)	0 (0)
1960–1969, n = 95	68 (71.6)	13 (13.7)	10 (10.5)	2 (2.1)	2 (2.1)
1970–1979, n = 97	77 (79.4)	7 (7.2)	5 (5.2)	5 (5.2)	3 (3.1)
Overall, N = 478	341 (71.3)	62 (13.0)	51 (10.7)	15 (3.1)	9 (1.9)

\*Participants lived in Beijing, Shanxi Province, Heilongjiang Province, Hubei Province, or Shenzhen.

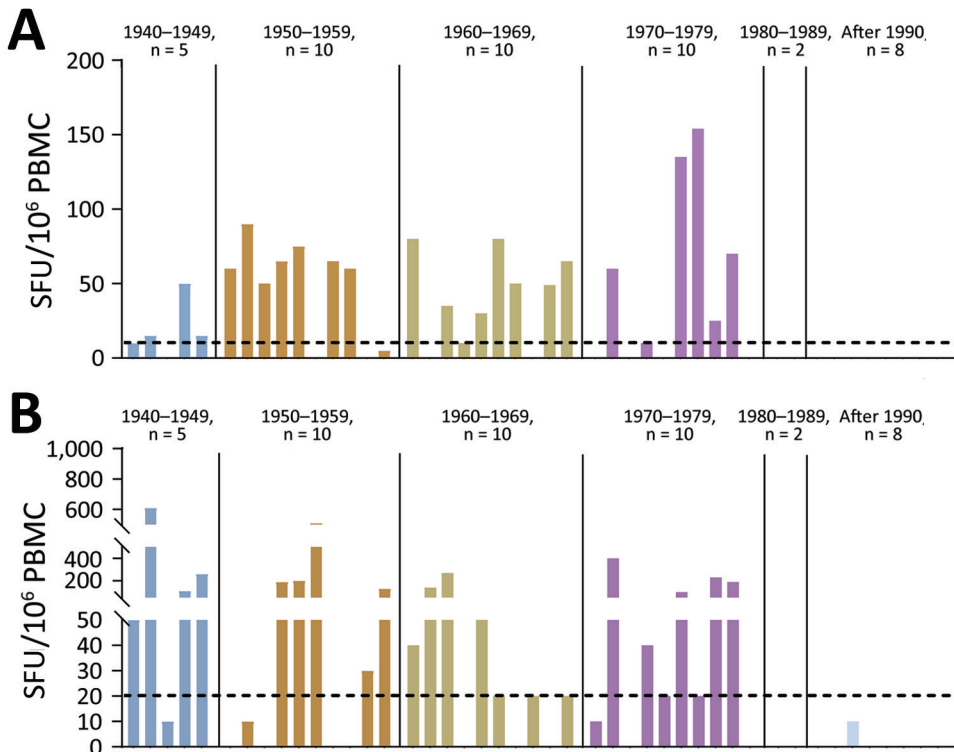
across the 4 birth decades was 80% (4/5) for 1940–1949, 70% (7/10) for 1950–1959, 80% (8/10) for 1960–1969, and 60% (6/10) for 1970–1979. PBMCs of all persons born after 1980 were negative for VTT-specific memory B-cells (Figure 2, panel A). We observed no significant correlations between VTT-specific memory B-cell magnitude and VTT IgG (Appendix Figure 4, panel A) or NAb (Appendix Figure 4, panel B) titers.

We further evaluated interferon- $\gamma$  (IFN- $\gamma$ ) responses to VTT in the same 45 participants (Appendix Figure 5). We detected VTT-specific memory T-cell responses in 65.7% (23/35) of persons across the 4 birth decades, distributed as 80% (4/5) for 1940–1949, 50% (5/10) for 1950–1959, 70% (7/10) for 1960–1969, and 70% (7/10) for 1970–1979. In contrast, T-cell IFN- $\gamma$  responses were below the detection limit in the 10 persons born after 1980 (Figure 2, panel B). We observed no correlations between the magnitude of VTT-specific memory T-cell responses and IgG (Appendix Figure 6, panel A) or NAb (Appendix Figure 6, panel B) titers.

As a control, we tested for influenza virus and Epstein Barr virus-specific memory T-cell responses, which we detected in persons born during 1940–2008 (Appendix Figure 7, panel A). Among the 35 persons born before 1980 and found to be positive for specific cellular immune responses, 28 (80%) had no detectable NAb (<1/4). However, 67.9% (19/28) persons showed IFN- $\gamma$  responses in the ELISpot assay (Appendix Figure 7, panel B).

