Court File No. CV-20-00652216-000

ONTARIO
SUPERIOR COURT OF JUSTICE

BETWEEN:

HER MAJESTY THE QUEEN IN RIGHT OF ONTARIO

Applicant/Respondent

AND

ADAMSON BARBECUE LIMITED
AND WILLIAM ADAMSON SKELLY

Respondents/Applicants

AFFIDAVIT OF WITNESS Dr. Mark Trozzi
sworn April 12, 2021

April 13, 2021

ELDERS WITHOUT BORDERS
237 Argyle Ave.
Ottawa, Ontario K2P 1B8
Tel: (613)563-7474
Fax: (613)563-9179
Email: spiritualelders@hotmail.com

Michael Swinwood (LSO #14587R)
Liza Swale (LSO #49683H)

Solicitors for the Respondents/Applicants

TO: **THE ATTORNEY GENERAL OF ONTARIO**
CONSTITUTIONAL LAW BRANCH
720 Bay Street, 4th floor
Toronto, Ontario M5G 2K1
Fax: 416-326-4015

Padraic Ryan
Email: padraic.ryan@ontario.ca

Zachary Green
Email: zachary.green@ontario.ca

Lawyers for the Applicant/Respondent

Court File No. CV-20-00652216-000

ONTARIO

SUPERIOR COURT OF JUSTICE

BETWEEN:

HER MAJESTY THE QUEEN IN RIGHT OF ONTARIO

Applicant/Respondent

AND

ADAMSON BARBECUE LIMITED
AND WILLIAM ADAMSON SKELLY

Respondents/Applicants

AFFIDAVIT OF WITNESS Dr. Mark Trozzi

I, MARK TROZZI, in the [redacted], MAKE OATH AND SAY:

1. I am a medical doctor; I graduated in 1990 from The University of Western Ontario. I have been practicing Emergency Medicine for the past twenty-five years, and I have been on call in multiple emergency units since the onset of the so-called "pandemic," including one ER designated specifically for COVID-19. I am an Advanced Trauma Life Support professor with the College of Surgeons of America, and I hold teaching positions at Sunnybrook Health Sciences in the Advanced Life Support Department, as well as with

both Queen's University and The University of Ottawa. I attach my curriculum vitae as Exhibit "A".

2. At the onset of this "pandemic," I was cautious and hence meticulous with N95 mask use, hand washing, social isolation and distancing etc. I studied coronavirus science and was deeply involved in many emergency department drills to modify our practice in profound ways to deal with the "killer virus" we were advertised. However, various things soon made me consider that we were being deceived and manipulated. Here are a few:

The First Wave

3. The "first wave" of the "pandemic" was absolutely the quietest time in my career. I have worked very hard and been very busy over the past twenty-five years in ER. However, both in my regular ER and my "COVID-19 designated" ER, there were almost no patients and almost no work. I had multiple long ER shifts without a single patient. Meanwhile, when I would go to the local grocery store, the propagandized public, God bless them, would usher me to the front of the antisocial distance line, thanking me for everything I was going through as a front-line emergency doctor. They believed that the ER's and hospitals were full of patients dying from COVID-19 and that I must be exhausted and at risk of dying myself from exposure. I began contacting doctors and friends all over Canada and the US and found the same pattern: empty hospitals and propaganda saying that they were full of patients dying of COVID-19.

4. Early in my studies, I investigated zinc and hydroxychloroquine, which, based on sound physiology, may genuinely help those rare persons who get very sick with this cold virus.

I was surprised that this treatment was simply brushed aside and dismissed by most of the medical community.

5. Researching the UN's World Health Organization, I learned that the Chinese dictatorship (PRC) had propped up communist and disreputable "Dr" Tedros, as the head of the WHO. I learned how the PRC had been involved in: the virus release; the cover-up for weeks; the disappearing and suppression of honest Chinese doctors and scientist; the spreading of the virus to the world (sparing Beijing where the PRC elite live); and dramatic abuse of the Chinese people in their well-timed lockdown, which was filmed and transmitted to the world to create the panic that herded all of us into surrendering our economies and civil rights.

6. I learned how Canada's chief public health officer Dr. Tam is on the WHO's oversight committee with Dr. Tedros. I think making her a double agent. I have listened to what I often found to be her bizarre dissertations to Canadians regarding COVID-19.

My Perception of the Situation

7. I perceive that at every level, the hospital administration has had no apparent choice other than to submit to the endless top-down roll-out from governments of questionable new rules, protocols, and procedures. My honest conversations with coworkers about my research and observations became a problem. Caught in this quandary, an important administrator whom I greatly respect told me that "my thoughts made others uncomfortable and made it difficult to keep everyone motivated and compliant" with all the new protocols and restrictions.

8. Sympathetic to the sad situation, I maintained my clinical position by promising to "bite my tongue any time I thought I was going to speak about COVID-19" in the hospital. This was ultimately ethically impossible for me. By mid-November 1, 2021, I began winding down my ER work and resigned from all my ER's by mid-February 2021 to avoid conflicts between my social, legal, and ethical responsibilities, and hospitals which I am actually fond of.

9. In my emergency department work, I have never seen a patient sick with COVID-19; I have seen some positive PCR tests in asymptomatic people and watched people be imprisoned in their own homes and isolated from family and friends.

10. My research into the PCR test has convinced me that it is misleading, manipulatable, and used to drain endless taxpayer money and increase future debt to enrich pharmaceutical companies dramatically. Ontario alone has performed ~50,000 PCR tests daily. Meanwhile, our federal government is bringing in hundreds of thousands of doses of potentially dangerous experimental injections of modified viral genetic material, calling them "vaccines," and having the military manage them. This is not reasonable for a predominantly mild and non-fatal viral illness.

11. I have watched the suppression of doctors and scientists who performed serum antibody studies, whose findings showed that the virus was much more widespread, yet generally non-fatal, and asymptomatic or very mild in most cases; and that in many regions, we had likely already achieved natural herd immunity by summer 2020.

12. This study performed in Wuhan, China, which shows that the virus was finished its harm there by June 1, 2020, just two months after their brief lockdowns ended, and no one was

spreading it, not even the very few people with a positive PCR "test" (and they were not sick). I attach as Exhibit “B” an article reviewing a post-lockdown Wuhan, China.

13. I perceive that many things we learned in medical school about infectious disease have been brushed aside and replaced by constantly expanding lists of often to-me-strange mandates by public health officials. Doctors, nurses, and teachers are especially important to the success of this COVID-19 deception, as we are leaders in society, and people trust our advice. So, it is no surprise that I have found free speech and thought have been very suppressed in our ranks. Rather than endure the punishments of dissenting, we can choose to experience the short-sighted perks like extremely quiet days in the ER, replacing our traditional hands-on work with Zoom sessions from home; and accessing a variety of new COVID-19 billing codes. At one point, an option existed to make more money than I normally make working in a busy ER, to just stay home and be available in case the COVID-19 swabbing nurse needed to video conference with me.

14. There are many positive and negative motivators used to manipulate Canadian doctors, nurses, and teachers to inadvertently participate in this grand COVID-19 deception; but this is destroying our society. To use a Titanic metaphor: "even the luxury suites on the Titanic end up at the bottom of the ocean when she sinks." Also, much of what is being done, including the experimental viral genetic injections, seem to violate the Nuremberg code regarding medical experimentation with full informed consent by the participants. I empathize with all my fellow doctors and nurses. We are all victims of the COVID-19 abuse.

15. I researched and perceived how corrupt oligarchs seem to have planned this crime against humanity. This planning included Event 201, which was a simulation of a coronavirus pandemic conducted by the Bill & Melinda Gates Foundation, the World Economic Forum and Johns Hopkins University in October 2019; and the Rockefeller Foundation's 2010 Viral outbreak simulation planning called "Operation Lockstep." Both these projects described how a viral outbreak would be used to bring in an authoritarian system with the loss of our human rights and freedoms. I also observed how their cohorts in big tech like Google, Facebook, Twitter and YouTube worked to censor and deceive us all; it's genuine propaganda.

16. The forced wearing of masks by most of the world's population is not unanimously supported by real science. These masks cause significant harm to our psychologic, social, dermatologic, dental and otolaryngologic health. Though I generally have great health, the masks have given me rashes and nasal symptoms whenever I have had to wear them for prolonged periods, which resolve whenever I do not wear them for a few days. What I find most disturbing is the elimination of facial expressions, and hence normal visual social interaction.

The Nuremberg Code:

17. During the Second World War, the Nazi's performed horrific medical experiments on imprisoned groups, most notably the Jewish people. Following the war, international groups worked to avoid such experimental abuse of people in the future, and the Nuremberg Code for medical experimentation was written. Here it is:

a. *The ten points of the code were given in the section of the verdict entitled "Permissible Medical Experiments":[5]*

b. *The voluntary consent of the human subject is absolutely essential.*

c. *The experiment should be such as to yield fruitful results for the good of society, unprocurable by other methods or means of study, and not random and unnecessary in nature.*

d. *The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem under study that the anticipated results will justify the performance of the experiment.*

e. *The experiment should be so conducted as to avoid all unnecessary physical and mental suffering and injury.*

f. *No experiment should be conducted where there is an a priori reason to believe that death or disabling injury will occur; except, perhaps, in those experiments where the experimental physicians also serve as subjects.*

g. *The degree of risk to be taken should never exceed that determined by the humanitarian importance of the problem to be solved by the experiment.*

h. *Proper preparations should be made and adequate facilities provided to protect the experimental subject against even remote possibilities of injury, disability, or death.*

i. *The experiment should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment.*

j. *During the course of the experiment, the human subject should be at liberty to bring the experiment to an end if he has reached the physical or mental state where continuation of the experiment seems to him to be impossible.*

k. *During the course of the experiment, the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of the good faith, superior skill and careful judgment required of him that a continuation of the experiment is likely to result in injury, disability, or death to the experimental subject.*

18. First, are the modified viral messenger RNA injections which are being called "vaccines," experimental? They have emergency use authorizations in the USA but are not FDA approved. Such injections have never been administered to human patients before. After reviewing much of the literature, I personally believe, like many experts, that these injections are experimental. On December 13, 2020 Doctors protested at CDC headquarters addressing the largest medical experiment in American history. I attach as Exhibit “C” an article on experimental covid vaccines.

19. Based on this, if I administered this injection to a patient as a simple "vaccine", without making it very clear to the recipient that this is an experiment, then I would find myself guilty of violating the first point of the Nuremberg Code.

20. Also, given the very low mortality of COVID-19, and my impression, like many experts, that we have likely achieved herd immunity many months ago, I think I would also be involved in a violation of points 2 and 6 of the Nuremberg Code.

21. If you consider the very negative effects of prior coronavirus experimental vaccines in laboratory animals, violations of points 3, 4 and 5 are also at issue here. I attach as Exhibit “D” a journal article reviewing the effects.

22. Though I want to cooperate and follow institutional rules and procedures wherever I work, I do not want to be part of unethical medical experimentation with the public, violations of the Nuremberg Code, and committing crimes against humanity.

23. Second, we should consider the PCR "test" as it relates to the Nuremberg Code. I propose that the same issues regarding violation of the Nuremberg Code exist with regards to this experimental procedure, of questionable value to society.

The Hippocratic Oath:

24. This famous physicians' oath, which graduates traditionally take at the end of medical school, includes this phrase: "I will follow that system of regimen which, according to my ability and judgment, I consider for the benefit of my patients, and abstain from whatever is deleterious and mischievous."

25. According to my judgement, after careful consideration, I do not consider experimental viral messenger RNA injections, PCR "tests", excessive and inappropriate use of masks, social isolation, state-mandated germophobic behaviour, and various other elements of the current COVID-19 practices, to be "for the benefit of my patients".

Personal Ethics and Morals:

26. We live in a diverse society that until recently honoured individual rights and freedoms, including freedom of religion. Though we have diverse historical and religious backgrounds, all reasonable religious and philosophical schools endorse the Golden Rule: do unto others as you would wish to have done unto you.

27. As it is my conclusion that many of the protocols relating to COVID-19 are not in the best interest of the patients or populace, I cannot endorse or participate in them without violating the highest and most fundamental law that I recognize, the Golden Rule.

Regarding COVID-19 Vaccinations:

28. The history of past attempts at vaccines for coronaviruses revealed some very dangerous side effects in animal models, and the efforts were abandoned. Why would we take a dangerous vaccine for a generally mild illness, to which we develop herd immunity anyway? The current roll-out of fast-tracked, expensive experimental "vaccines" is burying the taxpayers in endless debt to the rich and powerful villains of this story. Additionally, the so-called "vaccines" are not vaccines (unless we change the definition of vaccines). Rather they are injections of coronavirus genes.

29. Ivermectin has come to light as an extremely effective safe prevention, prophylaxis, and treatment for COVID-19; yet it has been suppressed by business and political interests, while very expensive and unlawful injections of the masses are underway. Big Pharma and its political consorts are pushing to administer these experimental injections into the world's population, even infants and children. In reality, the SARS-CoV2 poses very little threat to almost everyone but the very vulnerable persons who are similarly vulnerable to many viruses and other illnesses.

30. Children have zero risk of death from COVID-19 but are being severely damaged by the lockdowns, facial barriers ("masks"), isolation, and denial of many essential elements of childhood. Similarly, almost everyone but the very old or ill, have almost no risk of death or serious injury from coronavirus infection.

31. It is strongly my diligently researched conclusion that I do not want, nor do I recommend the experimental injections for the vast majority of people. Similarly, I do not support many measures like the "case" count generating PCR "test", lockdowns, and forced wearing of harmful facial barriers.

32. Big Pharma is promoting the idea that new variants of SARS-CoV2 will require injections for immunity updates, as well as more fear and lockdowns. However, the most distant variant currently is 99.7% genetically identical to SARS-CoV2; yet we now know that exposure to SARS-CoV1 17 years ago made people persistently immune to many coronavirus's including SARS-CoV2 to this day. Meanwhile, SARS-CoV1 and SARS-CoV2 are only 80% genetically identical. This makes it virtually impossible that immunity to SARS-CoV2 won't work for a subtle variant of it.

33. Research within the last year shows that many people, likely well above 50%, are immune to SARS-CoV2 without any experimental injection due to prior SARS-CoV1 exposure or exposure to SARS-CoV2 in the past year and a half. SARS-CoV2 is a very mild or asymptomatic experience for about 80% of people, and only a very small percent, less than 0.1 %, are at serious risk from this cold.

34. Pushing experimental injections on the masses as is currently happening severely violates the Nuremberg Code for medical experimentation. Many covid protocols are likewise crimes against humanity in violation of the Nuremberg Code and other legal standards such as civil liberties.

35. I make this Affidavit in support of the Respondents' Notice of Constitutional Question and for no improper purpose.

Sworn before me)
this 12 day of April, 2021 at)
15:00 in [redacted]

Commissi[redacted]

Exhibit "A".

This is the Affidavit of

Dr. Mark Trozzi

sworn before me this 12 day of April, 2021.

Commissioner of Oaths

[redacted]

Curriculum Vitae for Dr Mark Trozzi MD

Overview: I am a physician practicing emergency medicine for twenty-five years, with extensive rural emergency experience, a strong interest in trauma medicine, and extensive medical teaching experience.

Education and Professional Qualifications

- High School Ontario Scholar Gr13 Graduate 1983.
- Deans Honor List University of Western Ontario Sciences 1984 and 1985.
- University of Western Ontario Medical School Graduate 1985 to 1990.
- Ontario Medical Internship graduate University of Ottawa 1990 to 1991.
- College of Physicians and Surgeons of Ontario independent license since 1992.
- Certified in Advanced Trauma Life Support, Advanced Cardiac Life Support, and Pediatric Advanced Life Support.
- College of Surgeons of American Advanced Trauma Life Support Instructor.

Academic Appointments

- Head Preceptor Schulich School of Medicine Medquest Leamington program 2011.
- LDMH medical student preceptor 2007 to 2012.
- Advanced Cardiac Life Support Instructor 2008 to 2010.
- Teaching Support for medical students and residents at QHCNH 2011 to 2021.
- Assistant professor Queen's University 2013 to present.
- American College of Surgeons Instructor in Advanced Trauma Life Support 2014 to present.
- Sunnybrook Health Sciences Center Advanced Trauma Life Support Instructor 2014 to present.
- University of Ottawa lecturer 2018 to present.
- University of Ottawa Medical school ePortfolio coach 2018 to present.

Hospital Appointments

- Quinte Health Care North Hastings ER 2011 to 2021.
- St Francis Memorial Hospital ER 2009 to 2021.
- Hasting Highlands Health Services Center ER 2017 to 2021.

Hospital Appointments (past)

- Grace Hospital ER 1993 to 1994.
- Leamington District Memorial Hospital ER 1994 to 2013.
- Pembroke Regional Hospital ER 2010 to 2015.

Administrative Positions

- Quinte Health Care North Hastings Hospital, Head of Emergency Department 2014.
- Quinte Health Care North Hastings Hospital Chief of Staff 2014.

Professional Memberships/Associations:

- 1992 to present College of Physicians and Surgeons of Ontario.
- 1992 to present Canadian Medical Association.
- 1992 to present Ontario Medical Association.
- 1992 to present Canadian Medical Protective Association.
- 2005 to 2010 Canadian Association of Physicians for the Environment.
- 2015 Society of Obstetricians and Gynecologists of Canada.
- 1999 to 2000 Committee Membership Windsor Essex Air Quality Steering Committee.

