CLINICAL TRIAL PROTOCOL

PROTOCOL TITLE:	Using AS01 adjuvant to improve immune response in older adults through trained immunity
PROTOCOL NUMBER:	AS01-YF-01
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PRINCIPAL INVESTIGATOR:	Dr. Chan Yuen Yue Candice Senior Consultant Department of Infectious Diseases Singapore General Hospital

PROTOCOL SIGNATURE PAGE

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Declaration of Investigator

I confirm that I have read the above-mentioned protocol and its attachments. I agree to conduct the described study in compliance with all stipulations of the protocol, regulations and ICH E6 Guideline for Good Clinical Practice (GCP).

Chan Yuen Yue Candice

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Name of Principal Investigator:

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Signature of Principal Investigator:

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Date:

Singapore General Hospital

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Name of Study Site:

Note:

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Table of Contents

1. BACKGROUND AND RATIONALE

The innate immune system provides our body's initial defence against pathogens, responding within minutes to hours of infection through specialized myeloid cells like monocytes, neutrophils, macrophages and dendritic cells (DCs)[1]. These myeloid cells orchestrate to mount rapid non-specific immunity and activate longer-term specific adaptive immune responses mediated by B and T cells. However, the robustness of the innate immune system declines with aging, leaving older adults more vulnerable to infections and less responsive to vaccines[2]. The recent COVID19 pandemic highlighted the vulnerabilities of older individuals to both severe disease outcomes and reduced protection to vaccines, even highly efficacious mRNA vaccines[3,4].

Various efforts have been made to enhance immunity in older adults[5]. Among many strategies, two adjuvanted vaccines - the recombinant zoster vaccine (RZV; Shingrix™) and the respiratory syncytial virus (RSV; Arexvy™) vaccine - demonstrated over 90% and 80% efficacy respectively in large phase 3 clinical trials[6–9]. These results prove that robust immune responses can be induced in older adults. Both vaccines utilise AS01, a liposome-based adjuvant containing MPL (a toll-like receptor 4 agonist) and QS-21 (purified plant extract)[10–12]. AS01 triggers early inflammatory signals and recruits activated monocytes and DCs to the injection site and draining lymph nodes, leading to robust antibody and T cell responses[10–12]. Beyond its adjuvant role, emerging evidence suggests AS01 possesses broader immunological effects. A recent study using the AS01-adjuvanted Hepatitis B vaccine in young adults showed that the vaccine induced trained immunity (TI) phenotype in innate immune cells via epigenetic reprogramming[13]. TI has been associated with enhanced innate immune response against unrelated pathogens, also known as 'heterologous protection[14]. Supporting these findings, an epidemiological study during the COVID-19 pandemic found that recipients of RZV had a lower incidence of severe COVID-19[15]. Separately, our group recently revealed that the first RZV dose modulated the expression of genes that correlated with robust T cell response and restored the aged immune system's ability to mount robust response to the second dose (preliminary data, in review). While these results are encouraging, human studies have not yet revealed AS01's independent effects since it has only been studied in combination with vaccine antigens. Breaking through this limitation, Co-I Singhal's recent work with humanized mice demonstrated that AS01 alone could reprogram human hematopoietic stem cells (from the bone marrow of humanized mice) and innate immune cells at the epigenomic level, which is a characteristic of TI. This reprogramming manifested through alternations in gene expression across myeloid cells and altered chromatin accessibility at important genomic locus. Notably, AS01-alone promotes robust type I interferon (IFN) responses and improved antigen-presenting capability in DCs, with peak responses observed at 1-month. When challenged with *Mycobacterium tuberculosis* at one-month, AS01-treated mice showed protection against disease, indicative of heterologous protection. (preliminary data, in review).

These findings collectively reveal AS01's potential to epigenetically reprogram innate immune cells for enhanced adaptive immunity. During early disease outbreaks, AS01 could serve as a rapid and broadly protective immune booster for older populations before pathogen-specific interventions become available, offering a novel strategy to reduce disease severity. Despite AS01 and TI's promise as a new frontier in vaccinology, its effects have yet to be studied in humans, including the extent and durability of AS01-induced protection against pathogens.

This study is the first prospective placebo-controlled, randomized trial to evaluate the molecular mechanisms and durability of AS01-induced TI at one and three months in healthy older adults. Serial blood samples will be collected to analyze changes in immune cell populations and TI induction. To evaluate heterologous protection, we will employ a virus challenge study using a live-attenuated yellow fever vaccine virus strain (YF17D) that causes detectable viremia safely, to compare viral control between AS01 and placebo groups[16]. If successful, our findings could enable development of novel vaccination strategies by stimulating TI to elicit broadly protective immunity, especially during early outbreaks when pathogen-specific interventions are not yet available, helping to reduce mortality and morbidity and improve vaccine responses in vulnerable older adults.

The specific aims and hypotheses are as follows:

Specific aim 1: Examine the host response induced by AS01 and determine the duration of these changes for up to 3 months in healthy older adults aged 21 – 59 years old.
This aim **tests the hypothesis** that the AS01 adjuvant will induce significant and persistent changes in gene expressions and epigenetic markers associated with innate immunity in older adults.

Specific aim 2: Demonstrate that innate immune reprogramming post AS01 can provide cross-protection against heterologous YF17D viral infection.
This aim **tests the hypothesis** that AS01 recipients will have 50% reduction in detectable viremia versus placebo after YF17D challenge at 1 month, while also evaluating the longevity of this cross-protection at 3 months post-AS01.
Specific aim 3: Examine the T cell and B cell responses post YF17D challenge, and its correlation with YF viremia levels in AS01 group versus placebo.
This aim **tests the hypothesis** that the reprogramming of innate immune cells post-AS01 promotes enhanced YF viremia clearance without compromising humoral and cellular immune response to YF17D.

1.1. General Introduction

Current pandemic response limitations and aging populations: a dual challenge

This century has witnessed over ten transnational epidemics, underscoring the critical need for enhanced preparedness against novel pathogens[17]. With the global population rapidly aging[18], the COVID-19 pandemic exposed significant vulnerabilities in global readiness, with older populations being particularly susceptible due to severe disease due to immunosenescence – the age-related decline across all arms of the immune system[4]. While vaccines remain essential tools, they face inherent limitations in addressing emerging threats: development requires substantial time, protection against rapidly evolving pathogens is limited, and efficacy diminishes in older populations[19,20]. The reduced protection of even highly effective COVID-19 mRNA vaccines in older adults emphasized the urgent need for more robust and adaptable pandemic management strategies, particularly for vulnerable populations.

Heterologous protection by innate and trained immunity: Role of vaccines

The immune system consists of two distinct but interconnected components: innate and adaptive immunity. The adaptive immune system, through B and T cells, forms the basis of vaccinology by generating specific, long-term immune memory and improved responses to repeated exposures, though it requires weeks to reach optimal protection against new pathogens[21]. In contrast, the innate immune system serves as our first line of defence through its diverse components - myeloid cells (monocytes, macrophages), dendritic cells (DCs), innate lymphoid cells (ILCs) and neutrophils[1]. These cells provide rapid and broad-spectrum protection by recognizing conserved molecular patterns present across diverse microorganisms, while also being essential in activating adaptive immune responses.

Traditionally, the innate response was considered a temporary defence mechanism. However, this dogma was changed by the discovery of trained immunity (TI) by Netea et al[14,22,23]. An increasing body of evidence suggests that a set of innate cells (including myeloid cells, ILCs) can display adaptive characteristics of ‘de facto’ innate immune memory. These memory characteristics involve a priming event through infection or vaccination whereby upon first exposure, innate immune cells undergo sustained metabolic and epigenetic reprogramming, such that upon re-exposure to identical or heterologous stimuli, they displayed heightened response and host defense[14,22–24].

This paradigm-shifting finding was first evidenced through the live-attenuated Bacillus Calmette-Guérin (BCG) vaccine, where vaccinated children showed unexpectedly reduced overall mortality beyond its intended protection against tuberculosis[25,26]. Further research documented BCG’s broad protective effects across age groups, including enhanced protection against respiratory infections in the elderly[27,28], experimental malaria infection in young adults[29], and experimental yellow fever vaccine virus infection in young males[30]. Live-attenuated vaccines like measles, mumps and rubella (MMR) have demonstrated reduced incidence of non-targeted infections and immune cell epigenetic reprogramming in human studies[31–33], while the novel live-attenuated *Bordetella pertussis vaccine* showed protection against unrelated airway infections in mice[34]. However, repurposing vaccines for pandemic preparedness has limitations. For instance, live vaccines are contraindicated in immunocompromised individuals, and some have age restrictions or show reduced efficacy in older adults[35,36].

Adjuvant AS01 as a potential immune booster for the aging population

Yet, the RZV vaccine (containing the recombinant varicella zoster virus glycoprotein E (gE) with AS01) which has over 90% vaccine efficacy in adults aged 50 and older with protection lasting at least 10 years[6,7,37], as well as the RSV vaccine (containing the recombinant RSV prefusion F protein antigen and AS01) with 80% efficacy in those aged 65 and older[8]. The AS01 adjuvant, originally developed to enhance vaccine efficacy in older adults, represents a breakthrough in addressing both immediate and long-term protection needs. Its combination of liposomes, MPL (a TLR-4 agonist) and QS-21 (plant product), work synergistically to enhance both innate and adaptive immune responses[10]. In human studies,

AS01 stimulation of blood cells from both young and older adult vaccinees activates antigen-presenting cells (APCs) including monocytes and DCs, triggering inflammatory pathways and metabolic changes that enhance T cell response[11].