## Conclusions

We evaluated residual VTT immunity in the population of China across >5 birth decades. Our and other studies suggest that antibody responses against vaccinia virus after vaccination can be long-lived (5–7). We observed a low prevalence (28.7% [137/478]) of NAb against VTT in persons born before 1980, which is consistent with a previous study in the population of China (8). Our data demonstrate that 71.4% of the 35 tested participants



**Figure 2.** Vaccinia virus-specific memory B- and T-cell responses among 45 participants in a cross-sectional cohort study to determine IgG titers against vaccinia virus Tiantan strain (VTT), China. A) Magnitude of memory B-cell responses against VTT for each person. B) Magnitude of interferon- $\gamma$  T-cell responses against VTT for each person. Dotted lines indicate detection limit of assay. PBMC, peripheral blood mononuclear cells; SFU, spot-forming units.

born before 1980 had VTT-specific memory B-cell responses. Those memory B-cells can still rapidly differentiate into plasma cells and produce protective antibodies upon reinfection (9).

Smallpox vaccine-induced antibodies may protect against MPXV (10). Approximately 65.7% of the 35 participants born before 1980 that we tested had VTT-specific T-cell responses, which is consistent with previous reports that T-cell responses against vaccinia virus were maintained up to 51–75 years postimmunization and had a half-life of 8–15 years (5,7).

One limitation of our study is that it is a cross-sectional study. In addition, no information regarding smallpox vaccination or smallpox infection was available for the persons enrolled. Moreover, a small number of samples were tested for T- and B-memory cell responses.

In summary, we evaluated residual immune responses to VTT in the population of China and found that >65% of 35 tested persons born before 1980 showed memory B- and T-cell responses. However, the prevalence and NAb titers against VTT were low in this population. To protect the population from infection by MPXV and any other related pathogenic orthopoxviruses, safe and effective vaccines will be needed for all age groups.

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Author contributions: J.W. and F.G. conceived and designed the study, had full access to all the data in the study, and take responsibility for the integrity of the data and the accuracy of the data analysis. Y.H., L.G., F.X., and S.M. did the literature review. L.G., Y.H., F.X., Y.L., L.R., T.H., J.Z., Z.F., Y.Z., Y.X., and Q.Z. did the laboratory analysis. L.G., Y.H., F.X., F.G., and J.W. drafted the paper. Y.X. and X.W. collected the data. L.G., Y.H., F.X., L.R., L.X., and J.N. verified the underlying data in the study.

All authors read and edited the manuscript. All authors approved the final version, had full access to all the data, and had final responsibility for the decision to submit for publication.

### About the Author

Dr. Huang is a postdoctoral fellow at the Institute of Pathogen Biology, Chinese Academy of Medical Sciences. Her primary research interests include antiviral innate immunity, epidemic pathogens infectious disease surveillance, and applying these research to the prevention of epidemics.

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## Appendix

### Methods

#### Study design and participants

In this cross-sectional cohort study, we recruited 1070 healthy donors (583 males, 487 females) (Table 1), who came from Beijing city (n = 393), Shanxi province (n = 355), Heilongjiang province (n = 158), Hubei province (n = 101), or Shenzhen city (n = 63), China during regular health check-ups. 384 samples (243 males, 141 females) were collected in 2008, 576 samples (280 males, 296 females) were collected in 2014–2015, and 110 samples (60 males, 50 females) were collected in 2020–2021. Among these healthy donors, 478 (256 males, 222 females) were born in the period of 1930 to 1979, and 592 (327 males, 265 females) were born in the period of 1980 to 2008.

Venous blood was collected from participants and processed to isolate serum for immunological testing. Among these blood samples, 45 were used to isolate both plasma and peripheral blood mononuclear cell (PBMCs). All serum samples were inactivated at 56°C for 30 min before use.

Written informed consent was obtained from each individual. The study was approved by the Institutional Review Boards of Institute of Pathogen Biology, Chinese Academy of Medical Sciences (2013-IPB-03, IPB-2021–15).