Other

- Adequate Spanish speaking.
- Published author and musician.
- Active Ecosystem Conservationist 1999 to present.

Exhibit "B"

This is the Affidavit of

Dr. Mark Trozzi

sworn before me this 12 day of April, 2021.

Commissioner of Oaths

[redacted]

nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-020-19802-w OPEN

Post-lockdown SARS-CoV-2 nucleic acid screening in nearly ten million residents of Wuhan, China

Shiyi Cao[1,11], Yong Gan[1,11], Chao Wang[1,11], Max Bachmann[2], Shanbo Wei[3], Jie Gong[4], Yuchai Huang[1], Tiantian Wang[1], Liqing Li[5], Kai Lu[6], Heng Jiang[7,8], Yanhong Gong[1], Hongbin Xu[1], Xin Shen[1], Qingfeng Tian[9], Chuanzhu Lv[10]✉, Fujian Song[2]✉, Xiaoxv Yin[1]✉ & Zuxun Lu[1]✉

Stringent COVID-19 control measures were imposed in Wuhan between January 23 and April 8, 2020. Estimates of the prevalence of infection following the release of restrictions could inform post-lockdown pandemic management. Here, we describe a city-wide SARS-CoV-2 nucleic acid screening programme between May 14 and June 1, 2020 in Wuhan. All city residents aged six years or older were eligible and 9,899,828 (92.9%) participated. No new symptomatic cases and 300 asymptomatic cases (detection rate 0.303/10,000, 95% CI 0.270–0.339/10,000) were identified. There were no positive tests amongst 1,174 close contacts of asymptomatic cases. 107 of 34,424 previously recovered COVID-19 patients tested positive again (re-positive rate 0.31%, 95% CI 0.423–0.574%). The prevalence of SARS-CoV-2 infection in Wuhan was therefore very low five to eight weeks after the end of lockdown.

[1] Department of Social Medicine and Health Management, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. [2] Norwich Medical School, Faculty of Medicine and Health Science, University of East Anglia, Norwich, UK. [3] Wuhan Municipal Health Commission, Wuhan, Hubei, China. [4] Wuhan Centre for Clinical Laboratory, Wuhan, Hubei, China. [5] Department of Management Science and Engineering, School of Economics and Management, Jiangxi Science and Technology Normal University, Nanchang, Jiangxi, China. [6] Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, China. [7] Centre for Alcohol Policy Research, School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia. [8] Melbourne School of Population and Global Health, University of Melbourne, Melbourne, VIC, Australia. [9] School of Public Health, Zhengzhou University, Zhengzhou, Henan, China. [10] Department of Emergency, Hainan Clinical Research Centre for Acute and Critical Diseases, The Second Affiliated Hospital of Hainan Medical University, Haikou, Hainan, China. [11] These authors contributed equally: Shiyi Cao, Yong Gan, Chao Wang. ✉email: lyuchuanzhu@hainmc.edu.cn; Fujian.song@uea.ac.uk; yxx@hust.edu.cn; zuxunlu@yahoo.com

The Coronavirus Disease 2019 (COVID 19) was first reported in December 2019, and was classified as a pandemic by the World Health Organization on March 11, 2020[1]. Following strict lockdown measures, the COVID 19 epidemic was generally under control in China, and the whole country has progressed into a post lockdown phase. In this phase, countries face new problems and challenges, including how to accurately assess the post lockdown risk of the COVID 19 epidemic, how to avoid new waves of COVID 19 outbreaks, and how to facilitate the resumption of economy and normal social life. As the city most severely affected by COVID 19 in China, Wuhan had been under lockdown measures from January 23 until April 8, 2020. During the first 2 months after city's reopening, there were only a few sporadic COVID 19 cases in Wuhan (six newly confirmed cases from April 8 to May 10, 2020[2]). However, there was still concern about the risk of COVID 19 in Wuhan, which seriously affected the resumption of industrial production and social services, and hampered the normal lives of residents. In order to ascertain the current status of the COVID 19 epidemic, the city government of Wuhan carried out a comprehensive citywide nucleic acid screening of SARS CoV 2 infection from May 14, 2020 to June 1, 2020.

The citywide screening of SARS CoV 2 infection in Wuhan is a mass screening programme in post lockdown settings, and provided invaluable experiences or lessons with international relevance as more countries and cities around the world entering the post lockdown phase. In this study, we report the organisation process, detailed technical methods used, and results of this citywide nucleic acid screening.

Results

There were 10,652,513 eligible people aged ≥6 years in Wuhan (94.1% of the total population). The nucleic acid screening was completed in 19 days (from May 14, 2020 to Jun 1, 2020), and tested a total of 9,899,828 persons from the 10,652,513 eligible people (participation rate, 92.9%). Of the 9899,828 participants, 9,865,404 had no previous diagnosis of COVID 19, and 34,424 were recovered COVID 19 patients.

The screening of the 9,865,404 participants without a history of COVID 19 found no newly confirmed COVID 19 cases, and identified 300 asymptomatic positive cases with a detection rate of 0.303 (95% CI 0.270 0.339)/10,000. The median age stratified Ct values of the asymptomatic cases were shown in Supplementary Table 1. Of the 300 asymptomatic positive cases, two cases came from one family and another two were from another family. There were no previously confirmed COVID 19 patients in these two families. A total of 1174 close contacts of the asymptomatic positive cases were traced, and they all tested negative for the COVID 19. There were 34,424 previously recovered COVID 19 cases who participated in the screening. Of the 34,424 participants with a history of COVID 19, 107 tested positive again, giving a repositive rate of 0.310% (95% CI 0.423 0.574%).

Virus cultures were negative for all asymptomatic positive and repositive cases, indicating no "viable virus" in positive cases detected in this study.

All asymptomatic positive cases, repositive cases and their close contacts were isolated for at least 2 weeks until the results of nucleic acid testing were negative. None of detected positive cases or their close contacts became symptomatic or newly confirmed with COVID 19 during the isolation period. In this screening programme, single and mixed testing was performed, respectively, for 76.7% and 23.3% of the collected samples. The asymptomatic positive rates were 0.321 (95% CI 0.282 0.364)/10,000 and 0.243 (95% CI 0.183 0.315)/10,000, respectively.

The 300 asymptomatic positive persons aged from 10 to 89 years, included 132 males (0.256/10,000) and 168 females (0.355/10,000). The asymptomatic positive rate was the lowest in children or adolescents aged 17 and below (0.124/10,000), and the highest among the elderly aged 60 years and above (0.442/10,000) (Table 1). The asymptomatic positive rate in females (0.355/10,000) was higher than that in males (0.256/10,000).

The asymptomatic positive cases were mainly domestic and unemployed residents (24.3%), retired older adults (21.3%), and public service workers (11.7%) (Fig. 1).

The asymptomatic positive rate in urban districts was on average 0.456/10,000, ranging from 0.317/10,000 in Hongshan to 0.807/10,000 in Wuchang district. A lower rate of asymptomatic positive cases was found in suburban districts (0.132/10,000), ranging from 0.047/10,000 in Xinzhou to 0.237/10,000 in Jiangan district (Fig. 2).

Among the 7280 residential communities in Wuhan, asymptomatic positive cases were identified in 265 (3.6%) communities (only one case detected in 246 communities), while no asymptomatic positive cases were found in other 96.4% communities.

Testing of antibody against SARS CoV 2 virus was positive IgG (+) in 190 of the 300 asymptomatic cases, indicating that 63.3% (95% CI 57.6 68.8%) of asymptomatic positive cases were actually infected. The proportion of asymptomatic positive cases with both IgM (−) and IgG (−) was 36.7% (95% CI: 31.2 42.4%; $n = 110$), indicating the possibility of infection window or false positive results of the nucleic acid testing (Table 2).

Higher detection rates of asymptomatic infected persons were in Wuchang, Qingshan and Qiaokou districts, and the prevalence of previously confirmed COVID 19 cases were 68.243/10,000, 53.767/10,000, and 100.047/10,000, respectively, in the three districts. Figure 3 shows that districts with a high detection rate of asymptomatic positive persons generally had a high prevalence of confirmed COVID 19 cases ($r_s = 0.729$, $P = 0.002$).

Discussion

The citywide nucleic acid screening of SARS CoV 2 infection in Wuhan recruited nearly 10 million people, and found no newly confirmed cases with COVID 19. The detection rate of asymptomatic positive cases was very low, and there was no evidence of transmission from asymptomatic positive persons to traced close contacts. There were no asymptomatic positive cases in 96.4% of the residential communities.

Previous studies have shown that asymptomatic individuals infected with SARS CoV 2 virus were infectious[3], and might subsequently become symptomatic[4]. Compared with symptomatic patients, asymptomatic infected persons generally have low quantity of viral loads and a short duration of viral shedding, which decrease the transmission risk of SARS CoV 2[5]. In the present study, virus culture was carried out on samples from asymptomatic positive cases, and found no viable SARS CoV 2 virus. All close contacts of the asymptomatic positive cases tested negative, indicating that the asymptomatic positive cases detected in this study were unlikely to be infectious.

There was a low repositive rate in recovered COVID 19 patients in Wuhan. Results of virus culturing and contract tracing found no evidence that repositive cases in recovered COVID 19 patients were infectious, which is consistent with evidence from other sources. A study in Korea found no confirmed COVID 19 cases by monitoring 790 contacts of 285 repositive cases[6]. The official surveillance of recovered COVID 19 patients in China also revealed no evidence on the infectiousness of repositive cases[7]. Considering the strong force of infection of COVID 19[8 10], it is expected that the number of confirmed cases is associated with the risk of being infected in communities. We

Table 1 Characteristics of asymptomatic positive individuals.

	Total (%)	Asymptomatic positive persons (%)	Detection rate per 10,000 (95% CI)	*P* value
Total	9,899,828 (100.0)	300 (100.0)	0.303 (0.270 0.339)	
Sex				
Male	5,162,960 (52.2)	132 (44.0)	0.256 (0.214 0.303)	0.005
Female	4,736,868 (47.8)	168 (56.0)	0.355 (0.303 0.413	
Age (years old)				
≤17	969,014 (9.8)	12 (4.0)	0.124 (0.064 0.216)	<0.001
18 44	4,448,230 (44.9)	104 (34.7)	0.234 (0.191 0.283)	
45 59	2,492,943 (25.2)	96 (32.0)	0.385 (0.312 0.470)	
≥60	1,989,641 (20.1)	88 (29.3)	0.442 (0.355 0.545)	
Administrative Districts in Wuhan				
Wuchang	904,636 (9.1)	73 (24.3)	0.807 (0.633 1.015)	<0.001
Qingshan	414,312 (4.2)	23 (7.7)	0.555 (0.352 0.833)	
Qiaokou	583,440 (5.9)	32 (10.7)	0.548 (0.375 0.774)	
Hanyang	717,429 (7.2)	29 (9.7)	0.404 (0.271 0.581)	
Jianghan	524,224 (5.3)	19 (6.3)	0.362 (0.218 0.566)	
Hongshan	1,103,079 (11.1)	35 (11.7)	0.317 (0.221 0.441)	
East Lake High tech Development Area	782,987 (7.9)	19 (6.3)	0.243 (0.146 0.379)	
Jiangan	800,440 (8.1)	19 (6.3)	0.237 (0.143 0.371)	
Caidian	503,595 (5.1)	11 (3.7)	0.218 (0.109 0.391)	
Jiangxia	671,248 (6.8)	14 (4.7)	0.209 (0.114 0.350)	
Huangpi	979,920 (9.9)	14 (4.7)	0.143 (0.078 0.240)	
Hannan	417,022 (4.2)	4 (1.3)	0.096 (0.026 0.246)	
Dongxihu	777,204 (7.9)	5 (1.7)	0.064 (0.021 0.150)	
Xinzhou	634,408 (6.4)	3 (1.0)	0.047 (0.010 0.138)	
East Lake Scenic Area of Wuhan	85,884 (0.9)	0 (0.0)	0.000 (0.000 0.430)	

χ^2 test was used to assess the association between the detection rate of asymptomatic cases increased and sex and age. Urban districts of Wuhan includes Wuchang, Qingshan, Qiaokou, Hanyang, Jiangan, Jianghan, and Hongshan; Suburban districts of Wuhan includes Hannan, Caidian, Dongxihu, Xinzhou, Jiangxia, Huangpi, East Lake High-tech Development Area, and East Lake Scenic Area of Wuhan.

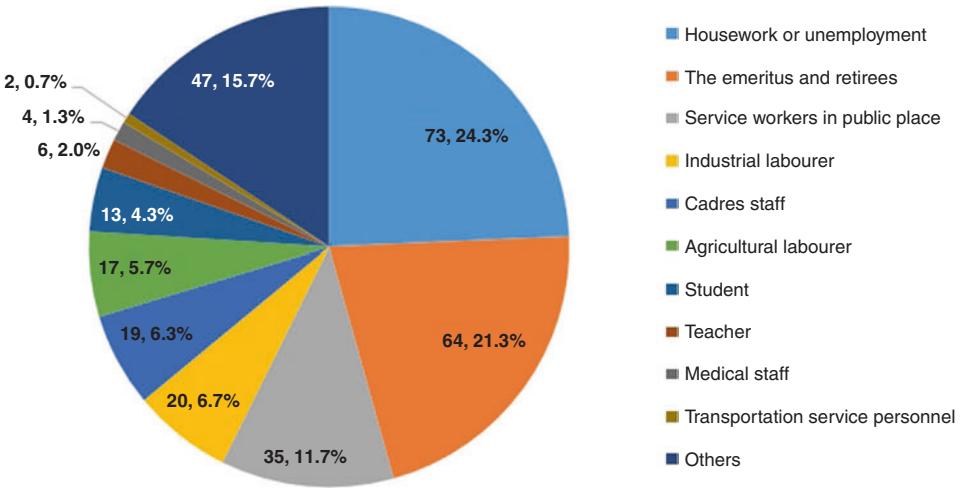

Fig. 1 The occupation distribution of asymptomatic positive cases (%). Note: Others included the self-employed, military personnel, and so on. (Source data are provided as s Source Data file.).

found that asymptomatic positive rates in different districts of Wuhan were correlated with the prevalence of previously con firmed cases. This is in line with the temporal and spatial evo lution (especially the long tailed characteristic) of infectious diseases[11].

Existing laboratory virus culture and genetic studies[9,10] showed that the virulence of SARS CoV 2 virus may be weakening over time, and the newly infected persons were more likely to be asymptomatic and with a lower viral load than earlier infected cases. With the centralized isolation and treatment of all COVID 19 cases during the lockdown period in Wuhan, the risk of residents being infected in the community has been greatly reduced. When susceptible residents are exposed to a low dose of virus, they may tend to be asymptomatic as a result of their own immunity. Serological antibody testing in the current study found that at least 63% of asymptomatic positive cases were actually infected with SARS CoV 2 virus. Nonetheless, it is too early to be complacent, because of the existence of asymptomatic positive cases and high level of susceptibility in residents in Wuhan. Public health measures for the prevention and control of COVID 19 epidemic, including wearing masks, keeping safe social dis tancing in Wuhan should be sustained. Especially, vulnerable populations with weakened immunity or co morbidities, or both, should continue to be appropriately shielded.

Findings from this study show that COVID 19 was well con trolled in Wuhan at the time of the screening programme. After two months since the screening programme (by August 9, 2020), there were no newly confirmed COVID 19 cases in Wuhan.

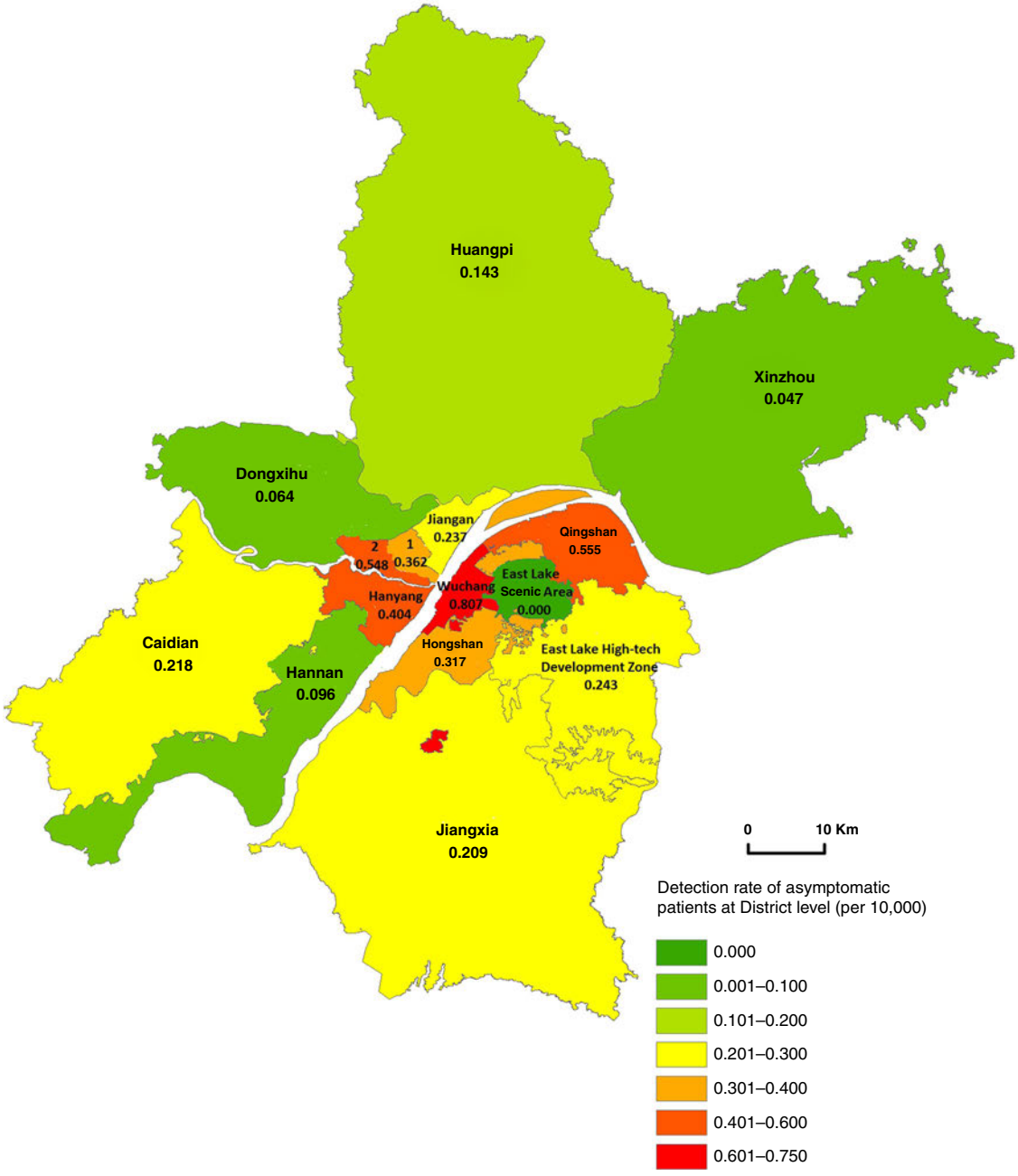

Fig. 2 The geographic distribution of the detection rate of asymptomatic positive cases. Note: 1 represents Jianghan district; 2 represents Qiaokou district. (Source data are provided as s Source Data file.).