Recent evidence suggests AS01's potential extends beyond its role as a vaccine adjuvant by demonstrating ability to induce TI changes in innate immune cells, conferring heightened protection against heterologous stimuli. Evidence of AS01's broader protective effects emerged when RZV recipients demonstrated a 32% lower risk of COVID-19 related hospitalizations over a 10-month period, suggesting potential TI induction[15]. A recent ex-vivo human study with AS01-adjuvanted hepatitis B vaccine demonstrated its ability to induce TI in innate immune cells, particularly monocytes and DCs, leading to reduced pro-inflammatory responses, improved response to cytokines and enhanced cell-mediated immunity[13].

Evaluating AS01 as a standalone immune booster for vulnerable older adults

While current TI-inducing vaccines present a promising avenue for enhanced pandemic preparedness, adoption into clinical practice still requires deeper understanding of TI's duration and population-specific responses. *In vitro* studies show TI effects lasting from days to weeks in myeloid cells, while epidemiological studies of BCG and MMR suggest protection lasting months to years[15,25]. Understanding the duration of TI that could be induced by AS01 in older populations remains crucial for determining optimal dosing intervals and establishing effective protection windows against emerging pathogens. To glean further insights into the immunological events and heterologous protection effects of AS01, our group and others have preliminary data showing AS01's potential as a versatile immune booster. However, a significant knowledge gap exists – all human studies have only tested AS01 with vaccine antigens, leaving its standalone effects unexplored. Building on the methods and findings from our previous studies, our CS-IRG proposal aims to address these critical knowledge gaps by investigating AS01-alone as an independent immune booster in older populations. In the short term (3 to 5 years), we aim to elucidate AS01's mechanisms in aging immune systems and evaluate its broad-spectrum protection potential and duration. Our long-term vision focuses on enhancing immune responses in older and other vulnerable adults. By understanding the mechanistic basis of TI and heterologous protection, we could develop novel approaches for preventing infectious diseases, potentially transforming our ability to protect at-risk groups during future pandemics.

1.2. Rationale and Justification for the Study

Preliminary studies in humanized mice and demonstrated that AS01 alone could reprogram human hematopoietic stem cells (from the bone marrow of humanized mice) and innate immune cells at the epigenomic level, which is a characteristic of TI. When challenged with *Mycobacterium tuberculosis* at one-month, AS01-treated mice showed protection against disease, indicative of heterologous protection. (preliminary data, submitted). Separately, our group recently revealed that the first RZV dose (which contains the AS01 adjuvant) modulated the expression of genes that correlated with robust T cell response and restored the aged immune system's ability to mount robust response to the second dose (preliminary data, in review).

Our findings collectively reveal AS01's potential to epigenetically reprogram innate immune cells for enhanced adaptive immunity. During early disease outbreaks, AS01 could serve as a rapid and broadly protective immune booster for older populations before pathogen-specific interventions become available, offering a novel strategy to reduce disease severity. Despite AS01 and TI's promise as a new frontier in vaccinology, its stand-alone effects have yet to be studied in humans, including the extent and durability of AS01-induced protection against pathogens.

This study is the first prospective placebo-controlled, randomized trial to evaluate the molecular mechanisms and durability of AS01-induced TI at one and three months in healthy older adults aged 21 –59 years old. Serial blood samples will be collected to analyse changes in immune cell populations and TI induction. To evaluate heterologous protection, we will employ a virus challenge study using a live-attenuated yellow fever vaccine virus strain (YF17D) that causes detectable viremia safely, to compare viral control between AS01 and placebo groups[16]. If successful, our findings could enable development of novel vaccination strategies by stimulating TI to elicit broadly protective immunity, especially during early outbreaks when pathogen-specific interventions are not yet available, helping to reduce mortality and morbidity and improve vaccine responses in vulnerable older adults.

1.2.1 Rationale for the Study Purpose

AS01 adjuvant induces TI in the bone marrow cells of humanized mice (*Negishi et al., submitted*)

To understand the long-term effects of AS01 adjuvant on the human bone marrow (BM) progenitor and myeloid cells, we administered AS01 intramuscularly to humanized NOG (hNOG) mice and collected BM cells at 4- and 8-week post-inoculation (**Fig 1A**).

Fig 1. AS01 induces type 1 IFN responses in diverse BM cells in humanized mice.
(A) Schematic of the study. NOG mice were transplanted with hCD34^{+} HSC and after 30 days AS01 and BCG was administered intramuscularly and intravenously, respectively to hNOG mice. 4- and 8-week later animals were sacrificed to isolate human myeloid BM cells, which were processed for scRNA-seq and scATAC-seq. (B) UMAP plot of scRNA-seq showing the annotation and colour codes for cell types in the BM environment of hNOG mice (C) Bubble plot showing enriched pathways, based on upregulated genes at 4-week in each cluster for AS01 (red) and BCG (blue). The size of the bubble is Bonferroni adjusted p-value. The intensity of the colour - fold enrichment in BCG or AS01 group. (D) Heatmap showing mean log2FC of type I IFN response genes in AS01 over control and BCG over control at 4 weeks post administration for each cluster in B. (E) Genomic views (hg38) at IFIT genes for cDC1, cDC2, monocyte and GMP. Blue shades indicate DARs in each cell type. Transcription factor (TF) binding track is TF binding sites derived from IRF1, STAT1 and STAT2 ChIP-seq experiments performed by the ENCODE project. The bottom track is *cis*-regulatory elements interactions predicted by Cicero co-accessibility algorithm.

In parallel, we performed intravenous BCG vaccination to analyse the effects of live BCG vaccine on human BM progenitors. Of note, the effect of BCG on BM progenitors has been already reported in humans and wild type (WT) mice[38–40]. From the BM of hNOG mice we flow-sorted human hCD45^{+}hCD3^{-}hCD19^{-}_ hCD20^{-}hCD56^{-} cells, which are devoid of mature T, B and natural killer (NK) cells, and performed single cell RNA sequencing (scRNA-seq) and single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) (**Fig 1A**). In scRNA-seq dataset we identified 12 transcriptionally distinct clusters, which were annotated based on the known cell-type markers (**Fig 1B**)[41,42]. We next identified differentially expressed genes (DEGs) in BM cell subsets from control, AS01-administered and BCG-vaccinated hNOG. At the 4-week time point we observed profound gene expression changes in many BM cell subsets of AS01-administered hNOG, which were dominated by genes involved in response to virus and response to type I IFN gene ontology (GO) terms (**Fig 1C**). In contrast, BCG-induced transcriptomic changes were mainly driven by genes in antigen processing and presentation, cytoplasmic translation and ribosome assembly (**Fig 1C**). Thus, our findings suggest that AS01-driven transcriptomic changes in BM populations are different compared to BCG-driven changes. Global scATAC-seq data also suggested enrichment of type 1 IFN response pathway in the BM cells of AS01-administered hNOG (data not shown). Analysing the expression of 35 genes in this pathway showed upregulation of several interferon-stimulated genes (ISGs) including MX1, ISG15, OAS2, and IFI, IFIT and IFITM families in all subsets of AS01-primed BM progenitor and myeloid cells compared to BCG-educated BM cells (**Fig 1D**). Assessment of genomic loci of IFIT and IFITM genes further highlight the shared and specific differential accessible regions (DARs) in AS01-primed and BCG-educated cDC1, cDC2, monocyte and GMP subsets with some of these peaks exhibiting co-accessibility profiles (**Fig 1E**). Motifs of IRF1, STAT1 and STAT2 occupy many of these differential accessible peaks, suggesting their role as master regulators of type 1 IFN response gene induction[43]. Of note, the expanded IFN response in AS01-primed BM cells at 4-week time point wanes off at 8-week time point (data not shown). Overall, our study indicates an epigenomic-mediated transcriptional control of type 1 IFN response pathway in AS01-primed BM cells.

Fig 2. AS01 reprograms BM monocytes that protect against pulmonary *Mycobacterium* infection.
(A) Schematic diagram of the AS01 or PBS administration (intramuscularly) in WT and CCR2-KO mice and challenge with either BCG (1x10^{6} CFU) or *Mtb* (~200 CFU). (B) BCG load in the lungs of AS01 and PBS administered WT mice at day 7 p.i. (C) Flow cytometry of lung myeloid cells. Dot plots of CD64^{+}F4/80^{+} macrophages are shown. (D) Median fluorescence intensity (MFI) of F4/80 expression. Left – overlay, right – bar graph of compiled data. (E) BCG load in the lungs of AS01 and PBS administered CCR2-KO mice at day 7 p.i. (F) *Mtb* load in the lungs of AS01 and PBS administered WT mice at day 7 and 22 p.i. Data is mean±SEM of n=5 mice/group/timepoint, unpaired t-test. Representative of 2-3 experiments.

AS01 adjuvant induces TI and monocyte-mediated protection against *Mycobacterium* infection in mice

Our monocyte chromatin accessibility data (not shown) indicates the induction of TI phenomenon in AS01-primed BM monocytes[40,44]. To investigate this, AS01 or PBS were administered intramuscularly to wild type mice and after 30 days mice were intratracheally infected with either BCG or *Mycobacterium tuberculosis* (*Mtb*) (**Fig 2A**). At 7- and 22-days post-infection (p.i), the lung bacterial load and myeloid cells profile was assessed. At 7 days p.i BCG lung burden was significantly lower in the lungs of AS01-administered wild type mice compared to PBS-treated control (**Fig 2B**). The reduced lung bacterial load corresponded with the decreased expression of F4/80 on $CD64^{+}F4/80^{+}$ lung macrophages in AS01-administered mice (**Fig 2C & 2D**). Decreased F4/80 expression has been similarly observed in trained peritoneal macrophages in subcutaneously BCG-vaccinated mice[45]. Protection against *Mtb* challenge in BCG-vaccinated mice has been attributed to the migration of BM-derived monocytes and macrophages to the lungs[40]. Thus, we examined the role of AS01-primed BM-derived monocytes using the CCR2-KO mice model, which lacks circulatory classical $Ly6c^{hi}$ monocytes (**Fig 2A**). The AS01-mediated protection against BCG was abolished in CCR2-KO mice (**Fig 2E)**. This indicated the necessity of monocytes / macrophages for AS01 mediated protection. We next used virulent *Mtb* to infect AS01-primed WT mice and lung CFU was assessed at days 7 and 22 p.i (**Fig 2A**). Compared to PBS control, lungs of AS01 administered mice showed significantly reduced CFU (**Fig 2F**). The AS01-mediated protection against *Mtb* was abolished in CCR2-KO mice (not shown). Together, these data demonstrate that intramuscular administration of AS01 alone can induce training in BM monocytes that migrate to lung and provide protection against *Mycobacterium* infection.

