**Induction of broadly neutralizing antibodies to HIV-Gp41-peptide-conjugated vaccine formulated in squalene emulsion but not aluminum hydroxide**

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**Abbreviations:**

Alum: aluminum hydroxide; bNAb: broadly neutralizing antibody; GC: germinal center; NGC: non-germinal center, SQE: squalene emulsion

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**Abstract (max 200 words)**

**Background:** Our potential novel HIV vaccine candidate, a 3S motif of the gp41 (W614A-3S), is based on the detection of natural anti-W614A-3S NAbs (neutralizing antibodies) in long-term, non-progressor HIV+ patients.

**Objective:** The efficacy of two vaccine formulations (in squalene emulsion (SQE) and in aluminum hydroxide (Alum)) in inducing broadly neutralizing Abs (bNAbs) against HIV strains in preclinical models was compared.

**Methods:** Animal models were used to screen the induction of bNAbs following immunization using W614A-3S vaccine formulated in SQE and Alum. W614A-3S-specific B cells were isolated for single-cell gene expression by qRT-PCR and single-cell V(D)J sequencing.

**Findings:** SQE adjuvant was more efficient than Alum in inducing W614A-3S-specific bNAbs. We showed that germinal center B cells were more frequent in SQE than in Alum, albeit with a different gene expression profile. These results suggest both qualitative and quantitative differences in B cell maturation and proliferation with these two formulations. The single-cell V(D)J sequencing of W614A-3S-specific BCR showed significant differences in BCR sequences and validates the dichotomy between SQE and Alum formulations.

**Conclusion:** Adjuvant formulations of our novel vaccine candidate W614A-3S impact on the quantity and quality of B cell immune responses at both gene expression level and BCR sequence.

**Introduction**

The disease caused by HIV remains a major public health problem. Although there is no cure for HIV infection, effective antiretroviral therapy aids in controlling the virus and helps to prevent transmission. HIV poses challenges for vaccine development. Over time, the virus has evolved a number of evasion mechanisms, particularly through the Env protein, by extensive amino acid variation and conformational instability. HIV-1 Env is the only target of antiviral antibodies (1). Ten to 30% of HIV+ patients have serum antibodies capable of neutralizing virus infection of cells, with some also able to neutralize a majority of different cross-clade viral strains (2). The first challenge is to design a vaccine formulation to neutralize virus entry against large multi-clade panels of genetically diverse HIV-1 Env-pseudotyped viruses, to maximize potential clinical benefit. However, Env has evolved a number of immune evasion strategies that create a major hurdle for vaccine design, in particular extensive amino acid variation, structural and conformational instability, and immunodominance of hypervariable regions (3, 4). The HIV-1 Env is a heavily glycosylated trimeric protein comprising three identical surface gp120 molecules, each noncovalently associated with a transmembrane gp41 molecule (3). The gp41 molecule is involved in the final steps of viral envelope fusion to the host cell membrane (5) and has been proposed in vaccination strategies.

We previously described a specific and highly conserved motif of HIV-1 gp41, named 3S “NH2-SWSNKS-COOH” (at residue position 613-618 ), which is absent in HIV-2 and SIV (6). We also previously demonstrated that a mutated form of the 3S motif, with a single amino acid change at position W614 to alanine (namely W614A-3S), had increased immunogenicity in preclinical models. Animal models including mice, rabbits and macaques showed immunogenicity of the W614A-3S peptide when coupled with cross-reacting material 197 (CRM197) carrier and adjuvanted in incomplete Freund adjuvant (IFA), a water-in-oil emulsion (7). Importantly, natural Abs against W614A-3S eluted from the plasma of HIV-1 patients showed neutralizing activity and were detected exclusively in patients with high CD4 counts and undetectable (<20 copies/mL) or controlled viral loads (7, 8). These specific Abs were immunoglobulin G isotypes (data not shown). Natural Abs directed against W614A-3S peptide can be detected in <5% of HIV+ patients, compared to more than 50% in 12-year, long-term non-progressors (9).