Table 2 Results of the detection of antibody in 300 asymptomatic positive persons.

IgM Results	IgG	Asymptomatic positive persons	% (95% CI)
	+	161	53.7 (47.8–59.4)
		110	36.7 (31.2–42.4)
+	+	29	9.7 (6.6–13.6)
+		0	0.0 (0.0–1.2)

"−" indicates negative; "+" indicates positive.

Further testing of SARS CoV 2 in samples collected from market environment settings in Wuhan were conducted, and found no positive results after checking a total of 52,312 samples from 1795 market setting during June 13 to July 2, 2020[12].

This study has several limitations that need to be discussed. First, this was a cross sectional screening programme, and we are unable to assess the changes over time in asymptomatic positive and reoperative results. Second, although a positive result of nucleic acid testing reveals the existence of the viral RNAs, some false negative results were likely to have occurred, in particular due to the relatively low level of virus loads in asymptomatic infected individuals, inadequate collection of samples, and limited accuracy of the testing technology[13]. Although the screening programme provided no direct evidence on the sensitivity and specificity of the testing method used, a meta analysis reported a

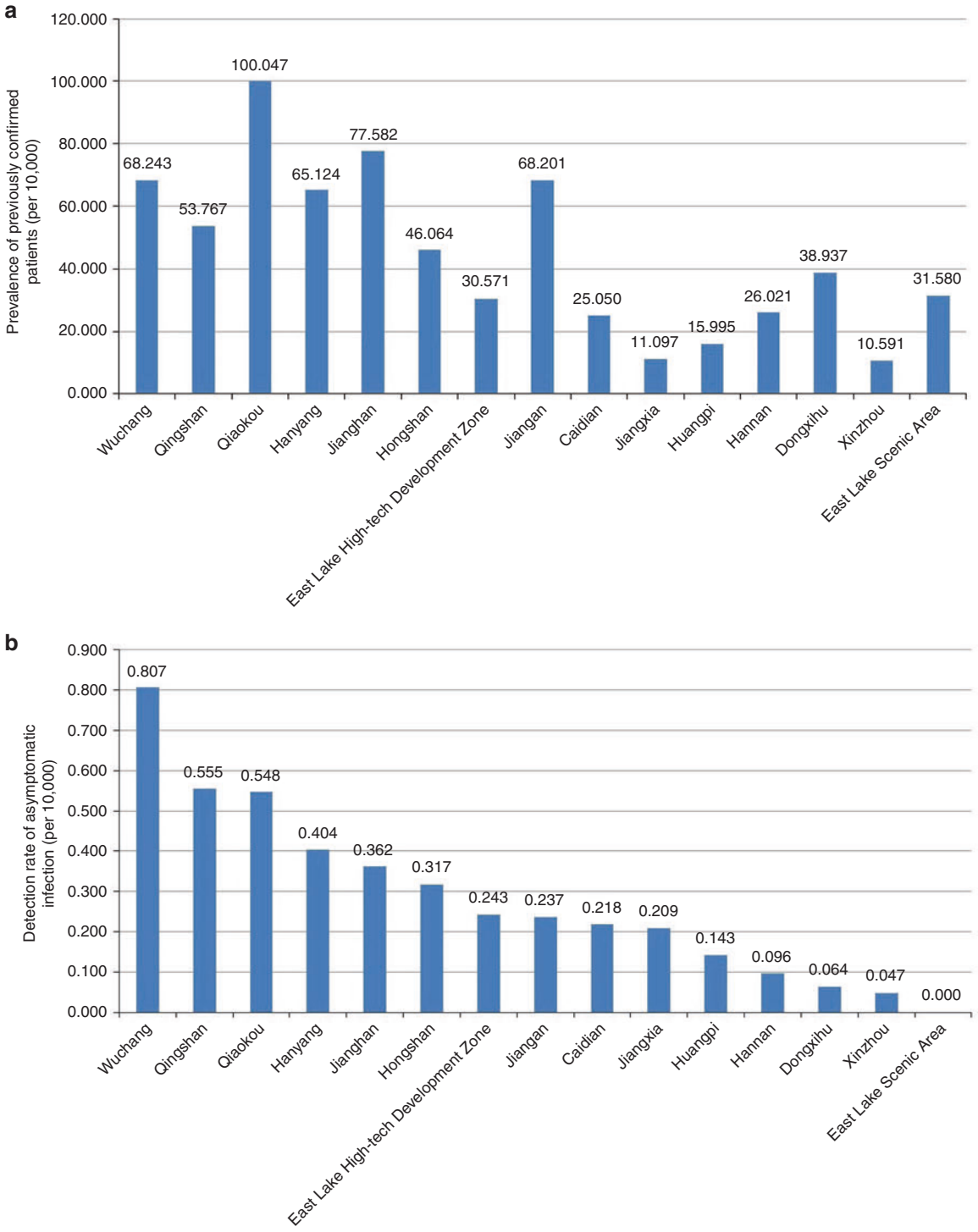

Fig. 3 The prevalence of previously confirmed patients and the detection rate of asymptomatic positive cases of COVID-19 in each district in Wuhan. **a** The prevalence of previously confirmed patients of COVID-19 in each district in Wuhan. **b** The detection rate of asymptomatic positive cases of COVID-19 in each district in Wuhan. (Source data are provided as s Source Data file.).

pooled sensitivity of 73% (95% CI 68 78%) for nasopharayngeal and throat swab testing of COVID 19[14]. Testing kits used in the screening programme were publicly purchased by the govern ment and these kits have been widely used in China and other countries. Multiple measures were taken to possibly minimise false negative results in the screening programme. For example, standard training was provided to health works for sample col lection to ensure the sample quality. The experiment procedures, including specimen collection, extraction, PCR, were according to official guidelines (Supplementary Note 1). For the real time RT PCR assay, two target genes were simultaneously tested. Even so, false negative results remained possible, particularly in any mass screening programmes. However, even if test sensitivity was as low as 50%, then the actual prevalence would be twice as high as reported in this study, but would still be very low. Around 7.1% of eligible residents did not participate in the citywide nucleic acid screening and the screening programme did not collect detailed data on reasons for nonparticipation, which is a limitation of this

study. Although there were no official statistics, a large number of migrant workers and university students left Wuhan before the lockdown, joining their families in other cities or provinces for traditional Chinese New Year. Therefore, it is likely that most nonparticipants were not in Wuhan at the time of the screening. The main objective of the screening programme was to assess the risk of COVID 19 epidemic in residents who were actually living in the post lockdown Wuhan. Therefore, the estimated positive rates are unlikely to be materially influenced by nonparticipation of residents who were not in Wuhan or some residents who did not participate in the screening for other reasons. Moreover, people who left Wuhan were the target population for monitoring in other provinces and cities and were required to take nucleic acid testing. Although there was no official statistics showing the positive rate of nucleic acid testing in this population, there was no report that shown a higher positive rate of nucleic acid testing than our findings.

In summary, the detection rate of asymptomatic positive cases in the post lockdown Wuhan was very low (0.303/10,000), and there was no evidence that the identified asymptomatic positive cases were infectious. These findings enabled decision makers to adjust prevention and control strategies in the post lockdown period. Further studies are required to fully evaluate the impacts and cost effectiveness of the citywide screening of SARS CoV 2 infections on population's health, health behaviours, economy, and society.

Methods

Study population and ethical approvals. Wuhan has about 11 million residents in total, with seven urban and eight suburban districts. Residents are living in 7280 residential communities (or residential enclosures, "xiao-qu" in Chinese), and each residential community could be physically isolated from other communities for preventing transmission of COVID-19.

The screening programme recruited residents (including recovered COVID-19 patients) currently living in Wuhan who were aged ≥6 years (5,162,960 males, 52.2%). All participants provided written or verbal informed consent after reading a statement that explained the purpose of the testing. For participants who aged 6 17 years old, consent was obtained from their parents or guardians. The study protocol for an evaluation of the programme based on anonymized screening data was approved by the Ethics Committee of the Tongji Medical College Institutional Review Board, Huazhong University of Science and Technology, Wuhan, China (No. IROG0003571).

Organizational guarantee and community mobilization. A citywide nucleic acid screening group was formed, with specialized task teams contributing to comprehensive coordination, technical guidance, quality control, participation invitation, information management, communication, and supervision of the screening. The city government invested 900 million yuan (RMB) in the testing programme. From 14 May to 1 June 2020, in the peak time, up to 2907 sample collection sites were functioning at the same time in Wuhan. Each sample collection site had an assigned sample collection group, including several health professionals (staffed according to the number of communities' residents), 2 4 community managers, 1 2 police officers, and 1 2 inspectors. The sampling sites were set up based on the number and accessibility of local residents. Local community workers were responsible for a safe and orderly sampling process to minimise the waiting time. In addition, mobile sampling teams were formed by primary health care professionals and volunteers to conduct door-to-door sampling for residents who had physical difficulties or were unable to walk.

About 50,000 health professionals (mainly doctors and nurses from community health centers) and more than 280,000 person-times of community workers and volunteers contributed to sample collection, transport of equipment and samples collected, arrangement of participation process, and maintaining order of sampling sites. Public information communication and participant invitation were implemented through mass media, mobile messages, WeChat groups, and residential community broadcasts, so as to increase residents' awareness and the participation.

Acquisition, preservation, and transport of samples. All sampling personnel received standard training for the collection of oropharyngeal swab samples. To minimise the risk of cross-infection, the sampling process strictly followed a disinfection process and environmental ventilation were ensured. The collected samples were stored in a virus preservation solution or immersed in isotonic saline, tissue culture solution, or phosphate buffer (Supplementary note 1). Then, all samples were sent to testing institutions within 4 h using delivery boxes for biological samples refrigerated with dry ice to guarantee the stability of nucleic acid samples.

Technical methods for laboratory testing of collected samples. A total of 63 nucleic acid testing laboratories, 1451 laboratory workers and 701 testing equipment were involved in the nucleic acid testing. Received samples were stored at 4 °C and tested within 24 h of collection. Any samples that could not be tested within 24 h were stored at 70 °C or below (Supplementary note 1). In addition to "single testing" (i.e., separate testing of a single sample), "mixed testing" was also performed for 23% of the collected samples to increase efficiency, in which five samples were mixed in equal amounts, and tested in the same test tube. If a mixed testing was positive for COVID-19, all individual samples were separately retested within 24 h[15].

Details regarding technical methods for sequencing and virus culture were provided in Supplementary note 1. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay method was used for the nucleic acid testing. We simultaneously amplified and tested the two target genes: open reading frame 1ab (ORF1ab) and nucleocapsid protein (N) (Supplementary Note 1). A cycle threshold value (Ct-value) less than 37 was defined as a positive result, and no Ct-value or a Ct-value of 40 or more was defined as a negative result. For Ct-values ranging from 37 to 40, the sample was retested. If the retest result remained less than 40 and the amplification curve had obvious peak, the sample was classified as positive; otherwise, it was reported as being negative. These diagnostic criteria were based on China's official recommendations[16].

For asymptomatic positive cases, virus culture was carried out in biosafety level-3 laboratories. The colloidal gold antibody test was also performed for asymptomatic positive cases (Supplementary note 1). All testing results were double entered into a specifically designed database, and managed by the Big Data and Investigation Group of the COVID-19 Prevention and Control Centre in Wuhan, which was established to collect and manage data relevant to the COVID-19 epidemic.

Participant data collection and management. Before sample collection, residents electronically (using a specifically designed smartphone application) self-uploaded their personal information, including ID number, name, sex, age, and place of residence. Then, the electronic machine system generated a unique personal barcode and stuck it on the sample tube to ensure the match between the sample and the participant. Then trained staff interviewed each individual regarding the history of COVID-19 and previous nucleic acid testing. There was a database of confirmed COVID-19 cases in Wuhan, which can be used to validate the self-reported previous COVID-19 infection. All information was entered into a central database. The testing results were continually uploaded to the central database by testing institutions. Contact tracing investigations were conducted on participants who tested positive for SARS-CoV-2, to track and manage their close contacts. The pre-existing unique identification code for each resident was used as the programme's identification number, to ensure information accuracy during the whole process of screening, from sampling, nucleic acid testing, result reporting, the isolation of detected positive cases, and tracing of close contacts of positive cases. All screening information was kept strictly confidential and was not allowed to be disclosed or used for other purposes other than clinical and public health management. Personal information of asymptomatic positive cases was only disclosed to designated medical institutions and community health centres for the purpose of medical isolation and identification of close contacts. Researcher was blind to the study hypothesis during data collection.

Biological security guarantee. Nucleic acid testing was performed in biosafety level-2 (BSL-2) laboratories, and virus culture was conducted in biosafety level-3 laboratories. Sampling and testing personnel adopted the personal protective measures according to the standard of biosafety level-3 laboratories. Participating laboratories implemented control measures to guarantee biological safety in accordance with relevant regulations[17].

Result query and feedback. Two to three days after sample collection, participants could inquire about their test results using WeChat or Alipay application by their unique ID numbers. The results included text descriptions of nucleic acid testing and coloured health codes. A green coloured health code refers to a negative result, and a red coloured health code indicates a positive result.

Definition and management of identified confirmed cases and close contacts. In this study, all confirmed COVID-19 cases were diagnosed by designated medical institutions according to National Guidelines for the Prevention and Control of COVID-19 (Supplementary Note 2). Asymptomatic positive cases referred to individuals who had a positive result during screening, and they had neither a history of COVID-19 diagnosis, nor any clinical symptoms at the time of the nucleic acid testing. Close contacts were individuals who closely contacted with an asymptomatic positive person since 2 days before the nucleic acid sampling[16]. Repositive cases refer to individuals who recovered from previously confirmed COVID-19 disease and had a positive testing again in the screening programme. All repositive cases, asymptomatic positive persons, and their close contacts were

isolated for at least 2 weeks in designated hotels managed by primary health care professionals, and they were released from isolation only if two consecutive nucleic acid tests were negative.

Statistical analysis. Detection rate of asymptomatic positive or repositive cases was calculated by dividing the number of individuals with a positive result of nucleic acid testing by the number of participants tested. Because of extremely low detection rates, we calculated 95% confidence intervals of estimated proportions using Pearson Klopper exact method, implemented through R package "binom" version 1.1-1[18]. SPSS version 22.0 was used for other statistical analyses. We analyzed the distribution of asymptomatic positive cases and assessed the Spearman correlation between the asymptomatic positive rate and the prevalence of previously confirmed COVID-19 cases in different districts of Wuhan. Differences in asymptomatic positive rates by sex and age groups were assessed using the χ^2 test. ArcGIS 10.0 was used to draw a geographic distribution map of asymptomatic positive cases. A value of $P < 0.05$ (two-tailed) was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Detailed data directly used to generate each figure or table of this study are available within the article, Supplementary Information and source data are provided with this paper.