Two doses of RZV are required to induce protective levels of T cells, despite the presence of pre-existing immunity in all subjects (Chan et al., in review)

Varicella zoster virus (VZV) infection initially causes chickenpox and establishes life-long latency in neurons. As immunity wanes with age, VZV can reactivate as herpes zoster (HZ)[46]. While RZV's two-dose regimen provides over 90% efficacy in protection against HZ reactivation[6,7], an intriguing question emerges: why are two doses necessary when here is widespread pre-existing immunity in older populations (>95%)?[46] Elucidating these mechanisms will help develop more effective vaccination strategies to improve immune responses in aging populations.

To study AS01-adjuvanted RZV immune responses, we conducted a randomized trial with 30 healthy adults (50-65 years) with prior chickenpox exposure. Participants received either two RZV doses (n=15) at day 0 and 60, or one dose of zoster vaccine live (ZVL) (n=15) as control. We collected blood samples to analyse gene expression, cytokines, antibodies, and T cells (**Fig 3A**). Compared to ZVL vaccination, anti-gE IgG response to a single dose of RZV vaccination was significantly higher (data not shown). The second dose of RZV did not boost antibody titres, suggesting that from a humoral response perspective, the second RZV dose was dispensable (data not shown). However, the second dose of RZV vaccination significantly increased gE-specific T cell counts at day 14 after dose 2 (**Fig 3B**). Given that the magnitude of VZV T cell response is known to correlate with protection from herpes zoster[47,48], and that two-doses of RZV produced 90% efficacy[6,7], we used the 10^{th} percentile of the gE-specific T cell counts after two doses of RZV to define a surrogate threshold of protection (**Fig 3B**). In addition, consistent with clinical findings that a single dose of RZV showed sub-clinical efficacy[49], only four individuals (27%) achieved T cell responses exceeding the threshold associated with protection (**Fig 3B**). These individuals, designated as 'fast responders' (FR), due to their rapid achievement protective T-cell response after a single dose, continued to have higher gE-specific T cell counts at day 60 post-vaccination. On the other hand, the

Fig 3. Fast responders achieve protective T cell levels after one dose of RZV while slow responders required two doses. (A) Study design. (B) Total gE-specific T cell responses to RZV measured on day 0, 14, 60, 74 using ELISpot. Slow responder (SR): purple (n = 11); Fast responder (FR): orange (n = 4).

majority of other subjects, referred to as 'slow responders' (SR), achieved protective T cell levels only after the second dose. Overall, the results indicate that despite the presence of pre-existing adaptive immune response against VZV in all subjects, there were heterogenous T cell responses among older individuals, and a two-dose regimen was needed for

Fig 4. First dose of RZV resets the baseline expression of transcriptomic correlates of T cell response. (A) Venn diagram of the shared number of protective T cell correlates based on transcripts identified. Red denotes up-regulated genes, blue denotes down-regulated genes. (B**)** Hierarchical clustering that had a log2FC = +/- 1.5 difference between D61 v 60 (Dose 2) and D1v0 (Dose 1) transcriptomic response of SR and also a FC = +/- 1.5 difference in D1v0 (Dose 1) transcriptomic response between FR (Orange) and SR (Purple). (C) Shared T cell correlates between protective T cell correlates for RZV in older adults (as in A) and T cell correlates for BNT162b2 vaccination in naïve adults. T cell correlates for BNT162b2 determined from correlating with ELISpot responses with D1v0 log2FC transcriptomic responses (Spearman's pairwise correlation; r = +/- 0.3). Common transcript highlighted in boxes where colours indicate the direction of association. Red and blue indicates positive and negative correlates. *Indicate coding transcripts. (D) Averaged expression (z-score) of the T cell correlates over time for FR and SR.

RZV to induce sufficient T cell responses in all subjects.

Gene expression changes associated with protective T cell levels after RZV administration

Next, we examined characteristics associated with FR and SR. T cell response differences were independent of age, gender, body mass index, adverse events (AE), pre-existing antibody titers and gE-specific T cell levels at the point of vaccination (Data not shown).

Given that demographics and baseline gE-specific T cell levels did not explain the differences in T cell response observed in FR and SR, we performed transcriptional profiling of whole blood from all 15 RZV recipients with the aim to identify early molecular signatures that could explain the differential responses between these groups. To do so, we employed a 3-step analysis approach. First, we compared gene expression between FR and SR after the first dose, identifying 1,145 DEGs (fold-change > 1.5) (data not shown). Second, since SR showed improved T cell responses after dose 2, we performed a similar analysis where we compared the fold-change differences between dose 2 and dose 1 in SR, revealing 615 genes with fold-difference >1.5 (data not shown). Finally, using Venn diagram analysis of these 2 gene sets, we identified 201 overlapping genes that were both differentially expressed between FR and SR after dose 1 and significantly changed after dose 2 (**Fig 4A**). These 201 overlapping genes likely represent key markers of T cell responses. Indeed, unsupervised hierarchical clustering confirmed that these 201 genes effectively distinguished FR and SR subjects (**Fig 4B**).

As all the recruited subjects had pre-existing adaptive immune response against VZV, the 201 genes that separate FR and SR would also contain gene expression changes from memory responses. Thus, to identify which of these genes drove T cell response to vaccination independent of memory responses, we leveraged on a study where SARS-CoV-2-naïve individuals were given the Pfizer-BioNTech BNT162b2 vaccine[50]. Further Venn diagram analysis between these 201 genes and genes correlating with naïve T cell responses to BNT162b2 vaccine identified 27 genes (**Fig 4C**). The magnitude of fold-change of these 27 genes at day 1 post RZV vaccination also significantly predicted protective T cell levels after either dose of RZV with receiver operating characteristic (ROC) curve with area under the curve (AUC) = 0.98 and $p < 0.0001$ (Data not shown). These genes included genes that encode for various cell functions, including the component of the receptor for the inflammatory cytokine IL-31 (IL31RA), endopeptidase (CAPNS2), membrane proteins (LY6G6C, MAL2), regulatory signalling proteins (INHBB, RGS7), and a cell cycle regulator (CEND1)[51].

First RZV dose induces gene expression changes that enhance T cell responses to the second dose

The higher magnitude of $\log_2$ fold-change differences observed between FR and SRs across these 27 genes could be potentially explained by two distinct possibilities. The first possibility is that both FR and SR shared the same baseline gene expression, but FR showed greater change in expression than SR. The other possibility is that the expression of these genes was different between FR and SR at baseline. We thus examined the gene expression in FR and SR at day 0 and day 1 post-RZV vaccination. The aggregate expression of the 27 genes was significantly different between FR and SR at day 0 and day 1 post-RZV vaccination (**Fig 4D**). These genes were more lowly expressed in FR compared to SR before vaccination, which was then associated with greater increase in expression at one day post-RZV vaccination. If a lower expression of these 27 genes at baseline could explain T cell response to RZV, then the first RZV dose should lead to a lower expression of these genes before the second RZV dose. Indeed, the expression of these genes were lowered following the first dose of RZV in SR to levels comparable to those in FR (**Fig 4D**). Concomitantly, the second dose in SR elicited a greater increase in gene expression at day 61 (**Fig 4D**). Collectively, our analysis revealed 27 signature genes correlated with T cell responses in older adults. The first dose of RZV induced critical expression changes in these genes, which helped overcome heterogenicity in T-cell responses and effectively restored the aging immune system's ability to mount strong T cell responses after the second dose for all subjects.

Collectively, our preliminary findings reveal AS01's potential to epigenetically reprogram innate immune cells for enhanced adaptive immunity. During early disease outbreaks, AS01 could serve as a rapid and broadly protective immune booster for older populations before pathogen-specific interventions become available, offering a novel strategy to reduce disease severity. Despite AS01 and TI's promise as a new frontier in vaccinology, its effects have yet to be studied in humans, including the extent and durability of AS01-induced protection against pathogens. Findings from this study will provide unique insights into TI as a rapid and broadly protective immune booster for older populations before pathogen-specific interventions become available, offering a novel strategy to reduce disease severity

1.2.2 Rationale for Doses Selected

Eligible volunteers will be randomised 1:1 to receive either 1 dose of AS01 (GSK) or Placebo (NaCl 0.9%) and then randomised again to receive one dose of YF17D (Stamaril, Sanofi-Pasteur) on either day 30 or day 90 according to manufacturer's instruction.

i. **One dose of AS01 (0.5ml) or placebo (NaCl 0.9%, 0.5ml) on day 0** is administered intramuscularly in the deltoid region of the upper arm. The AS01 adjuvant component (0.5ml, which contains 50mg of 3-O-desacyl-4'monophosphoryl lipid A and 50mg of Quillaja Saponaria Molina fraction 21) is supplied as a separate vial in the licensed recombinant zoster vaccine (Shingrix)[52].

ii. **One dose of YF17D (0.5ml) on either day 30 (YF17D 1-month group) or day 90 (YF17D 3-month group),** administered subcutaneously in the deltoid region of the upper arm[53].

A single dose of AS01 has been chosen based on previous studies and preliminary data demonstrating that one dose is sufficient to induce significant changes in immune cell populations, including epigenetic reprogramming[13].