Translation into human clinical trials planned in Q4 2021 required validation of vaccine formulations in preclinical studies. Using both mouse and rabbit preclinical models, we tested the ability of W614A-3S conjugated to a carrier protein and formulated in either squalene emulsion (SQE) or aluminum hydroxide (Alum) to induce neutralizing antibodies with wide reactivity against HIV-1 strains. We sought to elucidate the mechanism of B cell maturation by single-cell gene expression. We demonstrated the dichotomy between SQE-based adjuvant and Alum in the induction of germinal center (GC), leading to bNAbs versus non-NAbs, respectively. Single B-cell analyses validated this dichotomy, showing differential gene expression as well as BCR sequences of W614A-3S-specific B cells generated in SQE compared to Alum formulations.

**Results**

***Squalene emulsion adjuvant is more effective than Alum at eliciting broadly neutralizing antibodies in response to W614A-3S peptide-conjugated vaccine***

To evaluate the efficacy of W614A-3S-CRM197 conjugated vaccine formulated in SQE and Alum in inducing bNAbs, rabbits were immunized intramuscularly at week 0 (W0), W2, W4 and W10 to boost humoral response in two independent experiments (n=4-5) (**Figure 1A**). W614A-3S-specific Abs were detected in all rabbits regardless of the formulation (**Figure 1B)**. We then assessed the neutralization activities against a panel of Tier 1 and Tier 2 viruses by TZM-bl neutralization assay platforms using whole serum at W20 (two independent experiments, n=2-5). Only W614A-3S-CRM197 immunization using the SQE formulation induced neutralizing Abs; however, this remained variable among rabbits (**Supplementary Figure 1**). The breadth of antibody neutralization varied between 25 and 69% of virus strains tested in rabbits. Therefore, purified W614A-3S-specific rabbit IgG was tested in the TZM-bl neutralization assay. Out of a panel of 15 major HIV virus strains tested, the SQE formulation induced W614A-3S-specific rabbit IgG that neutralized 67 to 93% of virus strains (**Figure 1C**). The majority of rabbit purified Abs neutralized the five virus strains of the Montefiori global panel used for standardized assessment of NAb efficacy (10).

In order to further elucidate the immune mechanism of induction of NAbs versus non-NAbs using these two formulations, we validated our vaccine strategy using W614A-3S peptide coupled with either the KLH or CRM197 with SQE or Alum formulation. The immunization protocol is shown in **Figure 2A**. W614A-3S-specific IgG was detected in all mice immunized with W614A-3S peptide coupled with either CRM197 or KLH and formulated in SQE or Alum (data not shown). We pooled sera from five mice in the same group prior to W614A-3S-specific IgG purification. We confirmed detection of the neutralization of two Tier 2 viruses (JR-CSF and YU-2) by W614A-3S-specifc Abs following W614A-3S-CRM197 (**Supplementary Figure 2**) or W614A-3S-KLH immunization (**Figure 2B**). This result was obtained only after formulation with SQE emulsion and not when the vaccine was formulated in Alum.

In order to further study B cell differentiation and maturation, we measured W614A-3S-specific B cells in draining lymph nodes (dLNs), using W614A-3S-biotinylated ovalbumin (Ova). Flow cytometry analysis of W614A-3S-biotinylated Ova staining of B cells in either PBS or W614A-3S formulated in SQE or Alum is shown in **Figure 2C**. Despite background staining in control PBS-injected mice, we were able to identify the W614A-3S-specific B cells in mice over time with both the SQE and Alum formulations (**Figure 2C and 2D**). No significant difference between SQE and Alum conditions was observed an increase at W3 and W5 in the absolute numbers of W614A-3S-specific B cells (**Figure 2D**).

Thus, both formulations induced W614A-3S-specific B cells and W614A-3S-specific IgG responses. However, we found that SQE formulation of our vaccine candidate, W614A-3S conjugated with a carrier protein, induced bNAbs when neutralizing activity was nonexistent following vaccination using the Alum formulation. These data have been used in Go/No-go criteria for a phase I clinical study that started in September 2021. Here, we aim to elucidate the mechanism of B cell immune responses.

***Differential gene expression in W614A-3-specific B cell populations following W614A-3S-KLH vaccination using SQE and Alum formulations***

We used a single-cell quantitative RT-PCR approach to compare between the two formulations the quality of W614A-3S-specific B cell populations isolated from dLNs, one week after 2nd and 3rd immunizations (W3 and W5, respectively; **Figure 2C**).