Received: 18 August 2020; Accepted: 27 October 2020;
Published online: 20 November 2020

References

1. WHO. *Coronavirus disease 2019 (COVID-19) Situation Report—51. Data as reported by national authorities by 10 AM CET 11 March 2020* (WHO, 2020).
2. Prevention measures taken at Sanmin residential community in Wuhan Xinhua | English.news.cn http://www.xinhuanet.com/english/2020-05/11/c_139048342.htm (2020).
3. Gandhi, M., Yokoe, D. S. & Havlir, D. V. Asymptomatic transmission, the Achilles' heel of current strategies to control Covid-19. *N. Engl. J. Med.* **382**, 2158 2160 (2020).
4. He, D. et al. The relative transmissibility of asymptomatic COVID-19 infections among close contacts. *Int. J. Infect. Dis.* **94**, 145 147 (2020).
5. Arons, M. M. et al. Presymptomatic SARS-CoV-2 infections and transmission in a skilled nursing facility. *N. Engl. J. Med.* **382**, 2081 2090 (2020).
6. KCDC. *Findings from investigation and analysis of re-positive cases (notice). Division of Risk assessment and International cooperation 2020-05-19*. https://www.cdc.go.kr/board/board.es?mid=a30402000000&bid=0030 (2020).
7. National Health Commission. *News conference on the prevention and control of COVID-19. Beijing, 21-04-2020*. http://www.nhc.gov.cn/xcs/fkdt/202004/3e16b2976000411da737c70523e05522.shtml. (2020).
8. Li, Y. et al. Positive result of Sars-Cov-2 in faeces and sputum from discharged patient with COVID-19 in Yiwu, China. *J. Med. Virol*. https://doi.org/10.1002/jmv.25905 (2020).
9. Su, Y. C. F. et al. Discovery and Genomic Characterization of a 382-Nucleotide Deletion in ORF7b and ORF8 during the Early Evolution of SARS-CoV-2. *mBio* **11**, e01610-20 (2020).
10. Lin, Z. *Italian scientist: the virulence of SARS-Cov-2 is weakening, the newly infected person are almost asymptomatic* (Chinanews, 2020).
11. Ajelli, M. et al. Spatiotemporal dynamics of the Ebola epidemic in Guinea and implications for vaccination and disease elimination: a computational modeling analysis. *BMC Med.* **14**, 130 (2016).
12. Wuhan Municipal Health Commission. *All results were negative by checking 52312 samples from 1795 supermarket and other market environment setting for 20 days (news)*. http://wjw.wuhan.gov.cn/ztzl 28/fk/tzgg/202007/t20200702 1389323.shtml. (2020).
13. Woloshin, S., Patel, N. & Kesselheim, A. S. False negative tests for SARS-CoV-2 infection -challenges and implications. *N. Engl. J. Med.* **383**, e38 (2020).
14. Boger, B. et al. Systematic review with meta-analysis of the accuracy of diagnostic tests for COVID-19. *Am. J. Infect. Control* S0196-6553(20)30693-3. Advance online publication. https://doi.org/10.1016/j.ajic.2020.07.011 (2020).
15. Lohse, S. et al. Pooling of samples for testing for SARS-CoV-2 in asymptomatic people. *Lancet Infect. Dis.* **20**, 1231 1232. https://doi.org/10.1016/S1473-3099(20)30362-5 (2020).
16. National Health Commission of the People's Republic of China. *The prevention and Control Plan of COVID-19* 5th edition (National Health Commission of the People's Republic of China, 2020).
17. European Centre for Disease Prevention and Control (ECDC). *Laboratory support for COVID-19 in the EU/EEA*. (ECDC, 2020).
18. Dorai-Raj, S. *Package'binom'—Binomial Confidence Intervals For Several Parameterizations. Version 1.1-1*. https://cran.r-project.org/web/packages/binom/binom.pdf (2014).

Acknowledgements

We would like to thank all institutions and all citizens in Wuhan for their support for citywide nucleic acid screening work. We also would like to thank the Wuhan city government for this citywide nucleic acid testing, sampling and management, and thank the big data and investigation group of COVID-19 prevention and control institution in Wuhan (the data and investigation group of Wuhan Municipal Health Commission) for their efforts in the data collection. In addition, we would like to thank the National Social Science Foundation of China (Grant No. 18ZDA085) for supporting the fund.

Author contributions

S.Y.C., C.W., X.X.Y., and Z.X.L. conceived the study. C.W., Y.C.H., T.T.W., K.L., H.B.X., and X.S. participated in the acquisition of data. S.B.W. and J.G. were responsible for the on-site specimen collection, laboratory testing quality evaluation, and control. Y.C.H., T.T.W., and L.Q.L. analyzed the data. H.J., Y.H.G., and F.J.S. gave advice on methodology. Q.F.T. and C.Z.L. investigated the responses to the citywide nucleic acid testing among residents lived in outside of Wuhan city. S.Y.C., Y.G., C.W., and X.X.Y. drafted the manuscript, Y.G., M.B., and F.J.S. revised the manuscript, and M.B., C.Z.L., and F.J.S. critically commented and edited the manuscript. All authors read and approved the final manuscript. Z.X.L. is the guarantor of this study.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-19802-w.

Correspondence and requests for materials should be addressed to C.L., F.S., X.Y. or Z.L.

Peer review information *Nature Communications* thanks Junxiong Vincent Pang and the other, anonymous reviewer(s) for their contribution to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Exhibit "C"

This is the Affidavit of

Dr. Mark Trozzi

sworn before me this 12 day of April, 2021.

Commissioner of Oaths

[redacted]

Physicians: COVID vaccines are 'experimental' and should never be mandated or forced

'We will fight against any experimental therapy being forced on anyone.'

Wed Dec 16, 2020 - 4:42 pm EST

ATLANTA, Georgia, December 16, 2020 (LifeSiteNews) – A group of U.S. doctors held a protest in front of the Centers for Disease Control and Prevention (CDC) in Atlanta on Sunday warning the government against forcing millions of Americans to take an "experimental" COVID-19 vaccine that would amount to the "largest medical experimentation program in U.S. history."

Dr. Simone Gold, MD, JD, founder of America's Frontline Doctors, said that while the accelerated rollout of the COVID-19 vaccine according to President Trump's "Warp Speed" program is an "impressive" logistical accomplishment, the vaccination program itself "should be approached with caution."

"Vaccination mandates at the state, local, and private level are incompatible with civil liberties and subject millions of Americans to an experimental drug," Gold said in a press release.

"For this reason and more, America's Frontline Doctors is asking regulators to open up the process to additional public comment and transparency before millions more doses are manufactured and administered," she added.

Gold told LifeSiteNews that "most Americans should not consider an experimental drug for an illness with a 99.7 percent survival rate as the risk of the experimental treatment would exceed any benefit."

"Doctors take a Hippocratic Oath to do no harm. America's Frontline Doctors is calling for greater transparency for concerned citizens and better data for policy makers before we embark on the largest experimental vaccination program in history. Experimental vaccinations must always be an informed decision between a doctor and his or her patient that takes into consideration a plurality of risk factors including patient age, comorbidities, and exposure risks," she added.

The U.S. Food and Drug Administration (FDA) announced Friday that it has approved the emergency use of the Pfizer-BioNTech COVID-19 vaccine for individuals over 16 years of age. The CDC states that there is a "lack of data on the safety and efficacy" of the vaccine when it comes to various questions, such as if the vaccine is safe to use with other vaccines, if it should be used by persons with "HIV infection, other immunocompromising conditions," and if it can be safely taken by "pregnant people."

Gold said in a press release that her group is not "anti-vaccination." She added, however, that Americans should not be required to "sacrifice their constitutionally protected rights and, possibly, their health for an experimental vaccine that has not demonstrated its safety and effectiveness using the same rigorous scientific standards we demand of other drugs."

"We are therefore counseling caution among patients and their prescribing physicians and calling on the federal government to take these safety concerns seriously before administering the largest vaccination program in our country's history," she said.

The group of doctors released a position paper where they expressed their concerns about COVID-19 vaccines.

- The doctors call for more research into the vaccine's safety and effectiveness, including questions about "pathogenic priming resulting in sudden severe cytokine storm and possible fertility side effects in women of childbearing age."
- They lay out a number of already-known safety concerns regarding the mRNA vaccines developed by Pfizer/BioNTech and Moderna, including that these vaccines are a "brand new technology" from which "unexpected things must be expected," that there are no "independently published animal studies" on the vaccines, that there are "known complications" that are being minimized, that there are "unknown complications" regarding how the vaccines will affect an "enormous percentage of the population," that the creators of the vaccines are "immune from all liability," and finally, that the high rate of recovery of those who contract COVID-19 does not justify taking an experimental vaccine with unknown complications.
- The doctors lay out why the vaccines must be labeled properly as "experimental" since the pharmaceutical companies are clear in their advisories to the FDA that the products are "investigational."

The doctors in their paper point out that in medicine the guiding principle is "first, do no harm."

"Widely distributing a COVID-19 experimental vaccine before adequately addressing and clinically evaluating the above concerns is reckless," they write, adding, " This is especially true in adults under 50 years old who have an infection survival rate of about 99.98 percent, and even lower in those without high-risk comorbidities."

The doctors state that vaccination "must always be an informed decision between a doctor and his/her patient that takes into consideration a plurality of risk factors including patient age, comorbidities and exposure risks."

"Every patient is unique both in mind and body. It is in the sacrosanct relationship between a patient and doctor that these differences are explored, not by a politician or remote health authority that will never face a patient or grieving family member to report bad news from a medical intervention," they add.

America's Frontline Doctors has created a petition, signed by nearly 10,000 people, demanding that American citizens not be "intimidated or pressured into taking experimental vaccines."

“Your health and medical conditions are personal and private and nobody should be permitted to violate that, including an employer, an airline, or a government agency,” the petition states.

The petition states that any “business, employer, or school that mandates or otherwise attempts to force a vaccine” will be actively blacklisted and boycotted.

The group of doctors stated in a Dec. 13 Facebook post that the “most pressing issue is to disrupt the airline industry's decision to mandate this experiment on us as a requirement for travel.”

“Once the airline industry mandates proof of the experimental vaccine, freedom of movement in the USA is gone,” the doctors’ group states.

Exhibit "D"

This is the Affidavit of

Dr. Mark Trozzi

sworn before me this 12 day of April, 2021.

Commissioner of Oaths

[redacted]

Immunization with SARS Coronavirus Vaccines Leads to Pulmonary Immunopathology on Challenge with the SARS Virus

Chien-Te Tseng[1,2], Elena Sbrana[1], Naoko Iwata-Yoshikawa[1,2], Patrick C. Newman[1], Tania Garron[1], Robert L. Atmar[3,4], Clarence J. Peters[1,2], Robert B. Couch[3,4]*

1 Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, Texas, United States of America, **2** Center for Biodefense and Emerging Disease, The University of Texas Medical Branch, Galveston, Texas, United States of America, **3** Department of Medicine, Baylor College of Medicine, Houston, Texas, United States of America, **4** Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, United States of America

Abstract

Background: Severe acute respiratory syndrome (SARS) emerged in China in 2002 and spread to other countries before brought under control. Because of a concern for reemergence or a deliberate release of the SARS coronavirus, vaccine development was initiated. Evaluations of an inactivated whole virus vaccine in ferrets and nonhuman primates and a virus-like-particle vaccine in mice induced protection against infection but challenged animals exhibited an immunopathologic-type lung disease.

Design: Four candidate vaccines for humans with or without alum adjuvant were evaluated in a mouse model of SARS, a VLP vaccine, the vaccine given to ferrets and NHP, another whole virus vaccine and an rDNA-produced S protein. Balb/c or C57BL/6 mice were vaccinated IM on day 0 and 28 and sacrificed for serum antibody measurements or challenged with live virus on day 56. On day 58, challenged mice were sacrificed and lungs obtained for virus and histopathology.

Results: All vaccines induced serum neutralizing antibody with increasing dosages and/or alum significantly increasing responses. Significant reductions of SARS-CoV two days after challenge was seen for all vaccines and prior live SARS-CoV. All mice exhibited histopathologic changes in lungs two days after challenge including all animals vaccinated (Balb/C and C57BL/6) or given live virus, influenza vaccine, or PBS suggesting infection occurred in all. Histopathology seen in animals given one of the SARS-CoV vaccines was uniformly a Th2-type immunopathology with prominent eosinophil infiltration, confirmed with special eosinophil stains. The pathologic changes seen in all control groups lacked the eosinophil prominence.

Conclusions: These SARS-CoV vaccines all induced antibody and protection against infection with SARS-CoV. However, challenge of mice given any of the vaccines led to occurrence of Th2-type immunopathology suggesting hypersensitivity to SARS-CoV components was induced. Caution in proceeding to application of a SARS-CoV vaccine in humans is indicated.

Citation: Tseng C T, Sbrana E, Iwata Yoshikawa N, Newman PC, Garron T, et al. (2012) Immunization with SARS Coronavirus Vaccines Leads to Pulmonary Immunopathology on Challenge with the SARS Virus. PLoS ONE 7(4): e35421. doi:10.1371/journal.pone.0035421

Editor: Stefan Poehlmann, German Primate Center, Germany

Received January 31, 2012; **Accepted** March 15, 2012; **Published** April 20, 2012

Funding: Research performed by the authors and summarized in this report was supported by Public Health Service Contract NO1 AI 30039 from the National Institute of Allergy and Infectious Diseases. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E mail: rcouch@bcm.edu

Introduction

Severe acute respiratory syndrome (SARS) emerged in Guangdong, People's Republic of China, in late 2002, and spread to other countries in Asia and to Canada in the ensuing months [1 3]. Infection control efforts brought the infection under control by mid 2003 [4]. More than 8000 cases, including almost 800 deaths, were reported during the outbreak period [4]. Increasing age and comorbidity were risk factors for severe disease and death [5,6,7]. Since 2003, only sporadic cases have been reported; however, the possibility that SARS outbreaks could reemerge naturally or be deliberately released is a public health concern.

SARS is caused by a Coronavirus (SARS CoV) [8,9]. Limited data are available about the ecology of SARS CoV, but bats are thought to be the animal reservoir for the virus which may be transmitted to small mammals with exposure to these small animals as the source of human infections [10]. The clinical disease is similar to other severe acute respiratory infections, including influenza; the SARS case definition includes clinical, epidemiologic, and laboratory criteria [11,12]. A number of therapeutic efforts were employed for the disease in Asia and in Canada; however, no treatment of clear value was identified. Animal models were developed using mice, hamsters, ferrets and

nonhuman primates, and efforts to identify useful treatments and effective vaccines are ongoing.

Vaccine candidates for preventing SARS have been developed by various groups and include inactivated whole virus, spike (S) protein preparations, virus like particles (VLPs), plasmid DNA and a number of vectors containing genes for SARS CoV proteins [13 28]. Phase I studies in humans have been conducted with a whole virus vaccine and a DNA vaccine [29 30].

An early concern for application of a SARS CoV vaccine was the experience with other coronavirus infections which induced enhanced disease and immunopathology in animals when challenged with infectious virus [31], a concern reinforced by the report that animals given an alum adjuvanted SARS vaccine and subsequently challenged with SARS CoV exhibited an immunopathologic lung reaction reminiscent of that described for respiratory syncytial virus (RSV) in infants and in animal models given RSV vaccine and challenged naturally (infants) or artificially (animals) with RSV [32,33]. We and others described a similar immunopathologic reaction in mice vaccinated with a SARS CoV vaccine and subsequently challenged with SARS CoV [18,20,21,28]. It has been proposed that the nucleocapsid protein of SARS CoV is the antigen to which the immunopathologic reaction is directed [18,21]. Thus, concern for proceeding to humans with candidate SARS CoV vaccines emerged from these various observations.

The studies reported here were conducted to evaluate the safety, immunogenicity, and efficacy of different SARS CoV vaccines in a murine model of SARS.

Materials and Methods

Tissue Cultures and Virus

Vero E6 tissue cultures [obtained from The American Type Culture Collection (ATCC), CRL:1586] were grown in Dulbec co's modified minimum essential medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS). The Urbani strain of SARS CoV was obtained from T.G. Ksiazek at the Centers for Disease Control and Prevention (Atlanta, GA), and a working stock of this virus was prepared by serially passaging a portion of the seed virus three times (p3) in Vero E6 cultures. The culture fluid from infected cells was clarified by low speed centrifugation, filtered through a 0.45 µm filter, aliquoted, and stored at 80°C.

Vaccines

Four different SARS CoV vaccines were evaluated in these studies (Table 1). Two whole virus vaccines were evaluated; one was prepared in Vero tissue cultures, zonal centrifuged for purification, and double inactivated with formalin and UV irradiation, the DI vaccine (DIV); it was tested with and without alum adjuvant [16]. The other whole virus vaccine was prepared in Vero cells, concentrated, purified, inactivated with beta propiolactone and packaged with alum adjuvant (BPV) [13]. A recombinant DNA spike (S) protein vaccine (SV) was produced in insect cells and purified by column chromatography was tested with and without alum adjuvant [17]. The fourth vaccine (the VLP vaccine) was a virus like particle vaccine prepared by us as described previously; it contained the SARS CoV spike protein (S) and the Nucleocapsid (N), envelope (E) and membrane (M) proteins from mouse hepatitis coronavirus (MHV) [20].

Animals

Six to eight week old, female Balb/c and C57BL/6 mice (Charles River Laboratory, Wilmington, MA), were housed in cages covered with barrier filters in an approved biosafety level 3 animal facility maintained by the University of Texas Medical Branch (UTMB) at Galveston, Texas. All of the experiments were performed using experimental protocols approved by the Office of Research Project Protections, Institutional Animal Care and Use Committee (IACUC), University of Texas Medical Branch and followed National Institutes of Health and United States Department of Agriculture guidelines.

Study Design

Three different experiments, performed for comparing different vaccines, are reported here. Adjuvanted (alum) and non adjuvanted (PBS) vaccines were obtained from the NIH/BEI resource. Groups of mice (N 12 13 per group) were adminis tered various dosages of each vaccine intramuscularly (IM) on days 0 and 28; mice given only PBS, alum, trivalent inactivated influenza vaccine or live SARS CoV were included as controls in various experiments. On day 56, five mice from each group were sacrificed for assessing serum neutralizing antibody titers and lung histopathology; the remaining seven or eight mice in each group were challenged with 10^6TCID$_{50}$/60 µl of SARS CoV intrana sally (IN). Challenged mice were euthanized on day 58 for determining virus quantity and preparing lung tissue sections for histopathologic examination.