1.2.3 Rationale for Study Population

We will recruit 40 healthy adults 21 to 59 years of age, with a BMI ranging from ≥18.5 – 27.5 kg / m^2. The rationale for recruiting people aged 21-59 is twofold: First, although the study aims to improve vaccine development for older adults, the primary objective is to establish the molecular mechanisms elicited by AS01, which can be identified regardless of participant age. Second, expanding the age range increases the likelihood of successful recruitment, particularly when involving dengue seronegative subjects. The upper age limit of 60 is set because YF17D is not recommended for people above 60 years of age due to a higher risk of rare but potentially life-threatening AEs[54]. These restrictions will also ensure that our results are not confounded by extremes of BMI or poorly controlled diseases[55,56]. Subjects will be excluded if they have previously received yellow fever (YF) vaccines or AS01-adjuvanted vaccines, have a history of YF infection, dengue virus seropositivity, or known allergy to study vaccines, their components, or egg products. Dengue seropositivity is excluded because cross-reactive antibodies can confound yellow fever immunogenicity results[16,57]. Additional exclusion criteria that would be a risk factor for live-attenuated vaccines and blood taking, as established in our previous trials, will also be excluded[16,58].

(Please also refer to section on inclusion and exclusion criteria for details)

1.2.4 Rationale for Study Design

To test each of the three specific aims and corresponding hypotheses, we propose an experimental medicine study involving healthy older adult volunteers. We will administer AS01 and use the yellow fever live-attenuated vaccine (YF17D) to simulate a heterologous systemic viral infection. The overview of or proposed study is shown **(Fig 5A).**

The study will follow a pre-defined visit schedule and blood collection timepoints (**Fig 5B, C**). Eligible volunteers (n = 40) will be randomised in a 1:1 ratio to receive:

i) One dose of AS01 (n = 20) or NaCl 0.9% placebo (n = 20) on day 0: The AS01 suspension contains 50mg of 3-O-desacyl-4'monophosphoryl lipid A (MPL) and 50mg of Quillaja Saponaria Molina, fraction 21 (QS21), licensed by GSK, available as a separate vial from the licensed Shingrix vaccine[6]. The placebo will consist of 0.9% saline solution in equivalent volume. All AS01/placebo administration will occur in morning sessions to control for circadian rhythm effects on TI and immune responses[59]. Treatment allocation will be determined using a computer-generated randomization sequence, with assignments sealed in opaque envelopes. These envelopes will be opened by the study coordinator only at the time of intervention administration. This study will employ a single-blinded design, where subjects will be unaware of AS01/placebo allocation. Both treatments will be administered intramuscularly into the deltoid muscle. A single dose administration strategy has been chosen based on previous studies and preliminary data demonstrating that one dose of AS01 is sufficient to induce significant changes in immune cell populations, including epigenetic reprogramming[13].

Fig 5. Study Design. (A) Randomization. (B) Timeline for YF17D 1-month group and (C) YF17D 3-month group.

ii) One dose of YF17D (Stamaril, Sanofi-Pasteur) on either day 30 (YF17D 1-month group, n = 20. Fig 5B) or day 90 (YF17D 3-month group, n = 20. Fig. 5C): Research blood samples will be collected at pre-defined intervals by venipuncture to characterize host responses post-AS01/placebo for laboratory studies according to study schedule, as shown.

2. HYPOTHESIS AND OBJECTIVES

Specific aim 1: Examine the host response induced by AS01 and determine the duration of these changes for up to 3 months in healthy older adults aged 21 – 59 years old.
This aim **tests the hypothesis** that the AS01 adjuvant will induce significant and persistent changes in gene expressions and epigenetic markers associated with innate immunity in older adults.

Specific aim 2: Demonstrate that innate immune reprogramming post AS01 can provide cross-protection against heterologous YF17D viral infection.
This aim **tests the hypothesis** that AS01 recipients will have 50% reduction in detectable viremia versus placebo after YF17D challenge at 1 month, while also evaluating the longevity of this cross-protection at 3 months post-AS01.

Specific aim 3: Examine the T cell and B cell responses post YF17D challenge, and its correlation with YF viremia levels in AS01 group versus placebo.

This aim **tests the hypothesis** that the reprogramming of innate immune cells post-AS01 promotes enhanced YF viremia clearance without compromising humoral and cellular immune response to YF17D.

2.1. Potential Risks and Benefits

2.2.1 Potential Risks

Obtaining blood can cause bleeding, bruising, or swelling at the sight of needlestick, as well as thrombophlebitis. Infection at the site of needlestick and fainting rarely occurs. In addition, there are potential risks of adverse reactions from taking the vaccines.

For AS01 (According to published adverse events for Shingrix): Solicited local AEs in subjects aged 50 years and older were pain (78.0%), redness (38.1%), and swelling (25.9%). Solicited general AEs were myalgia (44.7%), fatigue (44.5%), headache (37.7%), shivering (26.8%), fever (20.5%), and gastrointestinal symptoms (17.3%)[60].

For YF17D, adverse events include mild headaches, myalgia, low-grade fevers, or other minor symptoms for 5 - 10 days. Local reactions include oedema, hypersensitivity, pain or mass at the injection site. Immediate hypersensitivity reactions, characterised by rash, urticaria, and/or asthma, anaphylaxis occur principally among persons with histories of allergy to eggs or other substances contained in the vaccine. Other adverse events include Yellow Fever Vaccine-Associated Neurotrophic Disease (YEL-AND) with incidence rate of 1 per 100,000, Guillain-Barre-syndrome, acute disseminated encephalomyelitis (ADEM) and bulbar palsy. Yellow Fever Vaccine-Associated Viscerotropic Disease (YEL-AVD) has been reported (Incidence 1 per 400,000 doses), with age $\geq$ 60 being a risk factor[53] and hence they will be excluded from this study.

2.2.2 Potential Benefits

Participants will receive a licensed yellow fever vaccine, which will help reduce the risk of developing yellow fever as well as associated complications should they travel to endemic countries in the future.

Novel knowledge gained on the induction of trained immunity by AS01 would help improve the development of broadly protective vaccines in the future for this group of population. This information is currently missing, and we believe that addressing this knowledge gap would revolutionise vaccine development. Since older adults are particularly susceptible to the complications of emerging infectious diseases, the development of trained immunity vaccines that confer broad protection will ensure they are better protected in future outbreaks.

3. STUDY POPULATION

3.1. List the number and nature of subjects to be enrolled.

We plan to recruit 40 adults with 21 to 59 years of age. (Please also refer to section on inclusion and exclusion criteria for details)

3.2. Criteria for Recruitment and Recruitment Process

Subjects will be recruited from SGH Clinical Trials and Research Centre (CTRC) healthy volunteer database and through poster advertisement. Subjects will be given a copy of the Participant Information Sheet and Informed Consent Form to read upon arrival. A briefing session on the study will be conducted by the Investigator. Thereafter, subjects will be ushered into a private room where written informed consent will be obtained and where they can freely ask questions about the study. Subjects will not be rushed into deciding to participate in the study and will be permitted to defer their decision (without any prejudice) after careful consideration and discussion with their family members.

3.3. Inclusion Criteria

Inclusion Criteria

1) Adults aged 21 to 59 years of age at time of screening.
2) BMI 18.5 – 27.5 kg / m^2 (BMI values for Asian population according to MOH guideline NIH Consensus Conference)[61].
3) Satisfactory baseline medical assessment as assessed by physical examination and a stable health status. For subjects with underlying comorbidities, the conditions must be deemed stable by the investigators, and they must not have any hospitalisation relating to these conditions in the last 6 months.
4) Voluntarily participate, understand and sign an informed consent form approved by the Ethical Review Board.
5) Subjects who are willing to comply with the requirements of the study protocol and scheduled visits. These requirements include completion of the subject diary, return for follow-up visits. Subjects should also be willing to make themselves available for the duration of the study, with access to a consistent means of contact.
6) Accessible vein at the forearm for blood taking.
7) Female subjects of non-childbearing potential due to surgical sterilisation (hysterectomy or bilateral oophorectomy or tubal ligation) or menopause. Post-menopausal subjects must have had at least 12 months of natural (spontaneous) amenorrhoea.

Exclusion Criteria

1) Previous vaccination against yellow fever, dengue either with a registered product or from participation in a previous vaccine study.
2) Previously received AS01-adjuvanted vaccines (e.g. Recombinant zoster vaccine, RTS,S/AS01, RSVPre-F3-AS01), either with a registered product or from participation in a previous vaccine study.
3) Planned administration of a AS01-adjuvanted vaccine or yellow fever vaccine other than the study vaccine during the study.
4) Subjects who have been unwell in the last 7 days prior to screening.
5) History of documented yellow fever and / or dengue infection.
6) Dengue seropositivity upon screening.
7) History of smoking within the last 1 year.
8) Planned travel to yellow fever endemic countries during the study.
9) Known allergy to AS01 and YF17D vaccine or their components (e.g. egg products).
10) Diagnosis of diabetes HBA1c $\geq$ 6.5 according to American Diabetes Association criteria[62].
11) Any medical condition that in the judgment of the investigator will make intramuscular injection unsafe (e.g. thrombocytopenia with platelet count < 50x10^9/L, coagulopathy, anti-coagulant therapy).
12) Risk factor for live-attenuated vaccines, including any confirmed or suspected primary or acquired immunodeficiency based on history and physical examination:
    - History of thymus gland disease
    - Haematologic neoplasms including leukaemia, lymphoma, myelodysplastic syndromes
    - Diagnosed with cancer or treatment for cancer (except for localised basal cell carcinoma) within 3 years prior to screening
    - Post-transplant: solid organ and haematopoietic stem cell transplant
    - Immunocompromised due to primary or acquired (including HIV/AIDS) immunodeficiency
    - Other significantly immunocompromising conditions
13) Administration of anti-inflammatory drugs for the past 7 days (e.g. NSAIDs, Paracetamol, aspirin).
14) Use of metformin for the last 1 month.