We purified W614A-3S-specific B cells by cell sorting of two groups of five mice per condition. Following quality control, we analyzed 73 detectable genes after data cleaning (M&M and **Supplemental Table 1**) in 747 antigen-specific B single cells (184-188 cells per condition). We used dimensional UMAP (Uniform Manifold Approximation and Projection) regression to compare B lymphocyte distribution in SQE and Alum immunization conditions (**Figure 3A**), at W3 (2nd injection) and W5 (3rd injection). Whereas no difference in B cell distribution was observed at W3, UMAP allowed segregation of B cells in the SQE formulation compared to the Alum formulation at W5 (3rd immunization), suggesting differential gene expression of W614A-3S-specific B cells.

A FlowSOM elbow metaclustering of all 747 cells allowed us to identify six populations of antigen-specific B cells that are represented by different levels of gene expression (**Figure 3B**). One cluster representing a few cells (38 of 747 cells) with very low gene expression was removed from the analyses. We identified germinal center (GC) B cells (expressing Bcl6, Fas, Efnb1 and Id3 genes) and plasma cell precursors (high expression of Prdm1, Irf4, Cd69 and Myc genes). We also identified three different memory B-cell populations (high expression of Cd38, Sell and Itga4 genes) that are differentiated by: 1) high expression of Ccr6, Il9r, Tlr7, Sox2 and Cd93 genes; 2) low expression of Rela and high expression of Il10ra; and conversely, 3) high expression of Rela and low expression of Il10ra genes. According to the literature, high-expressing CCR6 B cells might be memory precursors in the light zone of GCs with low antigen affinity (11). GC B cells and plasma cell precursor populations were individualized compared to three memory B-cell populations. The abundance of plasma cell precursors in Alum conditions and the abundance of GC B cells in SQE conditions were significant, as shown in Volcano plot (p-value threshold <0.1; **Figure 3C**). The percent of total number of cells (n=747) is represented in **Figure 3D**. We found that plasma cell precursors were present principally after the 3rd immunization (W5) in Alum conditions, whereas GC B cell numbers were higher in SQE conditions (**Figure 3D**). However, the proportions of the three memory B-cell populations were relatively similar between Alum and SQE formulations (**Figure 3D**).

This increase in GC B cell numbers after SQE immunization over time was validated by flow cytometry (**Figure 3E**). We observed significantly higher numbers of W614A-3S-specific GC B cells after the first and second injections (p=0.0495 and 0.0011, respectively), as detected by flow cytometry in SQE conditions compared to Alum conditions. When comparing single-cell analysis to flow cytometry, we found that both formulations induced GC B cells; however, their frequencies were significantly higher in SQE conditions compared to Alum, with similar gene expression profiles at W3. Interestingly, at W5, gene expression of W614A-3S-specific B cells was distinct in SQE conditions compared to Alum, suggesting that qualitative differences in W614A-3S-specific B cells would occur between the 2nd and 3rd injection.

***Significant diversity of B cell repertoire of germinal center and non-germinal center B cells following W614A-3S-KLH vaccination using SQE and Alum formulations***

To evaluate clonal diversity of W614A-3S-specific B cells using SQE and Alum formulations, we purified W614A-3S-specific B lymphocytes at W11, one week after the fourth immunization. We purified W614A-3S-specific IgG1+ GC (GL7+IgD-) and non-GC (NGC; GL7-IgD+) B cells for single-cell V(D)J sequencing (gating strategy is shown in **Figure 4A)**. We did not find any significant difference in the absolute numbers of W614A-3S-specific IgG1+ GC and NGC B cells between SQE and Alum conditions (**Figure 4B**).

Amino acid analysis of the variable part of heavy (H) and light (L) IgG1 chain sequences, the third complementarity-determining region (CDR3), was carried out for both conditions and in cell types with a total of 433 distinct sequences, regardless of their frequencies in samples. Clonotypes with exactly one heavy and one light chain were considered in the analysis. Venn diagram analysis showed the number of CDR3 sequences in each condition (SQE and Alum) and cell type (GC and non-GC B cells). We found one common clone with sequence similarities between GC and NGC cell populations in SQE and Alum conditions (**Figure 4C**). The proportions of cells with different lengths of CDRH3 were relatively similar in Alum and SQE conditions (**Figure 4D**). We represented the most frequent CDRH3 and CDRL3 sequences in the SQE condition compared to the Alum function (**Figure 4E and 4F, respectively)**. Interestingly, sequences generated in SQE were distinct from the one generated in the Alum condition. These results showed the major differences in amino acid alignment between conditions.