Neutralizing Antibody Assays

Mice were anesthetized with isoflurane and then bled from the retro orbital sinus plexus. After heat inactivation at 56°C for 30 minutes, sera were stored at 80°C until tested. Assays for virus specific neutralizing antibodies were performed on serial 2 fold diluted samples of each serum using 2% FBS DMEM as the diluent in 96 well tissue culture plates (Falcon 3072); the final volume of the serially diluted samples in each well was 60 µl after addition of 120 TCID$_{50}$ of SARS CoV in 60 µl into each well. The beginning dilution of serum was 1:20. The dilutions were incubated for 45 60 minutes at room temperature; then 100 µl of each mixture was transferred into duplicate wells of confluent Vero E6 cells in 96 well microtiter plates. After 72 hours of incubation, when the virus control wells exhibited advanced virus induced CPE, the neutralizing capacity of individual serum samples were assessed by determining the presence or absence of cytopathic effect (CPE). Neutralizing antibody titers were expressed as the reciprocal of the last dilution of serum that completely inhibited virus induced CPE.

Collection and Processing of Lungs for Histology and Virus Quantity

Two days post SARS CoV challenge, mice were euthanized and their lungs were removed. Lung lobes were placed in 10% neutral buffered formalin for histological examination and immunohistochemistry (IHC), as described previously [34,35]. For virus quantitation, the remaining tissue specimen was weighed and frozen to 80°C. Thawed lung was homogenized in PBS/ 10% FBS solution using the TissueLyser (Qiagen; Retsch, Haan, Germany). The homogenates were centrifuged and SARS CoV titers in the clarified fluids were determined by serial dilution in quadruplicate wells of Vero E6 cells in 96 well plates. Titers of virus in lung homogenates were expressed as TCID$_{50}$/g of lung ($\log_{10}$); the minimal detectable level of virus was 1.6 to 2.6 $\log_{10}$ TCID$_{50}$ as determined by lung size.

Table 1. Experimental Groups for Evaluation of SARS Coronavirus Vaccines.

Group	Exp 1[1] Vaccine Comparisons	Exp 2[1] Higher SV Dosage plus DIV and BPV Comparisons	Exp 3[1,3] Mouse and Vaccine Specificity
1	DIV/1 μg[2]	PBS	PBS PBS
2	DIV/0.5 μg	Live virus	PBS
3	DIV/0.25 μg	SV/9 μg	Live virus
4	DIV/0.125 μg	SV/3 μg	Flu vaccine
5	DIV/1 μg + alum	SV/1 μg	DIV/1 μg
6	DIV/0.5 μg + alum	SV/9 μg + alum	DIV/1 μg + alum
7	DIV/0.25 μg + alum	SV/3 μg + alum	BPV/undil + alum
8	DIV/0.125 μg + alum	SV/1 μg + alum	PBS PBS
9	SV/2 μg[2]	DIV/1 μg	PBS
10	SV/1 μg	DIV/0.25 μg (50 μl)	Live virus
11	SV/0.5 μg	DIV/1 μg + alum	Flu vaccine
12	SV/0.25 μg	DIV/0.25 μg + alum (50 μl)	DIV/1 μg
13	SV/2 μg + alum	BPV/undil + alum[2]	DIV/1 μg + alum
14	SV/1 μg + alum	BPV/undil + alum (25 μl)	BPV/undil + alum
15	SV/0.5 μg + alum		
16	SV/0.25 μg + alum		
17	VLP/2 μg[2]		
18	VLP/2 μg + alum		
19	Alum		
20	PBS		

[1]Design = All experiments in Balb/c mice except as noted in Exp 3. Each group contained 12 13 mice; all were given 100 μl of vaccine IM at dosages with or without alum as indicated on days 0 and 28 except as noted. Five mice in each group were sacrificed on day 56 for serum antibody; remaining mice were given 10^6 $TCID_{50}$ of SARS CoV intranasal on day 56 and sacrificed on day 58 for virus and lung histology.
[2]DIV/dosage = Vaccine DIV = Zonal centrifuge purified doubly inactivated (formalin and UV) whole virus SV/dosage = Vaccine SV = Recombinant baculovirus expressed S glycoprotein of SARS CoV VLP/dosage = Vaccine VLP = Virus like particles containing SARS CoV S glycoprotein and E, M, and N proteins from mouse hepatitis coronavirus BPV/dosage = Vaccine BPV = Purified beta propiolactone inactivated whole virus plus alum.
[3]Experiment 3 = Groups 1 to 7 were Balb/c mice; groups 8 to 14 were C57BL/6 mice. Flu vaccine was licensed trivalent 2009 10 formulation of high dosage vaccine (60 μg of HA of each strain). Groups 1 and 8 were given PBS (placebo) and challenged with PBS; all others were challenged with live SARS CoV.
doi:10.1371/journal.pone.0035421.t001

Histopathology

Evaluations for histopathology were done by pathologists masked as to the vaccine/dosage of each specimen source; numeric scores were assigned to assess the extent of pathologic damage and the eosinophilic component of the inflammatory infiltrates.

Statistical Analysis

Neutralizing antibody titers, lung virus titers, histopathologic lesion score and eosinophilic infiltration scores were averaged for each group of mice. Comparisons were conducted using parametric and nonparametric statistics as indicated.

Results

Experiments

The three experiments performed, vaccines and dosages used and controls for each experiment are shown in Table 1. The vaccines were evaluated for immunogenicity and efficacy; however, because of the previous report of immunopathology on challenge of ferrets and nonhuman primates that had been vaccinated with a whole virus adjuvanted vaccine and mice that had been vaccinated with a VLP vaccine, the primary orientation was to assess for immunopathology among animals in relation to type of vaccine, dosage, serum antibody responses, and virus infection. The vaccine preparations were made for human trials so identifying a preparation that was likely to be both safe and protective in humans was desired. The rationale for each experiment is described.

Comparison of Vaccines (Experiment 1). To differentiate between vaccines, three vaccine preparations were simultaneously evaluated, the double inactivated (formalin and UV) whole virus vaccine (DIV), the rDNA expressed S protein vaccine (SV), and the previously evaluated chimeric viral like particle vaccine (VLP) that had led to immunopathology with virus challenge [16,17,20].

Geometric mean serum neutralizing antibody titers for each group on day 56 are shown in figure 1A. Geometric mean titers for those given a nonadjuvanted or alum adjuvanted vaccine were not different for the double inactivated whole virus vaccine (DIV), and the VLP vaccine, ($p>0.05$, student's t test), but were different for the S protein vaccine (SV) (p 0.001, student's t test). Geometric mean titers for the different dosage groups given the DI vaccine (DIV) with alum and those for the groups given the S protein vaccine (SV) with or without alum were significantly different (p 0.007, p 0.028, and p 0.01, respectively, Kruskall Wallis) while the geometric means for those dosage groups given the DI vaccine (DIV) without alum were not ($p>0.05$, Kruskall Wallis). In a multiple regression analysis, postvaccination titers for the DI vaccine (DIV) were significantly increased by both alum and higher dosage (for alum, p 0.012, for dosage, $p<0.001$); for the S protein vaccine (SV), only alum increased responses (p 0.001).

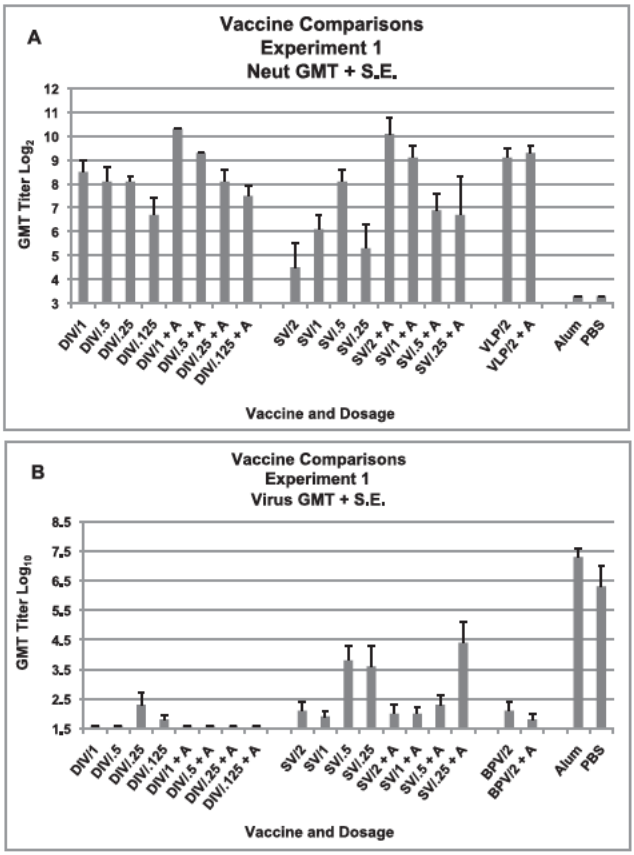

Figure 1. Vaccine Comparisons of Three SARS CoV Vaccines, Experiment 1. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer as log_2 and standard error of the mean (S.E.) on day 56 for each vaccine dosage group. Seven to eight mice per group. Vaccines: double inactivated whole virus (DIV), recombinant S protein (SV), viral like particle vaccine (VLP), with alum (+A). Five mice per group were given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS CoV challenge) for each vaccine dosage group. Analyses: A. GMT with compared to without alum: DIV p>.05, VLP p>.05, SV p = .001. GMT for different vaccine dosage: DIV with alum p = .007, DIV without alum p>.05, SV with alum p = .028, SV without alum p = .01. Multiple regression: GMT increased for alum p = .012 and dosage p<.001, for SV alum only p = .001. B. GMT for all DIV groups not different p>.05, GMT for SV group without alum p .008 and with alum p .023. GMT for VLP group is not different p>.05.
doi:10.1371/journal.pone.0035421.g001

Two days after challenge, lungs were obtained from all animals for virus quantitation and histology. CoV titers are shown in figure 1B. Geometric mean lung titers in the alum and PBS control groups were $10^{7.3}$ and $10^{6.3}$ $TCID_{50}$/g, respectively. All vaccine groups exhibited lower titers or no detectable virus on day two after challenge. None of the animals given any of the alum adjuvanted DI vaccine (DIV) dosages and only an occasional animal in the lower dosages of nonadjuvanted vaccine yielded virus (Kruskall Wallis and Mann Whitney U tests, p>0.05 for all comparisons). All groups given the S protein vaccine (SV) yielded virus after challenge and the differences between groups were significant (p 0.002 for all groups, p 0.023 for alum and p 0.008 for no adjuvant, Kruskall Wallis); also, geometric mean titers were higher for the groups given lower vaccine dosages. Geometric mean titers for the VLP vaccine groups were similar (p>0.05).

In the vaccine comparison experiment, lung lesion scores for histopathology were graded for individual animals on a scale of 0 to 4 where 0 2 represented degree of cellular infiltration and 3 4 represented the degree of bronchiolar epithelial cell necrosis and airway cellular debris (figure 2A). As shown, all animals exhibited pathologic changes after challenge including those animals with no measurable virus on day two suggesting virus infection had occurred but was not detectable on day two because of a short duration of infection or neutralization of virus by antibody in the lung during processing. The higher scores (>3) in some groups related primarily to the fact that virus infection had induced inflammatory infiltrates and epithelial cell necrosis with desqua mation of the epithelium and collection of cellular debris in airways of these animals. Mean score differences were noted among the various vaccines (p <0.001, Anova). Those groups given the DI vaccine (DIV) without alum had higher mean scores than did those given DI vaccine (DIV) with alum (p 0.001, Mann Whitney U); similarly, the group given the VLP vaccine without alum had a higher mean score than for those given VLP vaccine with alum (p 0.008, Mann Whitney U). Post hoc comparisons for the three different vaccines indicated that the DI vaccine (DIV) group overall had lower lesion scores than either the S protein vaccine (SV) group or the alum and PBS control groups (p 0.001 comparing the DI and S protein vaccines (DIV and SV) and p<0.001 for DIV vs. control groups, Tukey HSD and Dunnett t, respectively), but not the VLP vaccine group (p>0.05, Tukey HSD). The S protein vaccine group (SV) was also lower overall than the control groups (p 0.048, Dunnett t).

When the characteristics of the infiltrates were compared, animals given alum or PBS exhibited epithelial cell necrosis and peribronchiolar and perivascular mononuclear cell infiltrates consistent with epithelial cell infection and an inflammatory response seen in viral infections. In addition to mononuclear cells, however, infiltrates among vaccinated animals contained neutro phils and eosinophils that were not seen in the lesions of the animals that had been previously given PBS or alum only (figure 2B) suggesting a T helper cell type 2 hypersensitivity reaction; increased eosinophils are a marker for a Th2 type hypersensitivity reaction. Percent eosinophils was lower in these vaccinated animals (mean 1 3.2%) than had been seen in animals given VLP vaccines in the earlier study (mean 13.2±9.6% and 22±9.9% of cells for VLP with PBS or alum, respectively in that study) but no (0%) eosinophils were seen in the lung infiltrates of control animals in this experiment. This pattern of excess eosinophils in cellular infiltrates seen in lung sections from animals given vaccine and not in control animals was as seen in the earlier study with VLP vaccine and those later with other vaccines although the percent eosinophils was lower in this study.

The mean percent eosinophils differed between groups (p<0.001, Anova). Overall, the percent was lower for the groups given the DI and S protein alum adjuvanted vaccines than for the corresponding nonadjuvanted group (p 0.049 for DIV and 0.001 for SV, Mann Whitney U). For the vaccines, the eosinophil mean percentages were lower for the S protein vaccine (SV) than for either the DI vaccine (DIV) or VLP vaccine (DIV vs. SV, p 0.002; VLP vs. SV, p <0.001, Tukey HSD). Additionally, eosinophil percentages for all three vaccines, including the S protein vaccine, were significantly greater than the controls (SV, DIV and VLP vaccine, p<0.001 for each, Tukey HSD).

Higher Dosages of the S Protein Vaccine Plus the bp Inactivated Whole Virus Vaccine, Experiment 2. This experiment was conducted to verify the findings in the initial

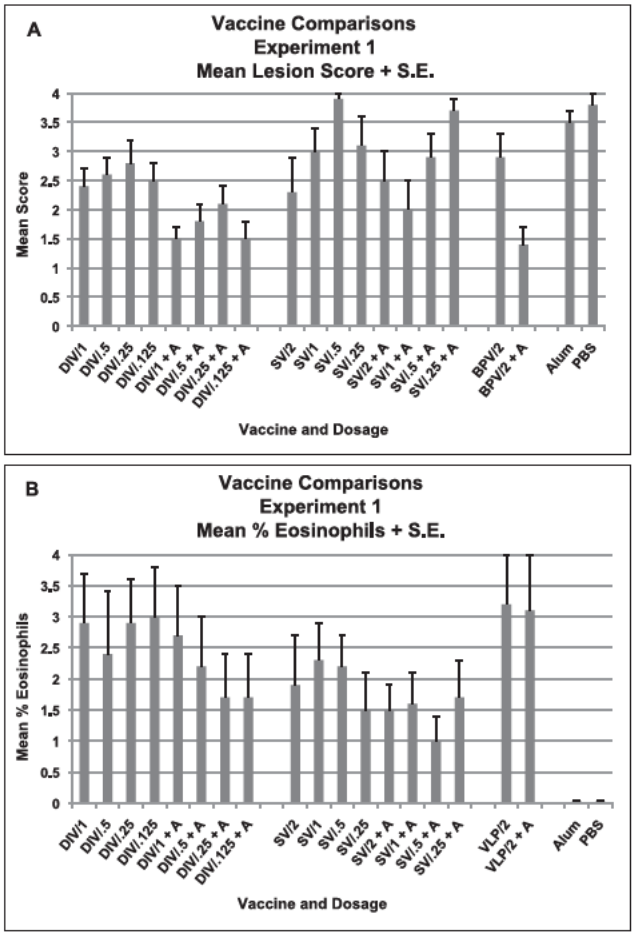

Figure 2. Vaccine Comparisons of Three SARS CoV Vaccines, Experiment 1. Mean lung cellular infiltration/lesion pathology and percent eosinophils in infiltrates for each vaccine dosage group two days after challenge with SARS CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. All mice exhibited lung histopathology. Scores are mean of scores for seven to eight mice per group. Scoring. 0 no pathology, 1 and 2 (1) minimal (2) moderate peribronchiole and perivascular cellular infiltration, 3 and 4 1 and/or 2 plus minimal (3) or moderate (4) epithelial cell necrosis of bronchioles with cell debris in the lumen. B. Mean percent eosinophils on histologic evaluation for seven to eight mice in each vaccine dosage group. Mean for each mouse is the mean percent eosinophils on five separate microscopy fields of lung sections. Analyses: A. Mean lesion scores were different p<.001. DIV without alum greater than with alum p = .001, VLP without alum greater than with alum p = .008. Posthoc comparisons: DIV lower than SV p = .001 and controls p<.001 but not VLP p>.05. SV lower than controls p .048. B. Mean percent eosinophils were different p<.001. Mean percent eosinophils lower for DIV with alum than without alum p = .049 and lower for SV with alum than without alum p = .001. Mean percent eosinophils lower for SV than DIV p = .002 or VLP. P = <.001. Mean percent eosinophils greater than controls for DIV, SV and VLP, all three vaccines p<.001.
doi:10.1371/journal.pone.0035421.g002

experiment of a hypersensitivity immunopathologic like reaction after SARS CoV challenge of vaccinated animals, to determine if a higher dosage of the S protein vaccine (SV) would suppress infection and still exhibit a similar reaction, and whether the original β propiolactone inactivated whole virus vaccine (BPV) that had shown an immunopathologic like reaction after challenge of vaccinated ferrets and nonhuman primates exhibited a similar immunopathologic reaction in the mouse model [13,14]. Additionally, a live virus "vaccination" group was added in this experiment for comparison of challenge results following vaccinations with inactivated vaccines to those following earlier infection.