15) Use of corticosteroids within the last 6 months prior to the first vaccine dose (defined as prednisolone ≥ 10 mg / day or equivalent for ≥ 2 weeks, or prednisolone ≥ 40mg / day or ≥ 1 week). Inhaled and topical steroids are allowed.
16) Received biologics (such as anti-TNF inhibitors, IL-1 inhibitors, co-stimulation blockers, B-cell depleting therapy) for the last 12 months.
17) Any condition (e.g. extensive psoriasis, chronic pain syndrome, severe hearing loss, cognitive impairment, dialysis, autoimmune disorders) that in the opinion of the investigator, would complicate or compromise the study or wellbeing of the subject, or prevent completion of the study.
18) Evidence of substance abuse, or previous substance abuse.
19) Clinically significant anaemia (Hb < 10 g/dL).
20) Blood donation exceeding > 450 ml in the past 3 months.
21) Participation in a study involving administration of an investigational or non-investigational compound within the past four months or planned participation during the duration of this study.
22) Administration of any licensed vaccine within 30 days before the first study vaccine dose or planned to receive such products within 30 days after the study vaccination.
23) Received immunoglobulin or any blood products within the 90 days preceding the first dose of study vaccine or planned to receive such products during the study period.

3.5 Subject Replacement

Subjects who drop out will not be replaced.

4 STUDY DESIGN

This is a single-centre, randomised, single-blind, placebo-controlled outpatient trial in healthy older adults. Study will be conducted at the Clinical Trials and Research Centre (CTRC) of Singapore General Hospital.

4.1 Contraception and Pregnancy Testing

All female subjects of child-bearing potential will need to undergo a urine pregnancy test at screening and on the day of administration of the vaccine. Only those with a negative urine pregnancy test will be considered eligible for the study, provided that other eligibility criteria are fulfilled.

4.2 Screening Visits and Procedures

40 healthy volunteers 21 to 59 years of age at time of screening will be recruited through CTRC database and screened for eligibility (Refer to section on inclusion and exclusion criteria). Informed written consent will be sought from subjects who fulfil criteria for enrolment. All consented subjects will undergo screening, which includes medical history, physical examination, urinary pregnancy test (for female subjects of child-bearing potential), screening blood tests (full blood count, dengue serology, HBA1c).

Randomisation and Study Arms:

All eligible and enrolled subjects will undergo two sequential randomisations (**Fig. 5A**):

i) First randomisation (1:1 ratio):
- AS01 group (intervention group): n = 20
- Placebo (NaCl 0.9%) group: n = 20

ii) Second randomisation (1:1 ratio):
- YF17D at 1 month (n = 20)
- YF17D at 3 months (n = 20)

Both randomisations will be computer-generated sequences with allocations sealed in opaque envelopes, opened by the study coordinator at the time of intervention administration.

4.2.1 Study Visits and Procedures

i) Initial intervention: Intramuscular administration of AS01 or placebo in deltoid muscle. Investigational bloods will be collected by venepuncture at pre-defined timepoints (**Fig. 5B, C**):
   a. YF17D 1-month group: Immediately before injection (day 0), day 1, day 7, day 14.
   b. YF17D 3-month group: Immediately before injection (day 0), day 1, day 7, day 14, day 21, day 30, day 60.

ii) Second intervention: YF17D administration at either 1 month (day 30) or 3 months (day 90). Investigational bloods will be collected by venepuncture at pre-defined timepoints (**Fig. 5B, C**):
   a. YF17D 1-month group: Immediately before YF17D (Day 30), day 32, day 33, day 34, day 35, day 36, day 37, day 44, day 60.
   b. YF17D 3-month group: immediately before YF17D (day 90), day 92, day 93, day 94, day 95, day 96, day 97, day 104, day 120.

The comprehensive blood sampling schedule in this study is essential to fully characterise the temporal dynamics and duration of trained immunity induced by AS01 and effect on YF vaccine-induced viraemia and immune (acute, adaptive) responses. The blood volume requested represents the minimum amount needed to perform the full panel of assays required to address the three specific aims, as supported by published studies in the field of trained immunity.

4.2.2 Final Study Visit

The last study day will be on day 60 for YF17D 1-month group and D120 for YF17D 3-month group.

4.2.3 Post Study Follow up and Procedures

There is no requirement for post-study follow-up and procedures.

4.3 Discontinuation/Withdrawal

4.3.1 Discontinuation Criteria

Subjects are free to withdraw consent and discontinue their participation at any time without prejudice to them or effect on their medical care. Subjects may also be withdrawn or removed, if necessary, to protect their health or integrity of the study. The investigator also reserves the right to withdraw participants from the study in event of inter-current illness, adverse events, protocol violations, administrative, or other reasons. Once a subject withdraws from the study, no new data will be collected for study purposes unless the data concerns safety or adverse events related to the study. All details available will be reported and recorded for any subjects that withdraws or is removed from the study.

The PI may stop subject's participation in the study at any time for one or more of the following conditions:

- Failure to follow the instructions of the investigators or study staff.
- The PI decides that continuing the participation could be harmful.
- Pregnancy
- Subject needs treatment not allowed in the study.
- The study is cancelled.
- Other unanticipated circumstances.

4.3.2 Discontinuation Visit and Procedures

Participants are free to withdraw consent and discontinue participation at any time without penalty and are not obliged to give their reason. Subjects may also be withdrawn or removed, if necessary, to protect their health or the integrity of the study. The investigator also reserves the right to withdraw participants from the study in event of inter-current illness, adverse events, protocol violations, administrative, or other reasons. Once a subject withdraws from the study, no new data will be collected for study purposes unless the data

concerns safety or adverse events related to the study. The subject will be asked to continue scheduled evaluations, complete an end of study evaluation, and be given appropriate care under medical supervision until the symptoms of any adverse events resolve or the subject's condition becomes stable. All details available will be reported and recorded for any subjects that withdraws or is removed from the study.

5 TRIAL MATERIALS

5.1 Trial Product(s)

i) AS01 Adjuvant

Content:
Throughout this protocol, we refer to the **AS01B** adjuvant system as "**AS01**". The GlaxoSmithKline (GSK) proprietary AS01B adjuvant system is composed of the plant extract Quillaja saponaria Molina, fraction 21 (QS-21) (50 micrograms) and 3-O-desacyl-4'monophosphoryl lipid A (MPL) from Salmonella minnesota (50 micrograms). The AS01 suspension is an opalescent, colourless to pale brownish liquid.

Dosage and administration:
The AS01 adjuvant is supplied as a component of the recombinant zoster vaccine (RZV; GSK), which normally contains 2 separate vials: a 0.5ml vial containing the AS01 adjuvant system and another vial containing the varicella zoster virus (VZV) glycoprotein E (gE) antigen. While these vials are typically reconstituted together for RZV administration, in this study, subjects will only receive the AS01 adjuvant vial (0.5ml) without reconstitution with the gE antigen. AS01 0.5ml will be given as intramuscular injection in the deltoid muscle.

Contraindications:
Do not administer Shingrix to anyone with a history of a severe allergic reaction (e.g. anaphylaxis) to any component of the vaccine or after a previous dose of RZV.

Adverse reactions (based on RZV data, which contains reconstituted AS01 plus VZV gE antigen):
Adverse reactions (AEs): Solicited local AEs in subjects aged 50 years and older were pain (78.0%), redness (38.1%), and swelling (25.9%). Solicited general AEs in subjects aged 50 years and older were myalgia (44.7%), fatigue (44.5%), headache (37.7%), shivering (26.8%), fever (20.5%), and gastrointestinal symptoms (17.3%).

Storage:
Adjuvant suspension component vials: Store refrigerated between 2°C to 8°C. Protect vials from light. Do not freeze. Discard if the adjuvant suspension has been frozen.

ii) Yellow Fever Vaccine (Stamaril)

Content:
Stamaril, the live-attenuated yellow fever vaccine, utilises the YF17D strain. Stamaril is supplied in the form of powder and solvent for suspension for injection in pre-filled syringe, Yellow fever vaccine (live). Before reconstitution, the powder is homogeneous, beige to orange beige, and the solvent is a clear and colourless solution.

Dosage and administration:
Stamaril (1 dose, 0.5ml after reconstitution) is injected by the subcutaneous route.

Contraindications:

- Hypersensitivity to the active substance or to any of the excipients listed in Section 6.1 or to eggs or chicken proteins.
- Severe hypersensitivity reactions (e.g., anaphylaxis) after a previous dose of any yellow fever vaccine.

- Immunosuppression, whether congenital, or acquired. This includes individuals receiving immunosuppressive therapies such as treatment with high-dose systemic corticosteroids (e.g. daily dose of 20 mg or 2 mg/kg body weight of prednisone or equivalent for 2 weeks or more or daily dose of 40 mg or more of prednisone for more than one week), any other medicinal products including biologicals with known immunosuppressive properties, radiotherapy, cytotoxic drugs or any other condition which may result in immunocompromised status.
- History of thymus dysfunction (including *myasthenia gravis*, thymoma).
- Thymectomy (for any reason).
- Symptomatic HIV infection.
- Asymptomatic HIV infection when accompanied by evidence of impaired immune function
- Moderate or severe febrile illness or acute illness.

Adverse reactions:
The most frequently reported reactions (between 12 – 18% of subjects) were headache, asthenia, injection site pain and myalgia. Adverse reactions usually occurred within the first three days following vaccination except pyrexia, which occurred between day 4 and day 14 and usually lasted for not more than 3 days. Both local and systemic reactions were usually of mild intensity; however at least one severe injection site reaction was reported in 0.8% of subjects and at least one severe systemic reaction was reported in 1.4% of subjects in general population. Other adverse reactions ($\geq$ 1 in 100 to < 1 in 10) include rash, nausea, and arthralgia. Very rarely (< 1 in 10,000), YEL-AND and YEL-AVD have been reported following vaccination with sequelae or with fatal outcomes and risk appears to be higher in those aged over 60 years of age.