### **Vaccinia virus Tiantan strain (VTT) Purification**

Vero cells were infected with VTT at MOI of 0.05 and incubated at 37°C for 2 h. After washing, DMEM medium containing 2% FBS was added, cells were incubated at 37°C until cytopathic effect (CPE) was observed. After aspirating supernatant, cell lysis buffer (10 mM Tris-HCl pH 8.0) was added to the cells. Cells were subjected to freeze (−80°C)-thaw (37°C) for three times, and then sonicated and centrifuged at 4°C, 1500 g for 15 min to remove the cell debris. The clarified supernatant was processed with serial ultracentrifugations (44,500 g for 80 min with 36% sucrose cushion; 24,000 g for 40 min with step-gradient of 24%, 28%, 32%, 36%, and 40% sucrose; 31,500 g for 60 min). The purified virus was suspended with 10 mM Tris-HCl, pH 8.0 and titrated by plaque assay.

### **Construction and purification of vaccinia virus recombinants rTV-Gluc**

Gaussia luciferase gene was inserted into the thymidine kinase (TK) locus in VTT genome under the transcriptional control of the early/late promoter p7.5 using a homologous recombination method in 293T cells. The recombinant, attenuated, replication-competent VTT carrying the Gaussia luciferase gene (rTV-Gluc) was propagated, plaque purified, and titrated by plaque assay in Vero cells.

### **Serum and PMBC isolation**

Venous blood was collected from participants and processed within 12 h to isolate serum. Among these blood samples, 45 were used for isolating both plasma and peripheral blood mononuclear cells (PBMCs). Plasma was separated by centrifugation at 300×g for 10 minutes and stored at −80°C until testing. PBMCs were isolated from blood using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL) according to the manufacturer's instructions (<https://www.fishersci.com/shop/products/ficoll-paque-plus-media-2/p-3753315>). Isolated PBMCs were frozen in 90% heat-inactivated fetal bovine serum (FBS, Hyclone, Northbrook, IL) supplemented with 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA), and stored in liquid nitrogen before analysis.

### **Enzyme-linked immunosorbent assay (ELISA)**

VTT-specific IgG antibody titers were evaluated using the enzyme-linked immunosorbent assay (ELISA). Briefly, VTT infected Vero cells were lysed using RIPA buffer (Solarbio, Beijing, China) and were quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Total 1 µg lytic proteins were used as coating antigens. Serum samples were diluted 1/100 in 0.2% bovine serum albumin (BSA) and incubated for 1 h at 37°C. After washing with PBST, horseradish peroxidase -coupled goat anti-human Fc specific polyclonal IgG (Sigma Aldrich, St Louis, MO, USA) antibodies were added to the plates at a dilution of 1/60,000 with 0.2% BSA. After 1 h incubation at 37°C, the plates were washed and developed with 100 µL TMB Two-Component Substrate solution in each well (Solarbio). Reaction was stopped by adding 50 µL of stop solution (Solarbio). Optical density at 450 nm (OD450) was determined with a multifunctional microplate reader SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). The same amount of lytic Vero cell without VTT infection was used as a negative control for each serum sample. VTT-specific IgG responses were calculated by subtracting the background before further analysis. To determine the cutoff values for the ELISA, we determined the mean values and SD of negative serum from individuals who were born after 1990. The cutoff values were determined by calculating the OD450 of the negative serum plus 3-fold the SD values, which were 0.1 for VTT-specific IgG.

### **Microneutralization Assay**

Neutralization was measured of serum samples by the reduction in Gaussia luciferase-based vaccinia neutralization assay. Briefly, a serial 2-fold dilutions of serum samples (starting at 1/4) was preincubated with an equal volume of rTV-GLuc virus (1000 plaque-forming units [pfu]) (Appendix Methods) for 1 h at 37°C. Then the virus-serum mixture was added to Vero cells (American Type Culture Collection number CCL-81) and incubated for 1 h. After washing, 200 µL of DMEM medium containing 2% FBS was added to Vero cells. After incubation for 24 h, 40 µL of culture supernatant was aspirated and mixed with 60 µL Coelenterazine-h substrate (Promega, Madison, WI, USA). The luminescence was measured using Steady-Glo Luciferase

Assay System (Promega). The neutralization titers (NT50) were defined as 50% luminescence reduction of the reciprocal of the highest dilution relative to the uninfected control cells.