In order to verify if the Ab sequences were specific to W614A-3S, we cloned and produced recombinant monoclonal IgG1 with completed V(D)J sequences among the most frequent ones in SQE conditions. All monoclonal Abs were specific to the W614A-3S peptide (**Figure 4G**). However, some of them (e.g., clone 691W11\_S\_GC, 573W11\_S\_GC) showed lower affinity. None of these selected, cloned and produced specific monoclonal Abs were able to neutralize HIV (data not shown). Despite these disappointing results, the single-cell V(D)J sequencing of W614A-3S-specific BCR validated the dichotomy in BCR sequences between SQE and Alum formulation of our vaccine candidate.

Altogether, both approaches using single-cell analysis of gene expression and BCR sequencing revealed that even though W614A-3S-specific B cells (IgG1+) were detected following injection of both formulations, gene expression, B cell differentiation and BCR selection were distinct.

**Discussion**

We propose here a gp41-based peptide HIV vaccine coupled with a carrier protein (CRM197) and formulated in SQE as a novel vaccine candidate capable of inducing broadly neutralizing antibodies. We demonstrated that the formulation of the novel candidate vaccine W614A-3S-CRM197 with either Alum or SQE impacts the quantity and quality of B lymphocytes. These differences result in: 1) the induction of non-neutralizing antibodies for the Alum formulation versus the production of NAbs for the SQE formulation, which were capable of neutralizing a wide range of HIV-1 strains including Tier 1 and Tier 2 viruses (73-93% breadth); 2) major differences in the proportion of differentiated B cells expressing GC B-cell-associated genes; and 3) a distinct amino acid CDR3 region.

Broadly neutralizing Abs (bNAbs) generally arise late in the course of HIV infection. The most mature bNAbs revealed one or more unusual features, such as high levels of somatic hypermutation (SHM), unusually long complementary-determining regions like CDRH3, and polyreactivity for non-HIV-1 antigens (12–14). While it is difficult to establish the generality of these observations, and if these features are required for the development of bNAbs, these features reveal a long and intensive process of B cell selection. It is necessary to elicit a similar antibody response by vaccination strategies. We have shown that anti-W614A-3S NAbs were rarely observed in HIV-1 progressors, but were significantly increased in untreated, long-term, non-progressor (LTNP) patients, of the ALT ANRS cohort who were infected for more than 7 years, but did not exhibit the disease (9). These anti-W614A-3S are found in strong association with both viral load and viral DNA, and are able to neutralize most of the HIV-1 clade B tested. HIV vaccine studies are currently based on the Env protein in a trimeric form. However, other approaches have provided evidence for a peptide epitope-based vaccine. The recent discovery of Abs with neutralizing breadth against the fusion peptide (FP) may lead to promising vaccine candidates (15). In mice, the FP elicited monoclonal Abs capable of neutralizing 31% of a cross-clade panel of 208 HIV-1 strains. Crystal structure and cryoelectron microscopy structures of these antibodies revealed FP diversity as a molecular explanation of cross-clade neutralization in macaque and guinea pigs (15).

We have chosen two main adjuvants that can be carried over into human clinical studies, Alum and SQE, as both are based on licensed human product formulations. Our SQE formulation is based on AddaVax™, which is a squalene-based, oil-in-water nano-emulsion based on the formulation of MF59® that has been licensed in Europe for adjuvanted flu vaccines (16). MF59® increases GC B cell differentiation and also induces persistent high-affinity functional Ab titers (18). In the context of pandemic influenza vaccine, MF59 adjuvant stimulates induction of broadly cross-reactive antibodies (17). SQE formulation of our HIV vaccine candidate induced bNAbs with 65-90% breadth of neutralization of Tier 1 and Tier 2 HIV strains, whereas the Alum formulation induced Abs without detectable neutralizing activities. This class of adjuvant is believed to act through a depot effect, enhancement of antigen persistence at the injection site, recruitment and activation of antigen-presenting cells, and direct stimulation of cytokine and chemokine production by macrophages and granulocytes (16).