Storage:
Store in a refrigerator (2°C - 8°C). Do not freeze. Keep the vial of powder and the syringe of solvent in the outer carton in order to protect from light.

5.2 Storage and Drug Accountability
Upon receipt of the vaccine product, the delegated research staff at CTRC will be responsible for taking an inventory of the investigational products. A record of this inventory must be kept and dispensation of vaccines must be documented on drug inventory forms. The record must be available for inspection by the medical monitor, IRB or regulatory authorities.

6 TREATMENT

6.1 Rationale for Selection of Dose
Yellow fever vaccine containing the YF17D strain (Stamaril) has been licensed and approved for use. The above dose and method of administration are chosen based on vaccine manufacturer's recommendations.

AS01 adjuvant has been licensed and approved for use together with the recombinant zoster vaccine (Shingrix). The above dose and method of administration are chosen based on vaccine manufacturer's recommendations, except VZV gE antigen will not be included in this study.

6.2 Study Drug Formulations
Live-attenuated yellow fever vaccine (Stamaril) and recombinant zoster vaccine (Shingrix) have been licensed and approved for use in Singapore.

6.3 Study Drug Administration

i) AS01 adjuvant or placebo
0.5ml AS01 or placebo (NaCl 0.9%) will be given as intramuscular injection in the deltoid muscle.

ii) YF17D (Stamaril) vaccine:
Stamaril (1 dose, 0.5ml after reconstitution) is injected by the subcutaneous route.

6.4 Specific Restrictions / Requirements

Subjects will be educated to refrain from self-administration of other prescription-only or over-the-counter oral or topical anti-inflammatory drugs, if possible, at any time throughout the study period. Subject should inform the CTRC or study team members if they are not feeling well for assessment before seeing General Practitioner. If the subject has seen a General Practitioner and was prescribed with drugs, they should be advised to inform study team members.

6.5 Blinding

Blinding:

- First intervention (AS01/placebo): single blinded – subjects are blinded to treatment allocation. Subjects will not be told their assignments unless unblinding is required to protect participant safety.

- Second intervention (YF17D timing): Open-label – no blinding.

Planned Unblinding:

- The investigators will receive unblinded data for analysis.

Emergency Unblinding:

- Emergency unblinding may occur in case of serious adverse events (SAEs) or medical emergencies where knowledge of the treatment is essential for subject care.
- The PI or designated physician may request emergency unblinding.
- All instances of emergency unblinding must be documented with:
    - Date and time of unblinding
    - Reason for unblinding
    - Name of person requesting unblinding
    - Name of person performing unblinding

6.6 Concomitant therapy

All vaccinations, medications (prescription and over the counter), vitamin and mineral supplements, and / or herbs taken by the participant should be documented.

7 SAFETY MEASUREMENTS

7.1 Definitions

An adverse event (AE) is any untoward medical occurrence in a trial participant administered a therapeutic products (TP)/ cell, tissue and gene therapy products (CTGTP)/ medicinal products (MP) and which does not necessarily have a causal relationship with this treatment.

An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a TP/CTGTP/MP, whether or not considered related to the TP/CTGTP/MP.

A serious adverse event (SAE) is any untoward medical occurrence that at any dose:

a. results in death
b. is life-threatening

c. requires inpatient hospitalisation or prolongation of existing hospitalisation
d. results in persistent or significant disability/incapacity, or
e. is a congenital anomaly/birth defect

7.2 Collecting, Recording and Reporting of Serious Adverse Events (SAEs) to CIRB

The reporting requirements will be in accordance to the reporting requirements published on CIRB website at the time when the event took place.

Only related SAEs (definitely/ probably/ possibly) will be reported to CIRB. Related means there is a reasonable possibility that the event may have been caused by participation in the clinical trial.

The investigator is responsible for informing CIRB after first knowledge that the case qualifies for reporting. Follow-up information will be actively sought and submitted as it becomes available.

Related AEs will not be reported to CIRB. However, the investigator is responsible to keep record of such AEs cases at the Study Site File.

7.3 Collecting, Recording and Reporting of Serious Adverse Events (SAEs) to the Health Sciences Authority (HSA)

The reporting requirements will be based on the reporting requirements published on HSA website at the time when the event took place.

7.4 Safety Monitoring Plan

The study may be evaluated by government inspectors/regulatory authorities who must be allowed access to electronic case report forms (e-CRFs), source documents, and other study files. The inspectors will review CRFs and compare them with source documents to verify accurate and complete collection of data and confirm that the study is being conducted according to the protocol, ICH-Good Clinical Practices (ICH-GCP) and all applicable regulations.

7.5 Complaint Handling

Subjects can complain to the Principal Investigator or Centralised Institutional Review Board (CIRB). The contact numbers are listed in the Participant Information and Informed Consent Form.

8 DATA ANALYSIS

8.1 Data Quality Assurance

The PI and Co-Is will periodically review the study for data and safety monitoring. Internal quality checks will be performed by two CRC who are study team members. The data entered by one CRC will be checked by another using the source documents. The study may also be evaluated by government inspectors / regulatory authorities who must be allowed to access to e-CRFs, source documents, and other study files. The inspectors will review CRFs and compare them with source documents to confirm accurate and complete collection of data. The inspectors will also ensure that the study is being conducted according to the protocol, ICH-Good Clinical Practices and all applicable regulations.

8.2 Data Entry and Storage

Demographic and clinical data will be collected using computer notebooks. The data will be kept confidential in password-protected computers accessible only by delegated research staff. Subject identifiers will be kept in a separate file in another office, and every effort will be made to protect the privacy of study participants. Data analysis will be conducted only on de-identified data. An electronic data capture system will be used.

9 SAMPLE SIZE AND STATISTICAL METHODS

9.1 Determination of Sample Size

Based on power calculation, we determined that 16 subjects per group (total 32) would provide 80% power to detect a true difference in gene expression of at least 2 standard deviations between vaccine groups, with a false discovery rate of ≤ 10%. This sample size is also sufficient for achieving reproducibility in gene set analysis[63]. To account for potential dropouts during the study period, we will enrol 40 subjects (20 per group).

9.2 Statistical and Analytical Plans

Specific Aim 1: Examine the host response induced by AS01 and determine the duration of these changes for up to 3 months in healthy older adults aged 21 – 59 years old.

Sample collection, laboratory investigations and safety considerations

Samples (PBMCs, serum, plasma) will be stored at – 80°C and analysed collectively. Subjects will undergo clinical review during visits, including medical history, vaccination history, drug history and physical examination. They will be instructed to observe for adjuvant-related AEs such as injection site reactions, fever, myalgia, fatigue and headache throughout the study period. Participants will maintain a diary to record any AEs and report to CTRC should they require medical attention.

CD56	CD29
CD1c	CD45
CD3	Viability
CD20	CD27
IgD	HLA-DR
CD14	CD66b
Vd2	CD8
CD172a	Lox-1
PD1	CD123
Vd1	FceR1a
CD16	CD15
CD11b	CD45Ra
CD141	CD33

Table 1: Markers for immunophenotyping

Early AS01-induced effects on immune cell populations will be analysed through flow cytometry, focusing particularly on myeloid cells (monocytes, DCs, plasmacytoid dendritic cells (pDCs), NK cells, and innate lymphoid cells (ILCs) due to their crucial role in innate immune response and TI. Flow cytometric analysis for immunophenotyping of blood leukocytes will be carried out using a 26-marker panel (Table 1), with PBMCs being thawed and stained according to our established protocols[64]. After staining, cells will be fixed in 2% paraformaldehyde before acquisition on BD Symphony cytometer. Flow cytometry data will be analyzed with FlowJo™ version 10.3 (Tree Star, Inc., USA).

Comprehensive analysis of gene expression profiles in individual immune cell types will be conducted using scRNA-seq on PBMCs, followed by comprehensive functional and pathway analyses over baselines (day 0) and post-vaccination timepoints (**Fig. 5B, C**). In brief, scRNA-seq library preparation and sequencing will be performed on thawed PBMCs resuspended in PBS + 1% BSA for processing by the Chromium Next GEM Single Cell 5' Kit (10x Genomics) v2. The Chromium Controller will be used to target droplet encapsulation of 5,000 cells, and gene expression libraries will be prepared following the manufacturer's protocol. The libraries will be quantified on the Agilent Bioanalyzer and sequenced at 2×151 cycles on Illumina NovaSeq 6000 at a targeted sequencing depth of 50,000 reads/cell. Impact of AS01 on epigenome will be investigated by genome-wide *scATAC-seq*. Processing and analytical pipelines for the single cell studies is already established in Co-I Singhal's group[65] (Nigeshi et al., submitted). scATAC-seq library preparation and sequencing will be performed on nuclei from thawed PBMCs and generated following the low cell input nuclei isolation option of the 10x Genomics Demonstrated Protocol CG000169, using 1x Lysis Buffer and 3 min lysis time. Nuclei will be processed by the Chromium Single Cell ATAC Kit (10x Genomics) v2 following the manufacturer's protocol, targeting the capture of 5,000-6,000 nuclei or fewer if the sample was limiting. After quantification on the Agilent Bioanalyzer, 2-3 ATAC-seq libraries will be pooled for sequencing per lane of Illumina HiSeq 4000.