### **Memory B cell ELISpot assays**

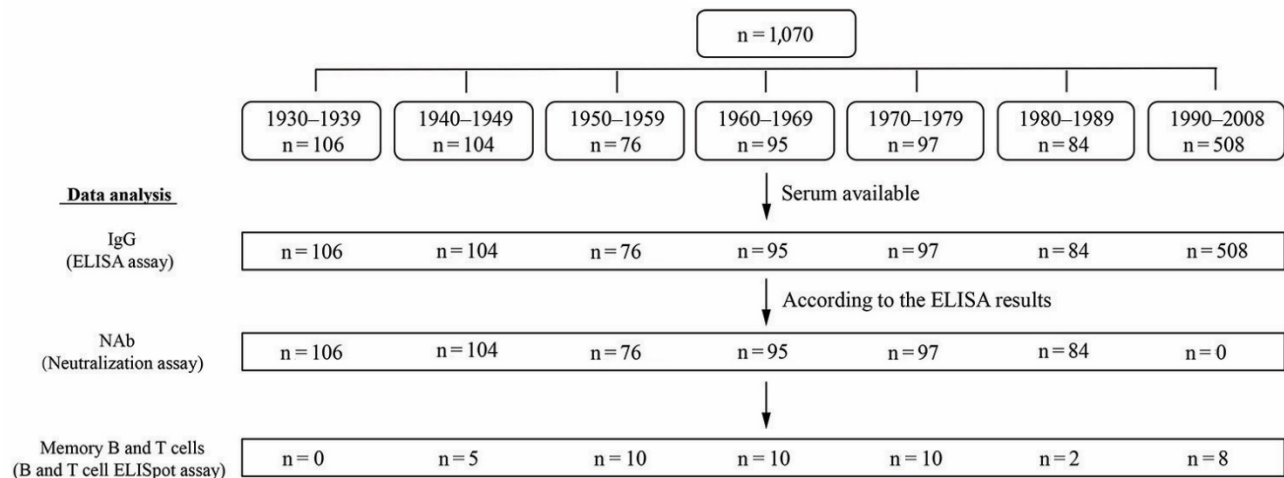
Memory B cell ELISpot assays were performed using cryopreserved PBMCs with an ELISpot Flex: Human IgG (ALP) kit (Mabtech, NS, Sweden) following the manufacturer's instructions. Briefly, cryopreserved PBMCs were thawed and washed once with the RPMI-1640 medium containing 10% FBS and rested overnight before assay. PBMCs were pre-stimulated with mixture of R848 at 1 µg/mL and rhIL-2 at 10 ng/mL for 48 h. After washing, total  $2 \times 10^5$  PBMCs per well were added to the ELISpot plate pre-coated with  $1 \times 10^7$  pfu heat-inactivated (56°C for 30 min) purified vaccinia virus. Lytic Vero cells were used as negative controls in each assay. Anti-human IgG mAb MT91/145 was used as positive control. Plates were incubated for 24 h at 37°C. After removing PBMCs, the plates were washed. Detection antibody diluted in 0.5% PBS-fetal calf serum was added and incubated for 1 h at room temperature. After washing, plate was added with streptavidin-ALP and incubated for 1 h at room temperature. Substrate solution were then added to the plate and developed until distinct spot emerges. The color development was stopped by washing in tap water. After the plate dried, the plate was inspected and counted spots in an AID ELISPOT reader system (AID Diagnostika GmbH, Strassberg, Germany). Mean spots of the negative control wells were subtracted from the test wells to quantify the intensity of VTT-specific memory B cell responses. The results were presented as Spot Forming Unit per  $10^6$  PBMC (s.f.u./ $10^6$  PBMC). Memory B cell responses were considered positive if the mean spot count was  $\geq 2$ -fold higher than the mean spot of the negative control and  $\geq 10$  s.f.u./ $10^6$  PBMCs.