We further investigated the impact of Alum and SQE formulations of W614A-3S-CRM197 vaccine on B cell differentiation. In non-human primates, higher frequencies of total and Env-specific GC-Tfh cells accompanied by larger and more diverse Env-specific B cell lineages were found after slow delivery of antigen (19). It has also been proposed that squalene oil-in-water emulsion facilitates a rapid antibody response in contrast to aluminum hydroxide due to different kinetics of delivery of antigen to the lymphoid organs (20). In the influenza vaccine, squalene-based emulsion adjuvants increase antibody affinity against the hemagglutinin-based vaccine and breadth of B cell responses, leading to protection across virus clades (21).

We used a single-cell qRT-PCR approach to compare gene expression of W614A-3S-specific single-B cells between the two formulations. We identified germinal center (GC) B cells (high expression of Bcl6 (22), Fas (23), Efnb1(24) and Id3 (25)), plasma cell precursors (high expression of Prdm1, Irf4, Cd69 and Myc), and three different memory B cell populations according to the literature definition (26–28). Our data showed that the proportion of GC B cells as defined by single-cell gene expression was significantly higher when W614A-3S vaccine was formulated in SQE, whereas plasma cell precursors were significantly higher following Alum formulation. Thus, qualitative and quantitative differences in the proportion of differentiated B cells were revealed by single-cell analyses.

The single-cell V(D)J sequencing of W614A-3S-specific BCR validates the dichotomy between SQE and Alum formulations of our vaccine candidate. Significant diversity of the B cell repertoire of germinal center and non-germinal center B cells was observed following W614A-3S vaccination using SQE compared to Alum formulations. We did not find any neutralizing activities among cloned Ab according to the defined sequence. There are some limitations in our study, notably in regard to BCR sequencing for detection of NAbs. Indeed, we used W614A-3S peptide conjugated with biotinylated ovalbumin in order to purify antigen-specific B cells that might impact the selection of certain BCR during this step. Of note, vaccination of animals with a W614A- 3S peptide in IFA induces neutralizing anti-HIV-1 Abs, among which we found a unique clone, F8, by hybridoma generation (29), suggesting the rarity of B cell clones or technical challenges in purification of B cells.

**Materials and methods**

*Animals*

BALB/cByJ female mice were purchased from Janvier Labs, housed at a free animal facility (Centre d’Experimentation Fonctionnelle (CEF) of Sorbonne University, France) under specified pathogen-free conditions and used for experiments at 6-10 weeks old. New Zealand rabbits were housed at Covalab company (France). All experimental protocols were approved by the French animal experimentation and ethics committee and validated by Service Protection et Santé Animales, Environnement, with the numbers 00954.02 and APAFIS#5863 – 2016062710255883 v3 for mouse experiments.

*Immunizations and vaccine antigens*

Rabbits received 50 μg of the peptide W614A-3S coupled with CRM197 by the intramuscular route at Week 0 (W0), W2, W4 and W10. Mice received 10 g of peptide coupled with keyhole limpet hemocyanin (KLH; Covalab) carrier protein or 11.7 g of W614A-3S peptide coupled with CRM197 (Minka Therapeutics) by intramuscular injections (in both quadriceps of left and right thighs) at the same kinetic (or W18 for mouse CRM197 experiments). W614A-3S peptide (NH2-PWNASASNKSLDDIW-COOH) is a mutated peptide of HIV-1 gp41 (7). The antigen was administered with PBS1X (Life Technologies), aluminum hydroxide (Alum; 100 g for mouse and 250 g for rabbit; Alhydrogel 2.0%; Invivogen) or squalene emulsion (2.5% v/v for mouse and rabbit; Polymun).