Plasma cytokines and chemokines will be measured at pre-vaccination baseline (Day 0) and post-vaccination timepoints (**Fig. 5B, C**) using the Olink Target 48 Cytokine panel as previously performed and published by our group[66]. Olink's proximity extension assay technology permits quantitative detection of 45 protein biomarkers

simultaneously, including cytokines and chemokines to depict the immune response following infection or vaccination. This analysis will complement our flow cytometry and sequencing data by providing insights into the early systemic immune environment that regulate cellular responses. The Olink analysis will be conducted at the Viral Research and Experimental Medicine Centre @ SingHealth Duke-NUS (ViREMiCS).

Analysis of scRNA-seq data

Raw FASTQ files data will be aligned to human reference genome GRCh38 by Cell Ranger (version 7.1.0, 10x Genomics)[67]. The output files will then be loaded into R package Seurat (version 4.4.0)[68]. Quality control (QC) will be performed for each library and low-quality cells will be removed. Cells containing less than 10% of mitochondrial reads will be retained in the analysis. After dimensional reduction using Principal Component Analysis (PCA) followed by Uniform Manifold Approximation and Projection (UMAP), unsupervised graph-based clustering (resolution=0.5) will be performed using *FindClusters* function of Seurat package. Major cell types will be annotated according to expression of canonical cell type markers, which will be further refined in downstream sub-clustering of each cell group. After identification of different cell types, DEGs will be evaluated for similar cell type across groups. *FindMarkers* function of Seurat package will be used with options *logfc.threshold=0* and *min.pct=0*. Genes with adjusted p-values less than 0.05 were considered as DEGs. The enriched pathways in DEGs were identified using R package clusterProfiler (version 4.2.0)[69]. The R package pheatmap (v1.0.12) will be used to create heatmaps[70]. The scRNA-seq data will be utilized to quantitatively infer cell-cell communication networks, for this R package CellChat[71] (version 1.6.1) will be used.

Analysis of scATAC-seq data

FASTQ files containing raw scATAC-seq reads will be mapped to human reference genome CRCh38 using Cell Ranger ATAC (version 2.0.0)[72]. The output files will be loaded into R package Signac (version 1.10.0)[68] for downstream processing. Nuclei with <200 genes detected, and high percentage mitochondria genes will be filtered out. Genes will be annotated to ATAC-seq peaks by *Annotation* function and Ensembl based annotation database EnsDb.Hsapiens.v86 (version 2.99.0)[73]. scATAC-seq data will be pre-processed and undergo dimensional reduction independently before calculating the weighted nearest neighbor (WNN)graph and cluster identification. This will be followed by performing differential accessible (DA) peak analysis. Peaks with adjusted p-value less than 0.05 will be considered as DA peaks. Pathway enrichment will be performed on the closet genes annotated to DA peaks using clusterProfiler package. Enriched transcription factor (TF) motifs will be determined using Signac [74] and chromVAR [75] to calculate per-cell accessibility scores for known motifs.

Statistical analysis

The raw data will be analyzed by GraphPad Prism (version 9.5.1). Generally, unpaired *t*-test will be applied to compare means between two groups of data after passing normality check using Shapiro-Wilk test and confirming equal variances using F test. If more than two groups are involved, Kruskal-Wallis test will be used with Dunn's multiple comparisons test.

Specific aim 2: Demonstrate that innate immune reprogramming post AS01 can provide cross-protection against heterologous YF17D viral infection

Recent evidence suggests that AS01's ability to induce TI could provide broad protection against heterologous infections through epigenetic and metabolic reprogramming of innate immune cells. However, the duration and extent of the AS01-alone mediated cross-protection in older populations remain undefined. This aim seeks to establish the protective capacity and temporal dynamics of AS01-alone induced immune enhancement.

Study design

To test this hypothesis, study participants from Specific Aim 1 who received either AS01 (n = 20) or placebo (n = 20) will be randomized in a 1:1 ratio to receive the live-attenuated yellow fever vaccine YF17D (Stamaril, Sanofi-Pasteur) at either 1 month (YF17D 1-month group) or 3 months (YF17D 3-month group) (**Fig 5B, C**). YF17D serves as an ideal experimental viral challenge model due to its well-characterized safety profile and reliable induction of measurable viremia, as demonstrated in our previous studies[16,76–78].

To assess the impact of AS01 on YF17D RNAemia, baseline blood samples will be collected on the day before YF17D administration (day 30 for the YF17D 1-month group, or day 90 for the YF17D 3-month group). This will be followed by daily collection on days 2 – 7 post-YF17D vaccination for measuring YF17D RNAemia. This sampling schedule follows our previously established protocols and aligns with known YF17D RNAemia dynamics and typical detection window for viral RNAemia[58,77]. Quantification of viral RNAemia in blood will be determined by viral RNA extraction followed by quantitative RT-PCR directed against the NS5 gene of YF17D. An *in vitro* transcribed RNA of the YF17D NS5 gene will be used to construct a standard curve to quantify viral RNAemia[77]. Viremia area under the curve (AUC) will be calculated as previously described[77].

Furthermore, we will characterize the immunological mechanisms underlying the protective effects of AS01 through comprehensive analysis of the host response. To understand the mechanisms of protection, YF17D RNAemia will be correlated with immune cell profiles and host responses induced 7 days post-YF17D challenge. Host response parameters to be evaluated include flow cytometry of immune cell populations, bulk RNA expression profiles, epigenetic changes and cytokine profiles. Notably, the sampling points selected for correlation analysis aligns with previous work from our group and others which demonstrates peak gene expression changes at these time points[77].

To perform bulk RNASeq, total RNA from PAXgene tubes will be extracted using the manufacturer's protocol (Qiagen) and quality-assessed via Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA libraries will be prepared using SMARTSeq v2 protocol with 500 pg total RNA, followed by indexed PE sequencing (2x51 cycles, Illumina Hiseq 2000)[64]. Reads will be mapped to the human genome using STAR, counted with featureCounts (Subread package)[79], and analyzed for differential expression using DESeq2 (R, version 3.3.3, $p < 0.05$)[80]. DEGs will undergo pathway enrichment analysis.

Specific aim 3: Examine the T and B cell responses post YF17D challenge, and its correlation with YF viremia levels in AS01 group versus placebo.

In the context of emerging pandemic threats, enhancing vaccine response is critical for establishing both individual protection and herd immunity to prevent sustained community transmission. This is particularly critical for older populations who face dual challenges: increased vulnerability to severe infections and reduced vaccine responsiveness due to immunosenescence.

Our preliminary data (Chan et al., submitted) demonstrates that a single dose of RZV can effectively modulate baseline expression of genes that correlate with T cell responses, leading to enhanced protective T cell responses following administration of the second RZV dose. Previous studies on BCG-induced TI in human monocytes have shown sustained immunological changes, particularly in T cell responses against ex-vivo experimental heterologous infections, suggesting important implications for improving overall vaccine responses[81]. Importantly, T cell immunity has been demonstrated to play an important role in protection against symptomatic infection and viral clearance for SARS-CoV2[82,83] and herpes zoster[47].

However, established studies from our group have shown that YF17D RNAemia duration positively correlates with both humoral and cellular immunity[16,84]. This raises a critical question: could the hypothesized reduction in vial RNAemia duration through AS01-induced innate immune reprogramming potentially impact the development of T cell immunity against YF17D?

For specific aim 3, we will investigate whether innate immune cell reprogramming post-AS01 administration can achieve enhanced YF17D RNAemia clearance while maintaining robust cellular immune responses to YF17D. This investigation will examine T cell responses following YF17D challenge and the correlation with YF17D RNAemia levels. The findings will provide crucial insights into the relationship between TI, viral RNAemia clearance, and the development of protective T cell responses *in vivo*.

Sample collection and laboratory investigations (**Fig. 5B, C**)

To quantify YF17D-specific T cells, peripheral blood samples will be collected at specific timepoints: days 30, 44, 90 for the YF17D 1-month group, and days 90, 104, and 120 for the 3-month group. PBMCs will be isolated and stored at – 80°C until further analysis as previously described[85]. YF17D-specific T cell response will be measured using YF17D specific IFNγ ELISPOT assays. In brief, PBMCs will be stimulated with YF17D peptide pools that span the structural (capsid, pre-membrane, envelope) and non-structural (NS1 to NS5) proteins. PBMCs will be stimulated with YF17D peptide pools inside IFNγ-coated ELISpot plates, followed by incubation with biotinylated anti-human IFNγ antibodies and treatment with streptavidin-alkaline phosphatase. Signal development will utilize BCIP/NBT phosphatase substrate. To quantify positive peptide-specific response, mean spots of the unstimulated wells are subtracted from the peptide-stimulated wells, and the results expressed as spot-forming units (SFU) per 10^6 PBMCs. Concomitant measurement of neutralizing antibodies induced by YF17D challenge will also be measured by plaque reduction neutralization test (PRNT), as previously described using sera obtained on day 0 and 1-month after YF17D administration[16]. Differences in YF17D-specific T cell response and YF17D neutralizing antibody titers between participants challenged with YF17D 1 month and 3 months post-AS01 administration will be performed using unpaired t-tests. Correlation analysis between viral RNAemia duration and T and B cell responses will be performed by nonparametric Spearman's correlation. Statistical significance is defined by p-value less than 0.05. To better understand the immunomodulatory role of AS01 on T and B cell responses against YF17D, we will also conduct comprehensive analyses integrating immune cell profiles, gene expression and epigenetic data between AS01 and placebo groups. This systematic evaluation will enable correlation of immunological parameters with adaptive immune responses, providing valuable insights into AS01's immunomodulatory effects and advancing our understanding of its immunomodulatory effects in older adults.

10 DIRECT ACCESS TO SOURCE DATA/DOCUMENTS

The investigator(s)/institution(s) will permit study-related monitoring, audits and/or IRB review and regulatory inspection(s), providing direct access to source data/document.

11 QUALITY CONTROL AND QUALITY ASSURANCE

The PI and Co-Is will conduct periodic reviews of the study for data and safety monitoring, with the frequency of these reviews to be determined by the committee and regulatory bodies based on their established policies. Internal quality control measures will be implemented through two CRCs who are members of the study team. To ensure data accuracy, a dual-verification system will be employed where data entered by one CRC will be independently verified by the second CRC using source documents.