Serum neutralizing antibody responses are shown in figure 3A. The bp inactivated vaccine (BPV), was only available at one dosage with alum so a smaller volume (25 µl) was given to one group for a dosage comparison. Geometric mean titers for the groups given the alum adjuvanted version of the DI and the S protein vaccines were greater than for the unadjuvanted vaccine (DIV P 0.014, SV p<0.001, student's t test). In multiple regression analysis, titers were also significantly increased after both the DI and S protein vaccines with use of alum (p≤0.01); no dosage effect was noted. The geometric mean neutralizing antibody titers of the two bp inactivated vaccine groups (BPV) were different (p 0.039, Mann Whitney U).

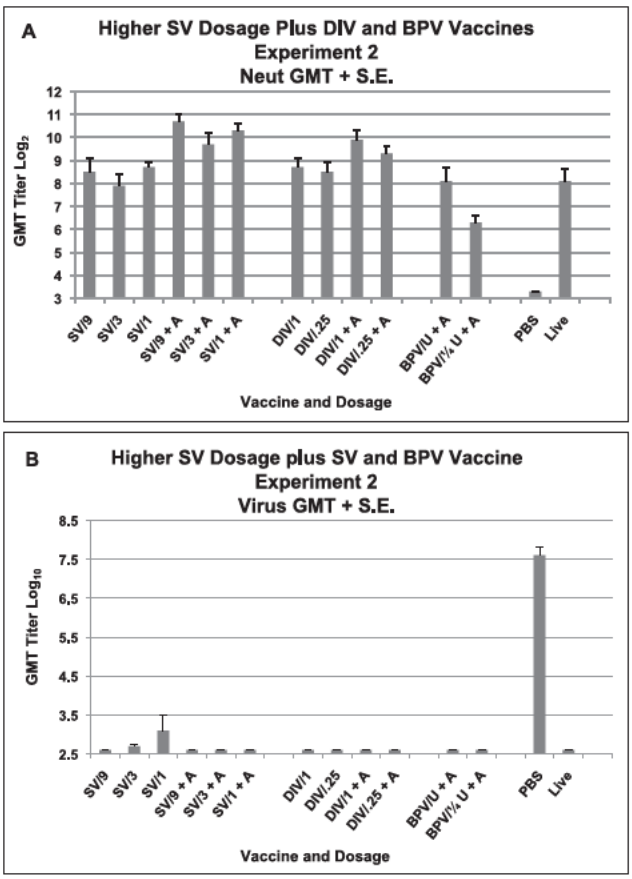

Figure 3. Higher Dosages of SV Vaccine plus DIV and BPV Vaccine Comparisons, Experiment 2. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer and standard error of the mean (S.E.) on day 56 for each vaccine dosage group. Five mice per group given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS CoV challenge) for each vaccine dosage group. Seven to eight mice per group. Vaccines: double inactivated whole virus (DIV), recombinant S protein (SV), β propiolac tone inactivated whole virus (BPV) with alum (+A). Analyses: A. GMT with alum greater than without alum: SV p<.001, DIV p = .014. GMT for the two BPV groups are different p = .039. Multiple regression: DIV and SV increased with alum p≤.01, no dosage effect p>.05.
doi:10.1371/journal.pone.0035421.g003

Two days after challenge with 10^6 $TCID_{50}$ of SARS CoV, titers in mice given PBS varied between $10^{7.0}$ and $10^{8.0}$ $TCID_{50}$ per g of tissue; one vaccinated animal in the group given the S protein vaccine (SV) at the 3 µg and the 1 µg dosage without alum yielded virus but all other animals in all other groups were culture negative for virus (figure 3B).

Shown in figure 4A are the mean lesion scores on histologic evaluations. The scoring system for experiments two and three were developed by a replacement pathologist who preferred a scale of 0 to 3 which corresponded to a judgment of mild, moderate or severe (figure 4A). Mean lesion scores for this grading system overall were significantly different from each other ($p<0.001$, Anova) and scores were lower for the S protein vaccine than for either of the whole virus vaccines (SV versus DIV and BPV, $p<0.001$ and p 0.006, respectively, Tukey HSD). Of interest is that those given live virus and then challenged with live virus two months later exhibited an infiltrative disease severity comparable to the PBS and vaccinated groups despite no detectable virus on day two, again suggesting some degree of infection may have occurred earlier.

The mean eosinophil scores for the lung infiltrations were lower for the S protein vaccine groups [SV vs. DIV $p<0.001$; SV vs. BPV, $p<0.001$, Tukey HSD]; however, they were clearly greater than seen in those given PBS or live virus earlier ($p<0.001$, Tukey HSD) (figure 4B).

Representative photo micrographs of lung sections from mice in this experiment two days after challenge with SARS CoV are shown in figure 5. The pathologic changes were extensive and similar in all challenged groups (H & E stains). Perivascular and peribronchial inflammatory infiltrates were observed in most fields along with desquamation of the bronchial epithelium, collections of edema fluid, sloughed epithelial cells, inflammatory cells and cellular debris in the bronchial lumen. Large macrophages and swollen epithelial cells were seen near lobar and segmental bronchi, small bronchioles and alveolar ducts. Necrotizing vasculitis was prominent in medium and large blood vessels, involving vascular endothelial cells as well as the tunica media, and included lymphocytes, neutrophils, and eosinophils in cellular collections. Occasional multinucleated giant cells were also seen. The eosinophil component of infiltrates was very prominent in animals vaccinated with the experimental vaccine preparations when compared to animals mock vaccinated using PBS, or those exposed earlier to live virus (figure 6); few to no eosinophils were seen in those lung sections. Thus, while pathology was seen in sections from the control mice, the hypersensitivity type pathologic reaction with eosinophils was not seen. The morphological identification of eosinophils in H&E stains was supported by using Giemsa stain to highlight intracytoplasmic granules in selected lung sections (not shown), and confirmed by immunostaining with antibodies against mouse eosinophil major basic protein (provided by the Lee Laboratory, Mayo Clinic, Arizona) [36].

The different groups of vaccinated animals showed similar trends in severity of pathology and of eosinophils in inflammatory infiltrates; however, the DIV and BPV preparations at high dosage tended to produce a greater infiltration with eosinophils.

Mouse and Vaccine Specificity (Experiment 3). Experiment 3 was performed to evaluate vaccine and mouse strain specificity. SARS CoV vaccines used were the DI vaccine (DIV) with and without alum and the bp inactivated vaccine (BPV), which contains alum, at the highest dosage. For mouse strain specificity, Balb/c mice were included for consistency between experiments; C57BL/6 mice were given the same vaccines and dosages as Balb/c mice for comparison as C57BL/6 mice do not exhibit a bias for Th2 immunologic responses as do

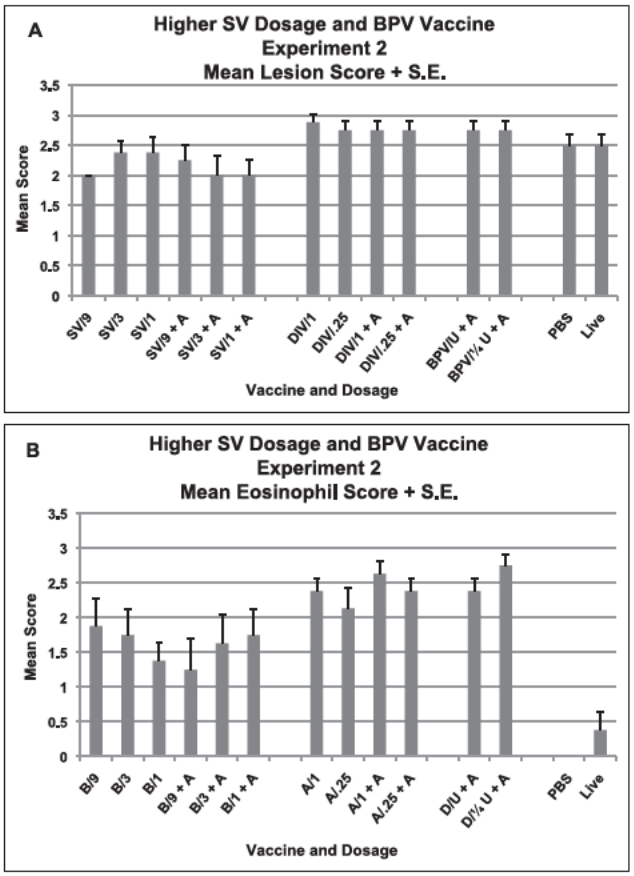

Figure 4. Higher Dosages of SV Vaccine plus DIV and BPV Vaccine Comparisons, Experiment 2. Mean lung cellular infiltration/lesion pathology and mean percent eosinophils in infiltrates for each vaccine dosage group two days after challenge with SARS CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring 0 no definite pathology, 1 mild peribronchiole and perivascular cellular infiltration, 2 moderate peribronchiole and perivascular cellular infiltration, 3 severe peribronchiolar and perivascular cellular infiltration with thickening of alveolar walls, alveolar infiltration and bronchiole epithelial cell necrosis and debris in the lumen. Ten to 20 microscopy fields were scored for each mouse lung. B. Mean score and standard error of the mean (S.E.) for eosinophils as percent of infiltrating cells for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring: 0 <5% of cells, 1 5 10% of cells, 2 10 20% of cells, 3 >20% of cells. Ten to 20 microscopy fields were scored for each mouse lung. Analyses: A. Mean lesion scores were different $p<.001$. Mean scores were lower for SV than DIV $p<.001$ and less than BPV $p=.006$. B. Mean eosinophil scores were lower for SV than DIV $p<.001$ and less than BPV $p<.001$. Eosinophil scores greater for SV than PBS or live virus $p<.001$.
doi:10.1371/journal.pone.0035421.g004

Balb/c mice [37 39]. PBS and live virus controls were again included and trivalent 2010 11 formulation influenza vaccine at a dosage of 12 µg per component was given to assess vaccine specificity.

Neutralizing antibody titers are shown in figure 7A. Geometric mean titers for the highest dose of the DI vaccine were higher for those vaccine groups in the Balb/c mice than the C57BL/6 mice but only the nonadjuvanted DI vaccine group was significantly higher (p 0.008, Mann Whitney U). The serum antibody responses after BPV and live virus administration were similar for the two mouse strains. After challenge, mean lung virus titers

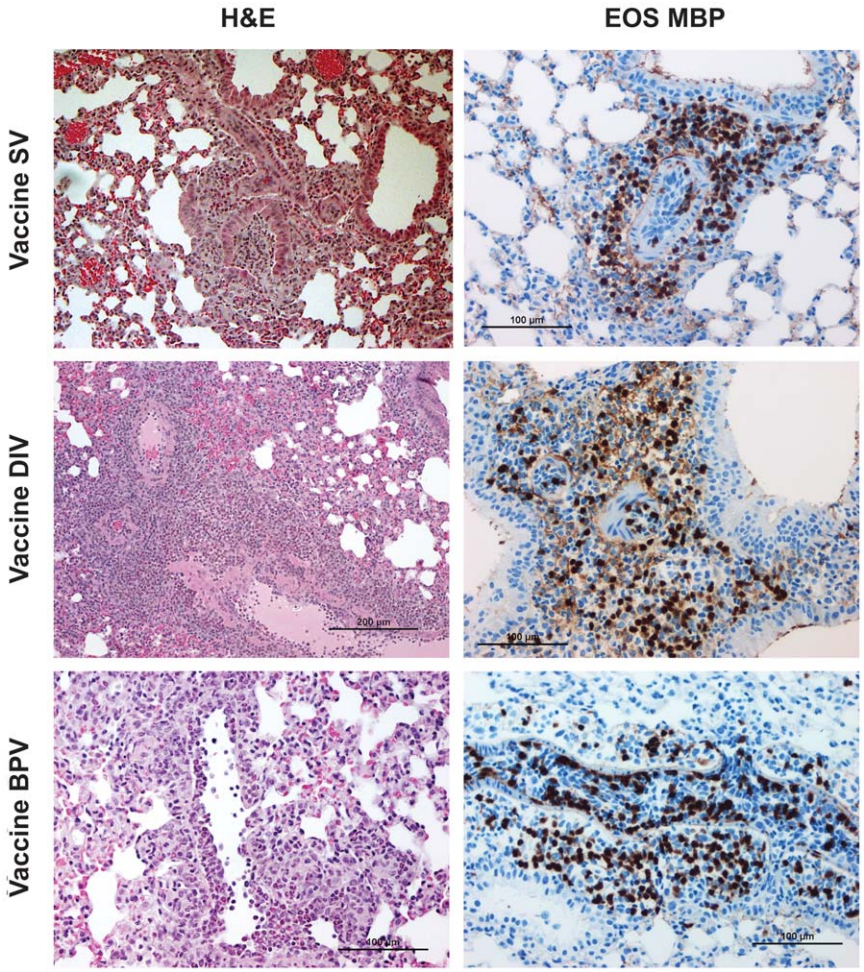

Figure 5. Photographs of Lung Tissue. Representative photomicrographs of lung tissue two days after challenge of Balb/c mice with SARS CoV that had previously been given a SARS CoV vaccine. Lung sections were separately stained with hematoxylin and eosin (H&E) and an immunohistochemical protocol using an eosinophil specific staining procedure with a monoclonal antibody to a major basic protein of eosinophils. DAB chromogen provided the brown eosinophil identity stain. The procedure and antibody were kindly provided by the Lee Laboratory, Mayo Clinic, Arizona. The H&E stain column is on the left and eosinophil specific major basic protein (EOS MBP) stain column is on the right. Vaccines: double inactivated whole virus (DIV), β propiolactone inactivated whole virus vaccine (BPV). As shown in the images, eosinophils are prominent (brown DAB staining) in all sections examined. Exposure to SARS CoV is associated with prominent inflammatory infiltrates characterized by a predominant eosinophilic component.
doi:10.1371/journal.pone.0035421.g005

were similar for the PBS control challenged mice of both mouse strains ($10^{6.7-7.3}$ $TCID_{50}$/g lung) (figure 7B). None of the Balb/c mouse groups given either vaccine or live virus earlier yielded virus after challenge but some virus was detected in C57BL/6 mice given the DIV without alum and the BPV with alum (C57BL/6 versus Balb/c, p 0.004, Mann Whitney U).

Mean lung lesion scores two days after challenge were similar for all groups and indicated a moderate to severe degree of cellular infiltration ($p>0.05$ for each, Anova) (figure 8A). However, eosinophil scores were significantly different between groups ($p<0.001$, Anova) with significantly lower scores for nonvaccine groups than for vaccine groups of both mouse strains ($p<0.001$ for all comparable group comparisons, Tukey's HSD). Eosinophil scores for the vaccine groups were not different between the two mouse strains ($p>0.05$, t test) (figure 8B). Photomicrographs of the different vaccine and mouse strain groups are shown in figure 9. Both vaccines in both mouse strains exhibited significant cellular infiltrations that included numerous eosinophils as shown in the MBP stained sections, a finding consistent with a hypersensitivity component of the pathology. Prior influenza vaccine did not lead to an eosinophil infiltration in the lung lesions after challenge.