*Antibody detection*

Sera were collected before first injection and at different time points after each immunization (W4, W6, W10, W12, W14 and W20 for rabbits; W3, W5, W8 and W20 for mice), and stored at -80°C prior to evaluation of W614A-3S-specific IgG titration by ELISA. MaxiSorp-plates (Corning) were coated overnight with 50 ng/well of W614A-3S peptide (Covalab). For W614A-3S-monoclonal Ab evaluation, plates were coated with 50 ng/well of W614A-3S-KLH vaccine. Wells were saturated with PBS1X + 1% bovine serum albumin (Life Technologies) prior to serum serial dilutions of 1/10 or different dilutions of W614A-3S-monoclonal Abs (32,000 to 0.97 ng/ml). All samples were tested in duplicate. Peptide-specific Abs were revealed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1/10,000; Southern Biotech) or detection antibody biotinylated anti-rabbit IgG (1/5,000; Jackson ImmunoResearch) followed by HRP-streptavidin (1/200; R&D Systems). Enzymatic activity was measured by adding tetramethylbenzidine (TMB; Pierce Endogen) and stopped by 1N H2SO4. Optical density (OD) of each well was monitored at 450 nm with a FlexStation 3 ELISA reader (Molecular Devices) and Ascent Software version 2.6. EC50 was calculated by sigmoidal dose response (Prism-9 software). For the NAb assay, serum-purified W614A-3S-specific Abs were obtained as described (7). A pool of 4-5 sera was used for mouse purified NAb evaluation. Viral titration and TZMbl neutralization assays were performed as previously described (30, 31).

*Cell phenotype and isolation*

Draining lymph nodes (dLN; inguinal) were harvested 7 days after each immunization. Cells were stained with W614A-3S coupled with biotinylated ovalbumin protein (Covalab) for 30 min at room temperature before cell surface antigen staining with a standard method after receptor Fc blocking with CD16/CD32 (clone 2.4G2; BD Biosciences), and the following anti-mouse Abs: CD3e (clone 145-2C11; eBioscience), CD45R/B220 (clone RA3-6B2), CD19 (clone 1D3), IgG1 (clone A85-1), IgD (clone 11-26c.2a), T- and B-cell activation antigen (clone GL7) and streptavidin (BD Biosciences). For cell analysis, dead cells were excluded by using the LIVE/DEAD fixable kit (Molecular Probes). Cells were analyzed by BD LSR Fortessa flow cytometry or isolated by BD FACSAria II sorter. A pool of 5 mice per condition at W3 and W5 was used for W614A-3S-specific B cell isolations (BioMark Dynamic array). A pool of 25 mice per condition at W11 was used for W614A-3S-specific IgG1+ germinal center (GC; GL7+IgDLow) and non-germinal center (NGC; GL7-IgD+) B cell isolations (chromium single cell V(D)J assay).

*Gene expression analysis of single cells*

Single-cell gene expression analysis was performed using the BioMark 96.96 Dynamic Array IFCs and the Biomark HD System from Fluidigm. Two-step single-cell gene delta gene expression was performed using EvaGreen Supermix according to Fluidigm real-time PCR protocol. Briefly, reverse transcription using a 2-step VILO cDNA synthesis kit (Invitrogen) was performed directly on single cells prior to specific target cDNA amplification.

Probes for 96 genes were selected from the catalog of delta gene expression assay (Fluidigm; **Table S1**). Processing of the IFCs and operation of the instruments were performed according to the manufacturer’s procedures. Thirty cycles of PCR were performed using the Biomark microfluidic chip (Fluidigm). Automated data analysis was performed with Singular Analysis Toolset (v3.6) compatible with R software. We merged data from two chips (of two experiments) per condition (n=184-188 cells). A multidimensional scaling analysis was performed without significant difference between chips. Results of negative control (no cells) or positive control samples (10 cells) and probe controls or non-amplified probes were removed before analysis using UMAP regression, heatmap after standard normalization, FlowSOM Elbow Metaclustering and volcano plot EdgeR analysis (OMIQ Data Science Platform).

*V(D)J repertoire and gene expression profiling*

W614A-3S-specific IgG1+ GC and NGC B cells at W11 were loaded according to the manufacturer’s instructions for the chromium single-cell V(D)J reagent kits (10X Genomics) to attain between 500 to 10,000 cells per well. Library preparation for V(D)J sequencing was performed according to the manufacturer’s protocol (chromium single-cell V(D)J enrichment kit, mouse B cells) prior to sequencing on the Illumina MiSeq. Quality of raw reads was assessed using FastQC 0.11.8 quality control tool and clonotype quantification was performed with the Cell Ranger 3.1.0 V(D)J pipeline. Only clonotypes with exactly one heavy and one light chain were considered. CDRH3 and CDRL3 sequence alignments were performed with Unipro UGENE software.