### **Ex-vivo Interferon- $\gamma$ (IFN- $\gamma$ ) ELISpot assays**

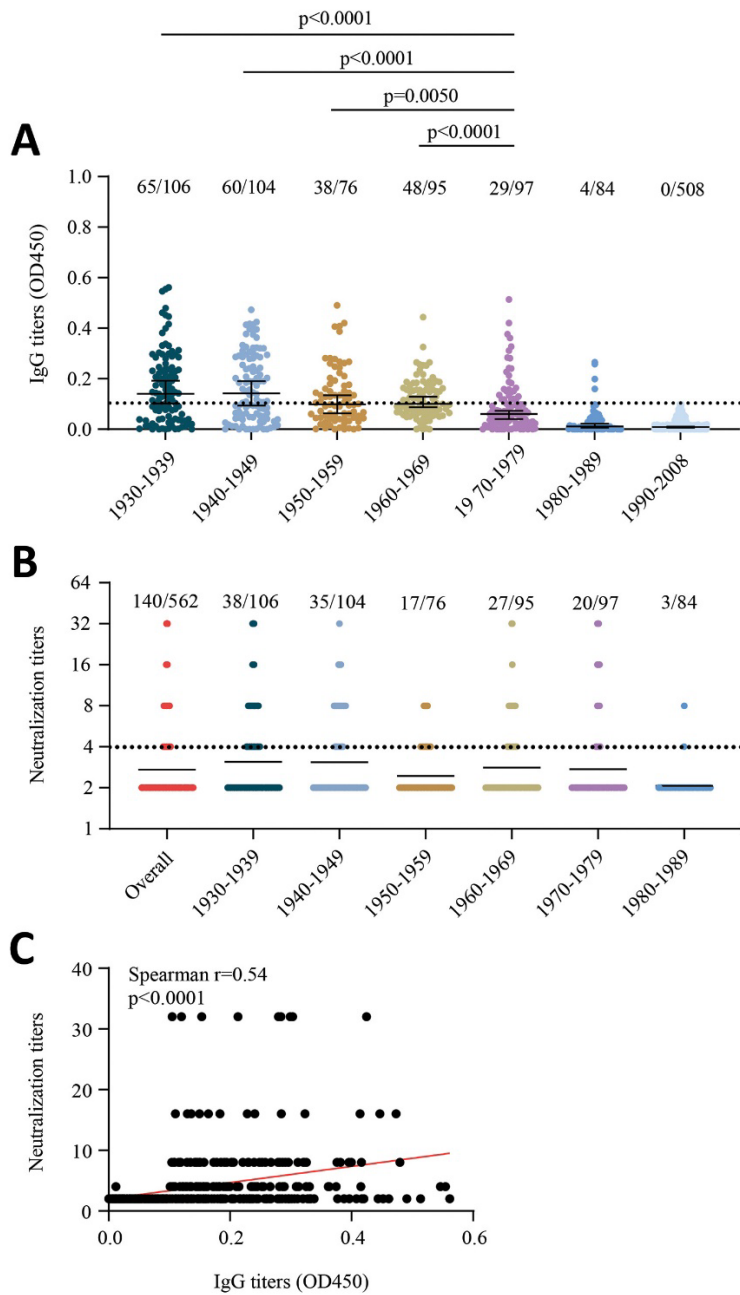
Memory T cell responses were evaluated using ex-vivo Interferon- $\gamma$  (IFN- $\gamma$ ) ELISpot assays with a Human IFN- $\gamma$  ELISpot kit (Mabtech) following the manufacturer's instructions. Briefly, cryopreserved PBMCs were thawed and rested overnight before assay. Plates pre-coated with human IFN- $\gamma$  antibodies were washed with phosphate-buffered saline (PBS), and blocked



with RPMI1640 medium containing 10% FBS for 30 min at room temperature. Total  $1 \times 10^5$  PBMC per well were stimulated in duplicates with  $10^5$  pfu inactivated ( $56^\circ\text{C}$  for 30 min) purified VTT for 24 h at  $37^\circ\text{C}$ . Lytic Vero cells were used as negative controls in each sample. Phorbol myristate acetate (PMA)/ionomycin (Multi Science, Hangzhou, China) and known T cell epitope pools of human influenza (Flu) and Epstein-Barr virus (EBV) were used as positive controls. Spots were counted using an AID ELISPOT Reader System. Mean spots of the negative control wells were subtracted from the test wells to quantify the intensity of VTT-specific T cell responses, and the results were presented as Spot Forming Unit per  $10^6$  PBMC (s.f.u./ $10^6$  PBMC). Memory T cell responses were considered positive if the mean spot count was  $\geq 2$ -fold higher than the mean spot of the negative control and  $\geq 20$  s.f.u./ $10^6$  PBMCs.