Discussion

The emergence of the disease SARS and the rapid identification of its severity and high risk for death prompted a rapid mobilization for control at the major sites of occurrence and at the international level. Part of this response was for development of vaccines for potential use in control, a potential facilitated by the rapid identification of the causative agent, a new coronavirus [8 9]. Applying the principles of infection control brought the epidemic under control but a concern for reemergence naturally or a deliberate release supported continuation of a vaccine development effort so as to have the knowledge and capability necessary for preparing and using an effective vaccine should a need arise. For this purpose, the National Institute of Allergy and Infectious Diseases supported preparation of vaccines for evalu ation for potential use in humans. This effort was hampered by the

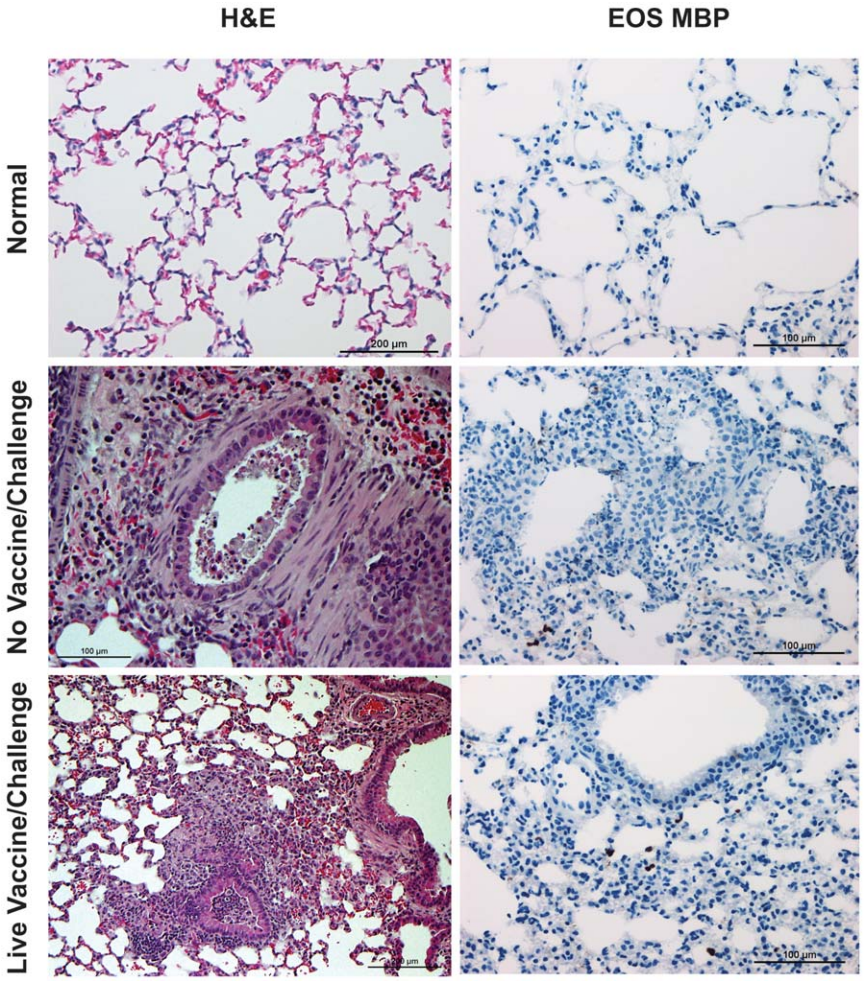

Figure 6. Photomicrographs of Lung Tissue. Representative photomicrographs of lung tissue from unvaccinated unchallenged mice (normal) and from Balb/c mice two days after challenge with SARS CoV that had previously been given PBS only (no vaccine) or live virus. H&E and immunohistochemical stains for eosinophil major basic protein were performed as described for figure 5. The H&E column is on the left and the Eos MBP column is on the right. Shown are sections from normal mice (no vaccine or live virus) and mice given PBS (no vaccine) or live SARS CoV and then challenged with SARS CoV. As shown in the middle and bottom row images, although exposure to SARS CoV elicits inflammatory infiltrates and accumulation of debris in the bronchial lumen, eosinophils in all groups remain within normal limits.
doi:10.1371/journal.pone.0035421.g006

occurrence in the initial preclinical trial of an immunopathogenic type lung disease among ferrets and Cynomolgus monkeys given a whole virus vaccine adjuvanted with alum and challenged with infectious SARS CoV [14]. That lung disease exhibited the characteristics of a Th2 type immunopathology with eosinophils in the lung sections suggesting hypersensitivity that was reminiscent of the descriptions of the Th2 type immunopathologic reaction in young children given an inactivated RSV vaccine and subse quently infected with naturally occurring RSV [32 33]. Most of these children experienced severe disease with infection that led to a high frequency of hospitalizations; two children died from the infection [33,40,41]. The conclusion from that experience was clear; RSV lung disease was enhanced by the prior vaccination. Subsequent studies in animal models that are thought to mimic the human experience indicate RSV inactivated vaccine induces an increased $CD4^+$ T lymphocyte response, primarily of Th2 cells and the occurrence of immune complex depositions in lung tissues [32,42,43]. This type of tissue response is associated with an increase in type 2 cytokines including IL4, IL5, and IL13 and an influx of eosinophils into the infected lung; [32,33,42,44]. Histologic sections of tissues exhibiting this type of response have a notable eosinophilic component in the cellular infiltrates. Recent studies indicate that the Th2 type immune response has both innate and adaptive immune response components [33,43].

In addition to the RSV experience, concern for an inappropri ate response among persons vaccinated with a SARS CoV vaccine emanated from experiences with coronavirus infections and disease in animals that included enhanced disease among infected animals vaccinated earlier with a coronavirus vaccine [31]. Feline infectious peritonitis coronavirus (FIPV) is a well known example of antibody mediated enhanced uptake of virus in macrophages that disseminate and increase virus quantities that lead to enhanced disease [31,45]. Antigen antibody complex formation with complement activation can also occur in that infection and some other coronavirus infections in animals. Thus, concern for safety of administering SARS CoV vaccines to humans became an early concern in vaccine development.

As a site proposed for testing vaccines in humans, we requested and were given approval for evaluating different vaccine candidates for safety and effectiveness. Two whole coronavirus

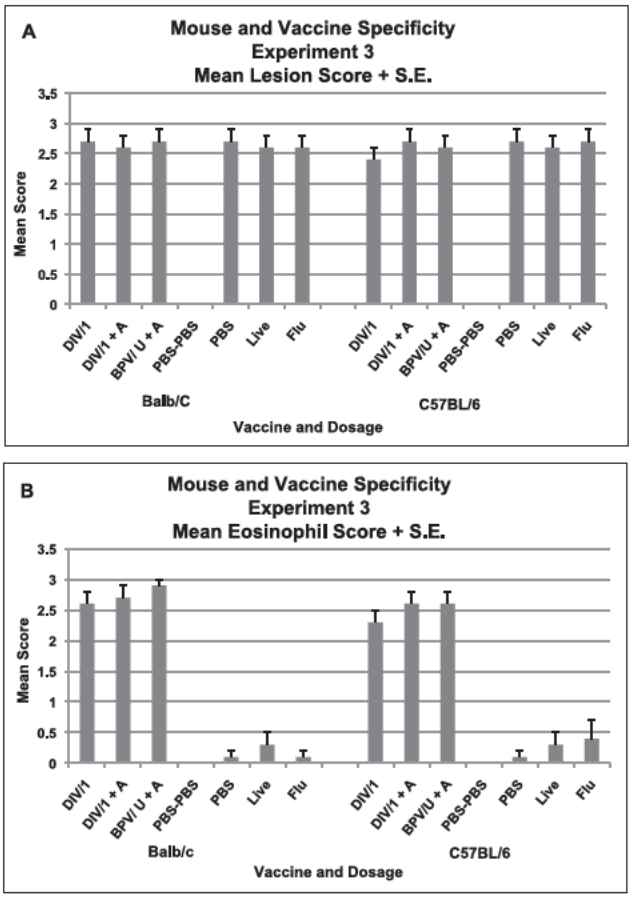

Figure 7. Mouse and Vaccine Specificity, Experiment 3. Serum neutralizing (neut) antibody and lung virus titers for each vaccine dosage group. A. Geometric mean serum antibody titer and standard error of the mean (S.E.) on day 56 for each vaccine dosage group for each mouse strain (Balb/c or C57BL/6). Five mice per group given 0.1 ml of vaccine intramuscularly on days 0 and 28. B. Geometric mean virus titer (log_{10} $TCID_{50}$/g) and standard error of the mean (S.E.) in lungs on day 58 (two days after SARS CoV challenge for each vaccine dosage group for each mouse strain. Seven to eight mice per group. Vaccines: Double inactivated whole virus, (DIV), β propiolactone inactivated whole virus (BPV), with alum (+A). Analyses: A. GMT for highest DIV dosage without alum greater for Balb/c than C57BL/6 p = .008 but not for alum p>.05. GMT for the BPV vaccine and live virus were not different for the two strains p>.05. B. GMT for PBS control mice were not different p>.05. GMT for DIV without alum and BPV with alum greater for C57BL/6 than Balb/c p = .004.
doi:10.1371/journal.pone.0035421.g007

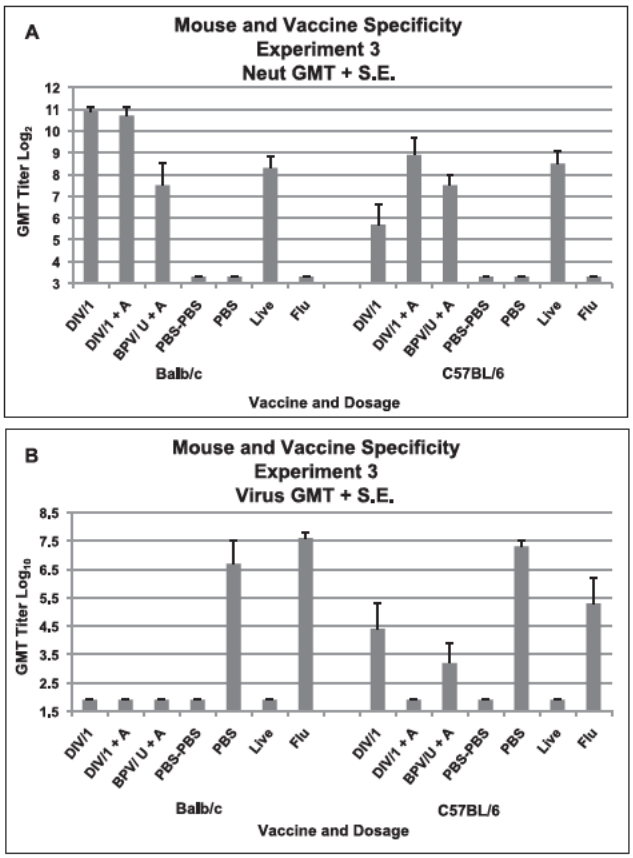

Figure 8. Mouse and Vaccine Specificity, Experiment 3. Mean lung cellular infiltration/lesion pathology and percent eosinophils in infiltrates for each vaccine dosage group for each mouse strain (Balb/c or C57BL/6) two days after challenge with SARS CoV. A. Mean lesion score and standard error of the mean (S.E.) for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring 0 no definite pathology, 1 mild peribronchiole and perivascular cellular infiltration, 2 moderate peribronchiole and perivascular cellular infiltration, 3 severe peribronchiole and perivas cular cellular infiltration with thickening of alveolar walls, alveolar infiltration and bronchiole epithelial cell necrosis and debris in the lumen. Ten to 20 microscopy fields were scored for each mouse lung. B. Mean score and standard error of the mean (S.E.) for eosinophils as percent of infiltrating cells for each vaccine dosage group. Scores are mean of scores for seven to eight mice per group. Scoring: 0 <5% of cells, 1 5 10% of cells, 2 10 20% of cells, 3 >20% of cells. Ten to 20 microscopy fields were scored for each mouse lung. Analyses: A. Mean lesion scores were not different p>.05. B. Mean eosinophil scores were different p<.001. Mean scores for vaccine groups greater than non vaccine groups for Balb/c and C57BL/6 p<.001 for all comparisons. Mean eosinophil scores for the same groups not different for Balb/c and C57BL/6 p>.05.
doi:10.1371/journal.pone.0035421.g008

vaccines, one rDNA expressed S protein vaccine and a VLP vaccine prepared by us were evaluated in a Balb/c mouse model, initially described by others, of SARS CoV [46,47]. The concern for an occurrence of lung immunopathology on challenge of mice vaccinated with an inactivated virus vaccine, as reported by Haagmans, et al. for ferrets and nonhuman primates, was seen by us after challenge of mice vaccinated with a SARS VLP vaccine [20]. This finding was duplicated in an experiment reported here and was also seen in mice vaccinated with a range of dosages of a double inactivated whole virus vaccine (DIV) and an rDNA S protein vaccine (SV) although the immunopathologic reaction appeared reduced among animals given the S protein vaccine when compared to those given the whole virus vaccine. In later experiments, these findings were confirmed and the vaccine utilized by Haagmans, et al. was also shown to induce the immunopathology in mice. Thus, all four vaccines evaluated induced the immunopathology; however, all four also induced neutralizing antibody and protection against infection when compared to control challenged animals.

The immunopathology in all experiments in the present study occurred in the absence of detectable virus in lungs of mice two days after challenge with infectious virus. In two experiments, a live virus group subsequently challenged with live virus was included. These challenged animals also exhibited similar histopathologic changes after challenge although no infectious virus was detected in lungs on day two; however, in the latter case, the infiltrates were nearly 100%

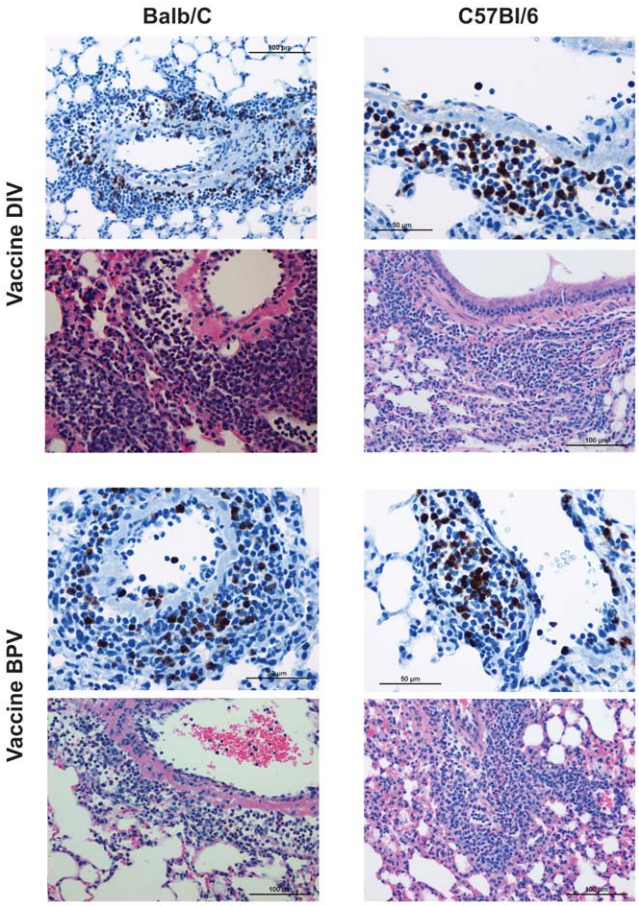

Figure 9. Photomicrographs of Lung Tissue. Representative photomicrographs of lung tissue two days after challenge of Balb/c and C57BL/6 mice that had previously been given a SARS CoV vaccine. Lung sections were separately stained with H&E (pink and blue micrographs) or the immunohistochemical stain for eosinophil major basic protein (blue and brown micrographs). Balb/c mice lung sections are in the left column and C57BL/6 are in the right column; doubly inactivated whole virus vaccine is in the upper four panels and those from mice given the β propiolactone inactivated whole virus vaccine are in the lower four panels. Pathologic changes observed (inflammatory infiltrates) are similar in Balb/c and C57BL/6 and eosinophils are prominent in both groups.
doi:10.1371/journal.pone.0035421.g009

monocytes and lymphocytes without the eosinophil component seen in the vaccinated challenged animals. In a separate test to assess the effects of the challenge inoculum, mice were given an IN challenge with 10^8TCID$_{50}$ of inactivated whole SARS CoV. Lungs of these animals revealed minimal or no histopathologic damage (data not shown). These findings suggest that virus replication probably occurred early after challenge, including in animals given live CoV earlier, and is required for development of pathology, including for the immunopathology. Infection would have been transient, below the limit of detection two days after challenge, or neutralized in lung homogenates before testing for virus.. Nevertheless, the Th2 type immunopathology pattern was seen only in animals given an inactivated vaccine earlier.

During the course of these experiments, a report appeared describing a similar immunopathologic type reaction with prominent eosinophils in SARS CoV challenged Balb/c mice that had been given Venezuelan equine encephalitis (VEE) vector containing the SARS nucleocapsid protein gene [18]. Those challenged animals exhibited infection similar to unvaccinated animals as well as Th2 type immunopathology. A similar experiment with a VEE vector containing only the S gene exhibited protection against infection and no immunopathology. More recently, this group has reported immunopathology with prominent eosinophil infiltration after SARS CoV challenge in Balb/c mice vaccinated with the same double inactivated whole virus vaccine used in our experiments [28]. They attribute the immunopathologic reaction following these SARS CoV vaccinations to presence of the nucleocapsid protein (N) in the vaccine.

In another report, vaccinia was used as a vector vaccine for immunizing Balb/c mice with each of the SARS CoV structural proteins (N, S, membrane, and envelope) and then challenged with SARS CoV [21]. Virus infection was present in all groups after challenge but reduced in the S vector vaccine group. Histopathology scores were high for the N containing vector group and low for the S containing group and for the vehicle control group. Eosinophilic infiltrates and IL 5 were increased in the N vaccine group but only IL 5 was increased in the S vaccine group.

To be certain the Th2 type immunopathology was elicited by the S protein vaccine in our studies and in hopes a greater immune response would result from higher dosages of the vaccine and induce greater protection against infection as well as reduce or prevent the immunopathology, our experiment 2 used up to 9 μg of the S protein for immunization. While increased titers of serum antibody were induced and no virus was detected day two after challenge in most animals, the Th2 type immunopathology occurred after challenge, and the immunopathology seen earlier after vaccination with the DI whole virus vaccine was seen again. This experiment also included the whole virus vaccine tested earlier in ferrets and nonhuman primates where the Th2 type immunopathology was initially seen. That vaccine, the BPV in this report, exhibited a pattern of antibody response, protection against infection and occurrence of immunopathology after challenge similar to the DI whole virus vaccine (DIV).

A final experiment was conducted to evaluate specificity. The Balb/c mouse was compared to C57BL/6 mice which do not exhibit the Th2 response bias known to occur in Balb/c mice. C57BL/6 mice in that same experiment exhibited results on challenge similar to those seen in Balb/c mice. Challenge of animals given prior influenza vaccine were infected and exhibited histopathologic damage similar to animals given PBS earlier; neither group exhibited the eosinophil infiltrations seen in animals given a SARS CoV vaccine.