*Monoclonal antibody production*

V(D)J sequences of W614A-3S-specific GC and NGC B cells were chosen from squalene conditions. Sixteen monoclonal antibodies were produced by the ProteoGenix company. An endotoxin-free DNA preparation was made for the pTXs1 expression constructions prior to XtenCHOTM transient expression. The recombinant Abs were purified by affinity vs. protein G. After production, specificity by ELISA and neutralization assay were performed.

*Statistics*

Statistical analyses and graph representation were performed using either GraphPad Prism 9, FlowJo X, OMIQ, Unipro UGENE software, or R 3.6.2. Two-way ANOVA test was used for kinetic peptide-specific IgG evaluation. Mann-Whitney tests were used to compare SQE and Alum conditions.

**Author contribution**

BC and VV designed the study. OB, CC, MT, MB, LB and VV carried out the experimental work, contributed to data acquisition, analysis and interpretation. SB, KA and JN compiled and analyzed the single-cell repertoire data. ES and DK were responsible for adjuvant production and quality control. MB and GS were responsible for neutralization assays and analysis. BC, PD, GS, DK and VV provided financial support. BC and OB wrote the manuscript. All authors contributed to reviewing the manuscript.

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**Figure legends**

**Figure 1: Broad NAb induction after squalene-adjuvanted W614A-3S peptide vaccination**

(**A**) Experimental schedule of rabbit immunizations and biologic samples. Animals were vaccinated at Week 0 (W0), W2, W4 and W10 (black arrows), and blood samples were collected (red arrows). (**B**) Rabbits were immunized withW614A-3S-CRM197 carrier non-adjuvanted (n=5), adjuvanted with aluminum hydroxide (Alum) (n=5) or squalene emulsion (SQE) (n=5). Kinetics of serum W6141-3S-specific IgG of rabbits, immunized withW614A-3S-CRM197 carrier adjuvanted with Alum (blue) or SQE (red). Graph represents EC50 mean +/- SEM of OD 450 nm; y-axis is in log 10 scale. Statistical analyses were performed with two-way ANOVA between two adjuvanted conditions. (**C**) W614A-3S-specific IgG per condition was serum-purified at W20, and neutralizing IC50 were evaluated against different virus strains: Montefiori Global panel (10), Tier2 and Tier1. Color codes of neutralization assay (IgG g/ml): green, >5; yellow, 4.9-2; orange, 1.9-1 and red, <0.9. Breadths represent percentage of total tested HIV strains neutralized by anti-W6141-3S IgG. T/F: Transmitted/Founder virus.

**Figure 2: Validation of neutralizing Abs in mouse models and detection of W614A-3S-specific B cells**

(**A**) Experimental schedule of mouse immunizations and biologic samples. Animals were vaccinated with W614A-3S-KLH carrier non-adjuvanted, adjuvanted with Alum or SQE at Week 0 (W0), W2, W4 and W10 (black arrows), and blood samples were collected or draining lymph node (dLN) analysis was made after each injection (red arrows). (**B**) Neutralizing IC50 of serum-purified W614A-3S-specific IgG was evaluated at W11 against Tier 2 virus JR-CSF strain. Purified peptide-specific IgG was obtained by pooling sera of 4-5 mice for each condition. Color codes of neutralization assay (IgG g/ml): green, >2; orange, 1.9-1 and red, <0.9. (**C**) Representative dot plots of W614A-3S-specific B cells in dLNs after three immunizations with PBS or W614A-3S-KLH adjuvanted with Alum or SQE. Numbers represent peptide+ cell percentages of B cells. (**D**) A dot plot with mean +/- SD (n=7-23) represents the absolute numbers of W614A-3S-specific B cells in two dLNs, one week after first (W1), second (W3) and third (W5) immunizations with PBS (black), W614A-3S-KLH adjuvanted with Alum (blue) or SQE (red). Statistical analyses were carried out using the Mann Whitney *U* test.