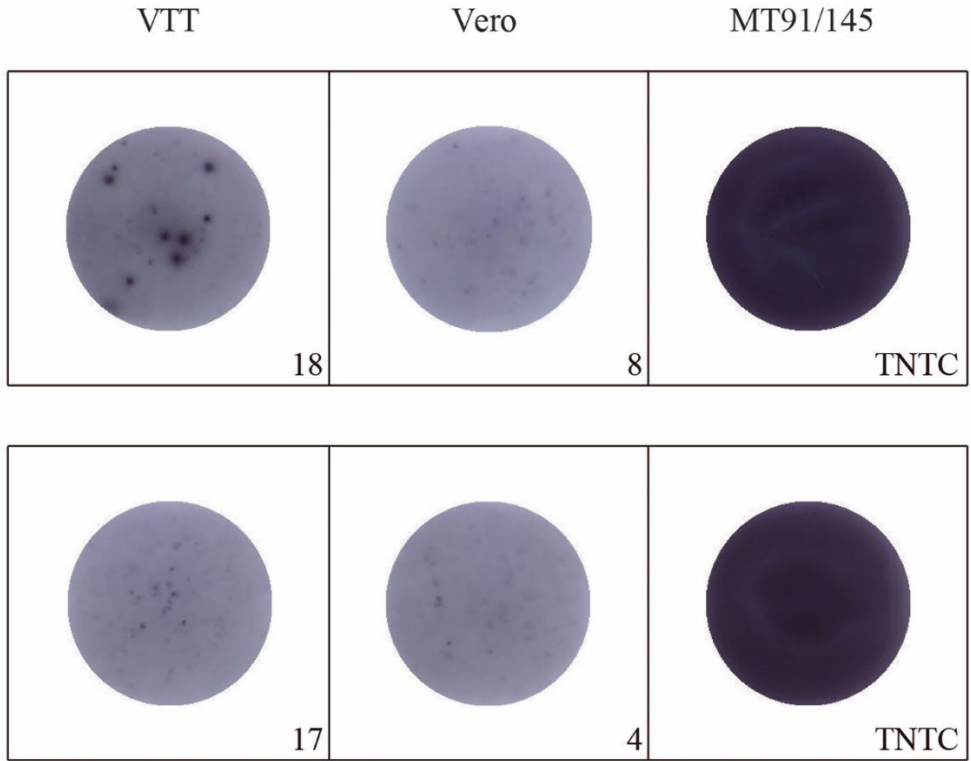
**Appendix Figure 1.** Flow diagram of the study.



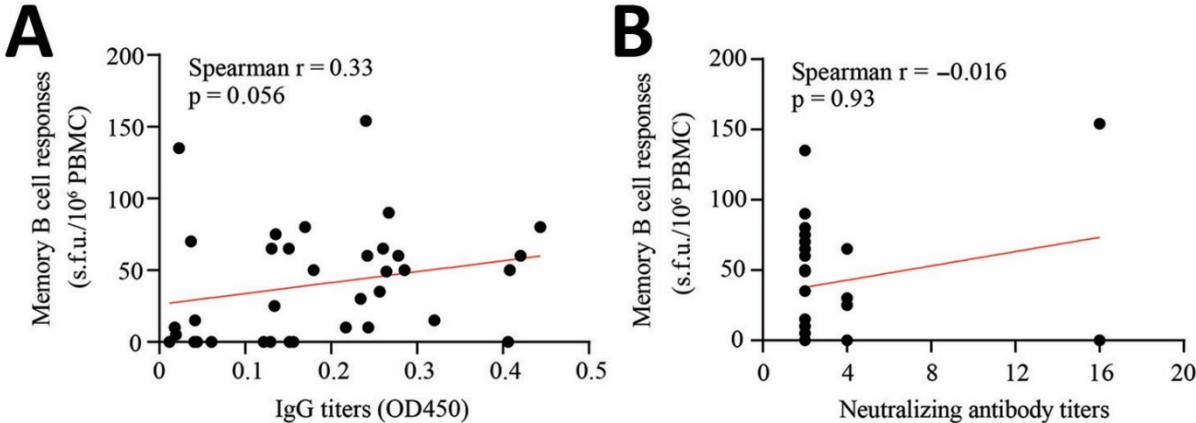
**Appendix Figure 2.** Serum IgG and neutralizing antibody responses against vaccinia virus Tiantan strain.

(A) IgG titers against VTT according to the year of birth. (B) Neutralizing antibody titers against VTT according to the year of birth. (C) Correlation between neutralizing antibody and IgG titers against VTT in individuals born before 1990 ( $n = 562$ ). IgG titers comparisons were performed using the Kruskal-Wallis one-way ANOVA test in different age groups. Spearman correlation analysis was performed.