In these various experiments alum was used as an adjuvant and this adjuvant is known to promote a Th2 type bias to immune responses [48]. However, the immunopathology seen in vaccinated challenged animals also occurred in animals given vaccine without alum. In an effort to determine whether an adjuvant that induced a bias for a Th1 type response would protect and prevent the immunopathology, we initiated an experiment where the DI PBS suspended vaccine was adjuvanted with Freund's complete adjuvant, a Th1 type adjuvant. However, this experiment was aborted by the September, 2008, Hurricane Ike induced flood of Galveston, Texas. An experiment with a SARS CoV whole virus vaccine with and without GlaxoSmithKline (GSK) adjuvant ASO1 in hamsters has been reported [25]. This adjuvant is thought to induce Th1 type immune responses [49]. The authors indicate no lung immunopathology was seen among animals after challenge, including the group given vaccine without adjuvant; however, whether the hamster model could develop a Th2 type immunopathology is uncertain. Finally, a number of other studies of vaccines in animal model systems have been reported but presence or absence of immunopathology after challenge was not reported.

Table 2. Summary of Reported Protection and Immunopathology in Animal Model Studies with SARS Coronavirus Vaccines.

Animal Model	Vaccine[1]	Protection[2]	Immunopathology[3]
Mice	Whole virus[tr]		
	w alum	Yes	Yes
	Whole virus[25,tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	VLP[17,tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	S Protein[tr]		
	w alum	Yes	Yes
	wo alum	Yes	Yes
	VEE Vector[15]		
	for N protein	No	Yes
	for S protein	Yes	No
	Vaccinia vector[18]		
	for N protein	No	Yes
	for S protein	Yes	?No
Ferrets	Whole virus[11]		
	w alum	Yes	Yes
Nonhuman Primate[4]	Whole virus[11]		
	w alum	Yes	Yes
Hamsters	Whole virus[22]		
	w ASO1	Yes	No

[1]Reference for each indicated; tr = this report; w = with, wo = without.
[2]Protection against infection (reduced lung virus after challenge).
[3]Th2 type immunopathology as indicated by cellular infiltrates with prominence of eosinophils.
[4]Cynomolgus monkeys.
doi:10.1371/journal.pone.0035421.t002

A summary of the SARS CoV vaccine evaluations in animal models (including the current report) that indicated an evaluation for immunopathology after challenge is presented in Table 2. As noted all vaccines containing S protein induced protection against infection while the studies with VEE and vaccinia vector containing the N protein gene only did not. Also shown is that a Th2 type immunopathology was seen after challenge of all vaccinated animals when evaluation for immunopathology was reported except the study in hamsters with a GSK whole virus vaccine. Thus, inactivated whole virus vaccines whether inacti vated with formalin or beta propiolactone and whether given with our without alum adjuvant exhibited a Th2 type immunopatho logic in lungs after challenge. As indicated, two reports attributed the immunopathology to presence of the N protein in the vaccine; however, we found the same immunopathologic reaction in animals given S protein vaccine only, although it appeared to be of lesser intensity. Thus, a Th2 type immunopathologic reaction on challenge of vaccinated animals has occurred in three of four animal models (not in hamsters) including two different inbred mouse strains with four different types of SARS CoV vaccines with and without alum adjuvant. An inactivated vaccine preparation that does not induce this result in mice, ferrets and nonhuman primates has not been reported.

This combined experience provides concern for trials with SARS CoV vaccines in humans. Clinical trials with SARS coronavirus vaccines have been conducted and reported to induce antibody responses and to be "safe" [29,30]. However, the evidence for safety is for a short period of observation. The concern arising from the present report is for an immunopatho logic reaction occurring among vaccinated individuals on exposure to infectious SARS CoV, the basis for developing a vaccine for SARS. Additional safety concerns relate to effectiveness and safety against antigenic variants of SARS CoV and for safety of vaccinated persons exposed to other coronaviruses, particularly those of the type 2 group. Our study with a VLP SARS vaccine contained the N protein of mouse hepatitis virus and Bolles, et al., reported the immunopathology in mice occurs for heterologous Gp2b CoV vaccines after challenge [25]. This concern emanates from the proposal that the N protein may be the dominant antigen provoking the immunopathologic reaction.

Because of well documented severity of the respiratory disease among infants given an inactivated RSV vaccine and subsequently infected with RSV that is considered to be attributable to a Th2 type immunopathologic reaction and a large number of studies in the Balb/c mouse model that have described and elucidated many components of the immunopathologic reaction to RSV vaccines, the similarity to the SARS CoV vaccine evaluations in Balb/c mice supports caution for clinical vaccine trials with SARS CoV vaccines in humans. Of interest are the similar occurrences in C57BL/6 mice and in ferrets and nonhuman primates that provide alternative models for elucidating vaccine induced mechanisms for occurrences of Th2 immunopathologic reactions after infection. As indicated, strong animal model evidence indicates expression of the N protein by SARS CoV vector vaccines can induce sensitization leading to a Th2 type immuno pathology with infection. In contrast to our results, those studies did not find clear evidence of the Th2 type immunopathology on challenge of mice given a vector vaccine for the S protein. The finding of a Th 2 type pathology in our studies in animals immunized with an rDNA produced S protein is unequivocal. In this regard, animal model studies with FIPV in cats and RSV in mice have indicated that viral surface proteins may be the sensitizing protein of inactivated vaccines for immunopathology with infection [32,45]. This suggests that presentation of the S protein in a vector format may direct immune responses in a different way so that sensitization does not occur.

Limitations of the present studies include their performance in mice only and uncertainty of the relevance of rodent models to SARS CoV vaccines in humans. Additionally, a more intense study for virus replication including quantitative RT PCR assays might have confirmed the probability that virus replication is required for induction of the immunopathology after vaccination. Evaluations of mechanisms for the immunopathology, including immunoglobulin and cytokine responses to vaccines and tests for antigen antibody complexes in tissues exhibiting the reaction, could have strengthened the Th2 type immunopathology finding. Finally, a successful study with a Th1 type adjuvant that did not exhibit the Th2 pathology after challenge would have confirmed a Th2 bias to immune responses as well as provide a potential safe vaccination approach for SARS.

Acknowledgments

We thank I. Darlene Kirk, CCRP, for aid in coordinating the study and preparing the manuscript. MBP antibodies were kindly provided by the laboratory of Drs. Jamie and Nancy Lee, Mayo Clinic Arizona; e mail address: jjlee@mayo.edu

Author Contributions

Conceived and designed the experiments: RBC CJP C TT. Performed the experiments: C TT ES NI Y PCN TG. Analyzed the data: RLA RBC C TT. Contributed reagents/materials/analysis tools: RBC C TT RLA ES. Wrote the paper: RBC C TT ES.

References

1. World Health Organization website (2003) Available: http://www.who.int/csr/media/sars wha.pdf. Accessed 2012 Apr 2. Severe acute respiratory syndrome (SARS): Status of the outbreak and lessons for the immediate future; unmasking a new disease. CSR/WHO, Geneva. 20 May 2003..
2. Tsang KW, Ho PL, Ooi CG, Yee WK, Wang T, et al. (2003) A cluster of cases of severe acute respiratory syndrome in Hong Kong. N Engl J Med 348: 1953 66.
3. Poutanen SM, Low D, Henry B, Finkelstein S, Rose D, et al. (2003) Identification of severe acute respiratory syndrome in Canada. N Engl J Med 348: 1953 66.
4. World Health Organization Website. Available: http://www.who.int/csr/sars/country/2003 04 04/en/index.html. Accessed 2004 April 21.
5. Lee N, Hui D, Wu A, Chan P, Cameron P, et al. (2003) A major outbreak of severe acute respiratory syndrome in Hong Kong. N Engl J Med 348: 1986 94.
6. Fowler RA, Lapinsky SE, Hallett D, Detsky AS, Sibbald WJ, et al. (2003) Critically ill patients with severe acute respiratory syndrome. JAMA 290: 367 80.
7. Peiris JSM, Yuen KY, Osterhaus ADME, Stohr K (2003) The severe acute respiratory syndrome. N Engl J Med 349: 2431 41.
8. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, et al. (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348: 1953 66.
9. Drosten C, Gunther S, Preiser W, van der WS, Brodt HR, et al. (2003) Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 348: 1967 76.
10. Li W, Shi Z, Yu M, Ren W, Smith C, et al. (2005) Bats are natural reservoirs of SARS-like coronaviruses. Science 310: 676 9.
11. World Health Organization website (2003) Case definitions for surveillance of severe acute respiratory syndrome (SARS). Geneva, Switzerland: World Health Organization, Available: www.who.int/csr/sars/casedefinition/en/. Accessed: 2012 Apr 2.
12. Centers for Disease Control and Prevention website (2003) Updated interim U.S. case definition for severe acute respiratory syndrome (SARS). Atlanta: Centers for Disease Control and Prevention, Available: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5217a5.htm. Accessed 2012 Apr 2.
13. Kusters IC, Matthews J, Saluzzo JF (2009) Manufacturing vaccines for an emerging viral infection Specific issues associated with the development of a prototype SARS vaccine. In: Barrett ADT, Stanberry LR, eds. Vaccines for biodefense and emerging and neglected diseases. City: Elsevier. pp 147 156.
14. Haagmans BL, Boudet F, Kuiken T, deLang A, Martina BE, et al. (2005) Protective immunity induced by the inactivated SARS coronavirus vaccine. Abstract S 12-1. Presented at the X International Nidovirus Symposium, Colorado Springs, CO.
15. See RH, Zakhartchouk AN, Petric M, Lawrence DJ, Mok CP, et al. (2006) Comparative evaluation of two severe acute respiratory syndrome (SARS) vaccine candidates in mice challenged with SARS coronavirus. J Gen Virol 87: 641 650.
16. Spruth M, Kistner O, Savidis-Dacho H, Hitter E, Crowe B, et al. (2006) A double-inactivated whole virus candidate SARS coronavirus vaccine stimulates neutralizing and protective antibody responses. Vaccine 24: 652 661.
17. Zhou Z, Post P, Chubet R, Holtz K, McPherson C, et al. (2006) A recombinant baculovirus-expressed S glycoprotein vaccine elicits high titers of SARS-associated coronavirus (SARS-CoV) neutralizing antibodies in mice. Vaccine 24: 3624 3631.
18. Deming D, Sheahan T, Heise M, Yount B, Davis N, et al. (2006) Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Medicine 3: 2359 2375.
19. Enjuanes L, DeDiego ML, Alvarez E, Deming D, Sheahan T, et al. (2008) Vaccines to prevent severe acute respiratory syndrome coronavirus-induced disease. Vaccine Research 133: 45 62.
20. Lokugamage KG, Yoshikawa-Iwata N, Ito N, Watts DM, Wyde PR, et al. (2008) Chimeric coronavirus-like particles carrying severe acute respiratory syndrome coronavirus (SCov) S protein protect mice against challenge with SCoV. Vaccine 26: 797 808.
21. Yasui F, Kai C, Kitabatake M, Inoue S, Yoneda M, et al. (2008) Prior immunization with severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV. J Immunol 181: 6337 6348.
22. See RH, Petric M, Lawrence DJ, Mok CPY, Rowe T, et al. (2008) Severe acute respiratory syndrome vaccine efficacy in ferrets: whole killed virus and adenovirus-vectored vaccines. J Gen Virol 89: 2136 2146.
23. Lamirande EW, DeDiego ML, Roberts A, Jackson JP, Alvarez E, et al. (2008) A live attenuated severe acute respiratory syndrome coronavirus is immunogenic and efficacious in golden Syrian hamsters. J Virol 82: 7221 7224.
24. Lu B, Huang Y, Huang L, Li B, Zheng Z, et al. (2010) Effect of mucosal and systemic immunization with virus-like particles of severe acute respiratory syndrome coronavirus in mice. Immunology 130: 254 261.
25. Roberts A, Lamirande EW, Vogel L, Baras B, Goossens G, et al. (2010) Immunogenicity and protective efficacy in mice and hamsters of a β-Propiolactone inactivated whole virus SARS-CoV vaccine. Viral Immunol 23: 509 519.
26. Du L, Zhao G, Chan CCS, Li L, He Y, et al. (2010) A 210-mer CHO-expressing receptor-binding domain of SARS CoV S protein induces potent immune responses and protective immunity. Viral Immunol 23: 211 219.
27. Liu YV, Massare MJ, Barnard DL, Kort T, Nathan M, et al. (2011) Chimeric severe acute respiratory syndrome coronavirus (SARS CoV) S glycoprotein and influenza matrix 1 efficiently form virus-like particles (VLPs) that protect mice against challenge with SARS-CoV. Vaccine 29: 6606 6613.
28. Bolles M, Deming D, Long K, Agnihothram S, Whitmore, et al. (2011) A double-inactivated severe acute respiratory syndrome coronavirus vaccine provides incomplete protection in mice and induces increased eosinophilic proinflammatory pulmonary response upon challenge. J Virol 85: 12201 12215.
29. Lin J-T, Zhang J-S, Su N, Xu J-G, Wang N, et al. (2007) Safety and immunogenicity from a Phase I trial of inactivated severe acute respiratory syndrome coronavirus vaccine. Antiviral Therapy 12: 1107 1113.
30. Martin JE, Louder MK, Holman LA, Gordon IJ, Enama ME, et al. (2008) A SARS DNA vaccine induces neutralizing antibody and cellular immune responses in healthy adults in a Phase I clinical trial. Vaccine 26: 6338 6343.
31. Perlman S, Dandekar AA (2005) Immunopathogenesis of coronavirus infections: Implications for SARS. Nature Rev Immunol 5: 917 927.
32. Castilow EM, Olson MR, Varga SM (2007) Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol Res 39: 225 239.
33. Collins PL, Graham BS (2008) Viral and host factors in human respiratory syncytial virus pathogenesis. J Virol 82: 2040 2055.
34. Tseng CT, Huang C, Newman P, Wang N, Narayanan K, et al. (2007) Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human Angiotensin-converting enzyme 2 virus receptor. J Virol 81: 1162 1173.
35. Yoshikawa N, Yoshikawa T, Hill T, Huang C, Watts DM, et al. (2009) Differential virological and immunological outcome of severe acute respiratory syndrome coronavirus infection in susceptible and resistant transgenic mice expressing human angiotensin-converting enzyme 2. J Virol 83: 5451 5465.
36. Protheroe C, Woodruff SA, DePetris G, Mukkada V, Ochkur SI, et al. (2009) A novel histological scoring system to evaluate mucosal biopsies from patients with eosinophilic esophagitis. Clin Gastroenterol Hepatol 2009 7: 749 55.
37. Hsieh C-S, Macatonia SE, O'Garra A, Murphy KM (1995) T cell genetic background determines default T helper phenotype development in vitro. J Exp Med 181: 713 721.
38. Gorham JD, Guler ML, Steen RG, Mackey AJ, Daly MJ, et al. (1996) Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses. Proc Natl Acad Sci U S A 93: 12467 12472.
39. Launois P, Maillard I, Pingel S, Gwihart KG, Xenarios I, et al. (1997) IL-4 rapidly produced by Vβ4 Vα8 $CD4^+$ T cells instructs Th2 development and susceptibility to Leishmania major in BALB/c mice. Immunity 6: 541 549.
40. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE (1969) An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus vaccine. Am J Epidemiol 89: 405 21.
41. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, et al. (1969) Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 89: 422 34.
42. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ (1996) Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J Virol 70: 2852 60.
43. Polack FP, Teng MN, Collins PL, Prince GA, Exner M, et al. (2002) A role for immune complexes in enhanced respiratory syncytial virus disease. J Exp Med 196: 859 65.
44. Power UF, Huss T, Michaud V, Plotnicky-Gilquin H, Bonnefoy J-Y, et al. (2001) Differential histopathology and chemokine gene expression in lung tissues following respiratory syncytial virus (RSV) challenge in formalin-inactivated RSV- or BBG2Na-immunized mice. J Virol 75: 12421 30.
45. Weiss RC, Scott FW (1981) Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. Comp Immunol Microbiol Infect Dis 4: 175 89.
46. Wentworth DE, Gillim-Ross L, Espina N, Bernard KA (2004) Mice susceptible to SARS coronavirus. Emerg Infect Dis 10: 1293 96.
47. Subbarao K, McAuliffe J, Vogel L, Fahle G, Fischer S, et al. (2004) Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J Virol 78: 3572 77.

48. Jordan MB, Mills DM, Kappler J, Marrack P, Cambier JC (2004) Promotion of B cell immune responses via an alum-induced myeloid cell population. Science 304: 1808 10.

49. Garcon N, Chomez P, Van Mechelen M (2007) GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 6: 723 9.

HER MAJESTY THE QUEEN IN RIGHT OF ONTARIO Applicant/Respondent	and	**ADAMSON BARBECUE LIMITED AND WILLIAM ADAMSON SKELLY** Respondents/Applicants	Court File No. CV-20-00652216-0000

ONTARIO
SUPERIOR COURT OF JUSTICE

Proceedings commenced at the City of Toronto

AFFIDAVIT OF WITNESS
DR. MARK TROZZI

(Sworn on April 12, 2021)

ELDERS WITHOUT BORDERS
Michael Swinwood (LSO #14587R)
237 Argyle Avenue
Ottawa, Ontario K2P 1B8
Tel: 613-563-7474; Fax: 613-563-9179
Email: spiritualelders@gmail.com

Liza Swale (LSO #49683H)
Email: lizaswale@gmail.com

Lawyers for the Respondents/Applicants