**Figure 3: Differential W614A-3S-specific B cell populations after adjuvanted vaccination**

Mice were immunized withW614A-3S-KLH adjuvanted with Alum (blue) or SQE (red) at W0, W2 and W4. Draining lymph nodes (dLNs; n=5 mice for each condition and for two independent experiments per condition) were harvested at W3 and W5, and used to isolate peptide-specific B cells and to perform single-cell gene expression analysis. (**A**) Uniform manifold approximation and projection (UMAP) plots were generated by concatenation of 747 single cells from Alum condition (n=375 cells) and SQE condition (n=372 cells) across W3 (left UMAP) and W5 (right UMAP) for expression of 73 genes. Each point depicts a single cell, colored according to condition designation. (**B**) Heatmap showing the medians of gene expression after concatenate W3 and W5 conditions for six different clusters, distinguished using FlowSOM algorithm. One cluster represented only a few cells (38 of 747 cells) and showed very low gene expression; it was removed of the rest of analyses (cells at the bottom right of UMAP). Blue indicates lower expression; red indicates higher expression. Population names were identified by specific over or under gene expression (white frame). (**C**) Volcano plot (log fold change (FC) versus -Log10 (p-value)) depicting five different clusters indicating empirical analysis of digital gene expression between adjuvanted conditions. Green points represent significant difference with p-value threshold <0.1. (**D**) Graph represents cell percentages for each condition (Alum at W3, solid blue; SQE at W3 solid red; Alum at W5, wire blue and SQE W5, wire red), based on 100% of evaluated cells (n=97 plasma cell precursors; n=224 memory B cell precursors; n=268 Il10ra+Rela- memory B cells (MBCs); n=64 Il10ra-Rela+ MBCs; and n=56 germinal center (GC) B cells). (**E**) A dot plot with mean +/- SD (n=7-13) represents the absolute numbers of W614A-3S-specific GC B cells in two dLNs, at W1, W3 and W5. Statistical analyses were carried out using the Mann Whitney *U* test and statistical significance is indicated between adjuvanted conditions: \*p<0.05; \*\*p<0.01.

**Figure 4: Repertoire diversity of germinal center and non-germinal center B cells after adjuvanted vaccination**

Mice were immunized withW614A-3S-KLH adjuvanted with Alum (blue) or SQE (red) at W0, W2, W4 and W10. (**A**) Representative dot plots of gating strategy for W614A-3S-specific IgG1+ germinal center (GC) and non-germinal center (NGC) B cells sorting after four immunizations. Numbers represent peptide+ cell percentages of B cells. (**B**) A dot plot with mean +/- SD (n=7-13) represents the absolute numbers of W614A-3S-specific IgG1+ GC and NGC B cells in two dLNs, one week after fourth immunizations (W11). Statistical analyses were carried out using the Mann Whitney *U* test. (**C**) For W614A-specific IgG1+ B cell repertoire analysis, 2 dLNs of 25 mice per condition were pooled at W11. Venn diagram of differentially abundant CDRH3 (heavy chain) + CDRL3 (light chain) sequences (number and percentage) per condition (GC B cells of Alum condition, blue; NGC B cells of Alum condition, yellow; GC B cells of SQE condition, green; NGC B cells of SQE condition, red). (**D**) Distribution of CDRH3 amino acid (aa) lengths for all clonotypes of GC B cells (left graphs) and NGC B cells (right graphs) for Alum condition (blue; upper graphs) and SQE condition (red; lower graphs). (**E**) Representative sequences of CDRH3 and CDRL3 of higher clonotype frequencies for SQE condition. The V(D)J of these clonotypes were used for production of 16 clonal IgG. (**F**) Representative sequences of CDRH3 and CDRL3 with clonotype frequencies greater than one for Alum condition. (**G**) Anti-W614A-3S specificity of 16 clonal IgG1 titers (from SQE condition) were measured by ELISA. Graph represents OD450 nm versus log10 of IgG1 concentration (ng/ml). Eight clonal IgG1 issued from GC B cells (black) and eight clonal IgG1 issued from NGC B cells (green) were produced and evaluated.