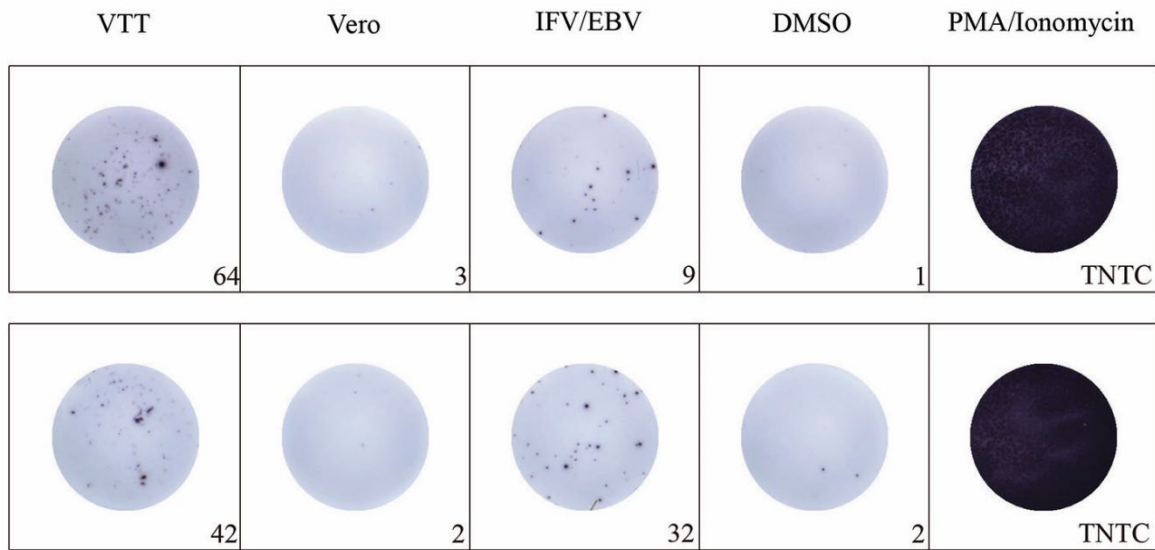
VTT = vaccinia virus Tiantan strain, OD = optical density.



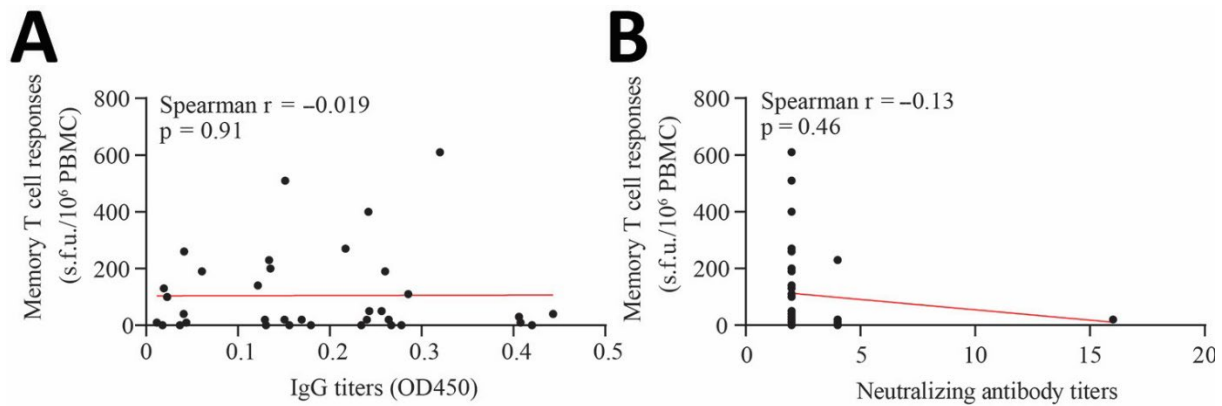
**Appendix Figure 3.** VTT-specific memory B cell responses. Two representative sample of the memory B cell ELISpot responses. Vero cell was used as the negative control. Anti-human IgG mAb MT91/145 was included as the positive control. TNTC = too numerous to count.