The study will be subject to evaluation by government inspectors and regulatory authorities, who will be granted full access to e-CRFs, source documents, and other study files. These inspectors will conduct thorough reviews of the CRFs, comparing them against source documents to verify accurate and complete

data collection. Additionally, they will assess study conduct to ensure compliance with the protocol, International Conference on Harmonisation-Good Clinical Practices (ICH-GCP), and all applicable regulations. The frequency and scope of these regulatory inspections will be determined by the respective regulatory bodies according to their standard procedures and policies.

12 ETHICAL CONSIDERATIONS

This study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with the Good Clinical Practice and the applicable regulatory requirements.

This final Clinical Trial Protocol, including the final version of the Participant Information and Consent Form, must be approved in writing by the Centralised Institutional Review Board (CIRB), prior to enrolment of any patient into the study.

The principal investigator is responsible for informing the CIRB and HSA of any amendments to the protocol or other study-related documents, as per local requirement.

12.1 Informed Consent

The informed consent process will begin with potential subjects being identified through the CTRC healthy volunteer database, followed by initial contact from CTRC to assess interest and availability. Eligible candidates, determined through preliminary telephone screening against basic inclusion and exclusion criteria, will be invited for a physical screening visit at CTRC. Prior to any study procedures, blood sample collection, randomisation, or interventions, written informed consent will be obtained by the study PI or CO-I from subjects who meet all inclusion criteria. For participants who are not fluent in English, qualified interpreters or translators will assist in the informed consent process to ensure comprehensive understanding of study procedures and participant rights.

In accordance with Good Clinical Practice (GCP) guidelines and the ethical principles outlined in the Declaration of Helsinki, the investigators will maintain strict compliance throughout the consent process. Two separate consent forms will be utilized: one for study participation and another for future use of specimens, ensuring clear differentiation between immediate study involvement and potential future research applications. This dual consent approach allows participants to make informed decisions about both their current participation and the future use of their biological samples.

12.2 Confidentiality of Data and Patient Records

All information collected for this study will be kept confidential. The subject records, to the extent of the applicable laws and regulations, will not be made public available. Only investigators and delegated study team members will have access to the confidential information. Singapore General Hospital, Regulatory Agencies, Institutional Review Board and Ministry of Healthy may be granted direct access to the original medical records to check study procedure and data, without making any of the information public. Hardcopy research data will be kept under lock and key in a secured office and can only be accessed by the study team. In publications, identifiable information will not be used.

13 PUBLICATIONS

In the event of any publications pertaining to this study, identifiable information will not be used. PI, CO-I and collaborators will oversee all publications that arise from this study. The publication process encompasses manuscript preparation, reviews and revisions, with authorship granted to substantial contributors.
Publications will follow the journal's review timelines. All publications shall acknowledge trial participants, the funding support provided by Grantor and where appropriate, the scientific and other contributions of other Institutions and Research Personnel in accordance with established norms.

14 RETENTION OF TRIAL DOCUMENTS

Records for all participants, including CRFs, all source documentation (containing evidence to study eligibility, history and physical findings, laboratory data, results of consultations, etc.) as well as IRB records and other regulatory documentation should be retained by the PI in a secure storage facility. The records should be accessible for inspection and copying by authorized authorities. They will be kept for 15 years then subsequently destroyed.

15 FUNDING AND INSURANCE

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List of Attachments

Appendix 1 – Study Schedule

YF17D 1-month group

	Screening D-14 to D-1	D0[h]	D1	D7	D14	D30[i] (±3 day)	D32 (±3 day)	D33 (±3 day)	D34 (±3 day)	D35 (±3 day)	D36 (±3 day)	D37 (±3 day)	D44 (±5 day)	D60 (±5 day)
Informed consent	X													
Eligibility check	X													
Medical history & demographic [a]	X													
Physical examination [b]	X													
Vital signs [c]	X	X	X	X	X	X	X	X	X	X	X	X	X	X
FBC[d], Dengue serology, HBA1c	X													
Urine Pregnancy Test[e]	X	X[f]				X[g]								
Research bloods		X[f]	X	X	X	X[g]	X	X	X	X	X	X	X	X
Randomisation		X												
AS01 Adjuvant		X												
YF17D (Stamaril) vaccine						X								
Adverse event		X	X	X	X	X	X	X	X	X	X	X	X	X
Concomitant medication check		X	X	X	X	X	X	X	X	X	X	X	X	X

[a] Demographic includes DOB, gender, ethnicity, height, weight, and BMI

[b] Full physical examination will be performed at Screening. On Day 0, 1, 7, 14, 30, 32, 33, 34, 35, 36, 37, 44 and 60 visits, brief PE will be performed by investigator as needed.

[c] Vital signs include temperature (oral or tympanic), blood pressure, pulse rate, and respiratory rate.

[d] Full blood count (FBC) includes haemoglobin, RBC, WBC, haematocrit, platelet count, neutrophil, lymphocyte, monocyte, eosinophil, basophil.

[e] For female subjects of child-bearing potential only.

[f] Samples to be collected before AS01 / placebo (NaCl 0.9%) administration on Day 0.

[g] Samples to be collected before YF17D administration on day 30.

[h] AS01 or placebo (NaCl 0.9%) intramuscular injection to be given.

[i] YF17D subcutaneous injection to be given.

YF17D 3-month group

	Screening D-14 to D-1	D0[h]	D1	D7	D14	D21	D30 (±3 day)	D60 (±3 day)	D90[i] (±3 day)	D92 (±3 day)	D93 (±3 day)	D94 (±3 day)	D95 (±3 day)	D96 (±3 day)	D97 (±3 day)	D104 (±5 day)	D120 (±5
Informed consent	X																
Eligibility check	X																
Medical history & demographic [a]	X																
Physical examination [b]	X																
Vital signs[c]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
FBC[d], Dengue serology, HBA1c	X																
Urine Pregnancy Test[e]	X	X[f]							X[g]								
Research blood		X[f]	X	X	X	X	X	X	X[g]	X	X	X	X	X	X	X	X
Randomisation		X															
AS01 Adjuvant		X															
YF17D (Stamaril) vaccine									X								
Adverse event		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Concomitant medication check		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

[a] Demographic includes DOB, gender, ethnicity, height, weight, and BMI

[b] Full physical examination will be performed at Screening. On Day 0, 1, 7, 14, 21, 30, 60, 90, 92, 93, 94, 95, 96, 97, 104 , and 120 visits, brief PE will be performed by investigator as needed.

[c] Vital signs include temperature (oral or tympanic), blood pressure, pulse rate, and respiratory rate.

[d] Full blood count (FBC) includes haemoglobin, RBC, WBC, haematocrit, platelet count, neutrophil, lymphocyte, monocyte, eosinophil, basophil.

[e] For female subjects of child-bearing potential only.

[f] Samples to be collected before AS01 / placebo (NaCl 0.9%) administration on Day 0.

[g] Samples to be collected before YF17D administration on day 90.

[h] AS01 or placebo (NaCl 0.9%) intramuscular injection to be given.

[i] YF17D subcutaneous injection to be given.

Appendix 2 - Blood Sampling Summary

YF17D 1-month group

	Collection Tube	Volume	Screen	D0#	D1	D7	D14	D30#	D32	D33	D34	D35	D36	D37	D44	D60
FBC, HBA1c	EDTA tube (clinical)	3ml	X													
Dengue serology (Panbio)	Plain tube (research)	3.5ml (BD 367983)	X													
YF PRNT								X								X
Gene expression (Bulk RNAseq)	Tempus tube	3ml		X	X	X	X	X			X			X	X	X
Gene expression (scRNASeq)	10ml EDTA tube	20ml		X	X	X	X	X			X			x	X	X
Epigenetic studies (scATACseq)				X	X	X	X	X			X			X	X	X
Flow cytometry				X	X	X	X	X			X			X	X	X
YF17D T cells								X							X	X
Cytokines (Olink)	3ml EDTA tube (research)	3ml		X	X	X	X	X			X			X	X	X
YF17D RNAemia								X	X	X	X	X	X	X		
Total volume each visit			6.5ml	26ml	26ml	26ml	26ml	29.5ml	3ml	3ml	26ml	3ml	3ml	26ml	26ml	29.5ml
Total volume 259.5 ml																

#Day 0, Day 30 bloods are collected pre-vaccination

YF17D 3-month group

	Collection Tube	Volume	Screen	D0#	D1	D7	D14	D21	D30	D60	D90#	D92	D93	D94	D95	D96	D97	D104	D120
FBC, HBA1c	EDTA tube (clinical)	3ml	X																
Dengue serology (Panbio)	Plain tube (research)	3.5ml	X																
YF PRNT											X								X
Gene expression (Bulk RNAseq)	Tempus tube	3ml		X	X	X	X	X	X	X	X			X			X	X	X
Gene expression (scRNASeq)	10ml EDTA tube	20ml		X	X	X	X	X	X	X	X			X			X	X	X
Epigenetic studies (scATACseq)				X	X	X	X	X	X	X	X			X			X	X	X
Flow cytometry				X	X	X	X	X	X	X	X			X			X	X	X
YF17D T cells											X							X	X
Cytokines (Olink)	3ml EDTA tube (research)	3ml		X	X	X	X	X	X	X	X			X			X	X	X
YF17D RNAemia											X	X	X	X	X	X	X		
Total volume each visit			6.5ml	26ml	26ml	26ml	26ml	26ml	26ml	26ml	29.5ml	3ml	3ml	26ml	3ml	3ml	26ml	26ml	29.5ml
Total volume 337.5 ml																			

#Day 0, Day 90 bloods are collected pre-vaccination