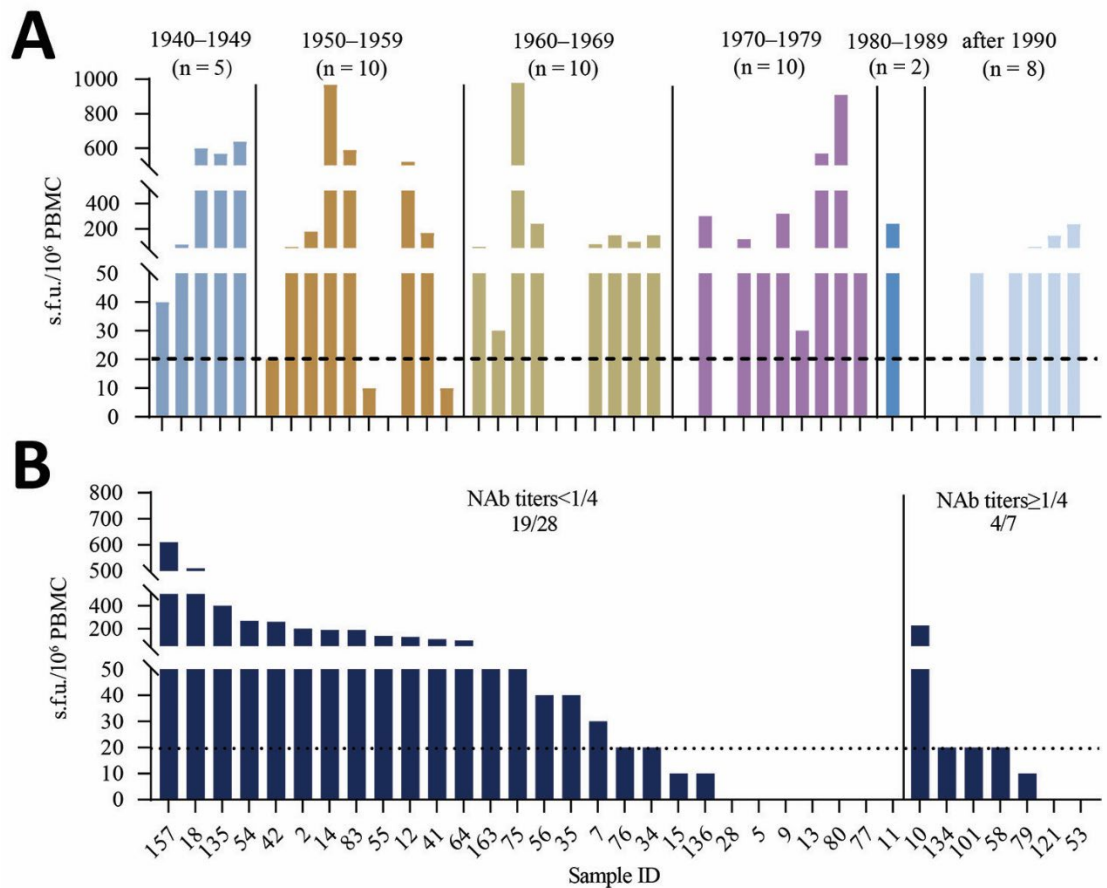
**Appendix Figure 4.** Correlation between memory B cell responses and antibody titers. Correlation between the magnitudes of memory B cell responses and IgG (A), and neutralizing antibody (B) titers against VTT (n = 35). Spearman correlation analysis was performed.



**Appendix Figure 5.** Two representative samples of the IFN $\gamma$  ELISpot responses. Uninfected Vero cell serves as the negative control of vaccinia virus infection. DMSO serves as the negative control of IFV/EBV peptide pool. PMA/Ionomycin was assayed as the positive control.



**Appendix Figure 6.** Vaccinia virus-specific memory T cell responses. (A) Magnitude of IFN $\gamma$  T-cell responses against IFV/EBV for each individual (n = 45). (B) Magnitude of IFN- $\gamma$  T cell responses in ELISpot for 35 individuals who had been evaluated of neutralizing antibody levels and were born before 1980. The dotted lines indicate the detection limit of the assays. IFV = Influenza virus, EBV = Epstein-Barr virus, TNTC = too numerous to count.



**Appendix Figure 7.** Correlation between memory T cell responses and IgG titers. Correlation between the magnitude of IFN $\gamma$  ELISpot responses and IgG (A) and the neutralizing antibody (B) titers against VTT (n = 35). Spearman correlation analysis was performed.