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Research Article:

A self-emulsified adjuvant system containing alpha-tocopherol induced higher neutralizing antibody responses than a squalene only emulsion against a recombinant Cytomegalovirus (CMV) pentamer antigen

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Running title: CMV pentamer antigen co-administered with self-emulsified adjuvant systems containing alpha tocopherol.

Abstract

Research for novel vaccine adjuvants remains a critical need to improve the immune responses to a recombinant vaccine antigen. Emulsion adjuvants such as AS03 and MF59, particularly in the area of Influenza vaccines, have shown antigen dose sparing and allowed reduced immunizations. It was previously demonstrated that these emulsion adjuvants can be formulated using a simpler, low shear process of self-emulsification. The role of alpha tocopherol as an immunomodulator in emulsion adjuvants is evident from the success of AS03 in the on-going covid-19 pandemic. Although, it was a challenge to formulate alpha tocopherol with a low shear process to get closer to the AS03 composition, the self-emulsified version referred as self-emulsifying adjuvant systems (SE-AS) showed comparable immune responses to AS03 when co-

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administered with a Quadrivalent influenza virus (QIV) vaccine. In this paper, we first optimized the SE-AS with alpha tocopherol referred to as SE-AS44 to allow sterile filtration. We compared the in vitro cytokine profile with self-emulsifying adjuvant 160 (SEA160), a squalene-only self-emulsified adjuvant with composition similar to MF59. We compared SE-AS44 and SEA160 co-administered with a recombinant cytomegalovirus (CMV) pentamer antigen, which is a less immunogenic antigen, in vivo to compare the antibody and T-cell responses, in different adjuvanted groups, in C57BL/6 mice.

Keywords: Adjuvants, emulsions, self-emulsification, alpha-tocopherol

Introduction

Emulsion adjuvants are well established as safe and efficacious when combined with variety of antigens, particularly influenza vaccines for both seasonal and pandemic responses [1-3]. Given their key attribute of minimizing antigen dose and reducing the number of immunizations needed; emulsion adjuvants, mainly MF59 and AS03, have been the adjuvant of choice during a pandemic setting as seen in the 2009 H1N1 influenza pandemic [4-6] but were also investigated and now approved, in several countries, as a component of protein-based vaccines in response to SARS-CoV2 pandemic [7-9]. These adjuvants can be manufactured at a large scale in response to increase in demand, despite complex manufacturing facility needs, as well as detailed equipment maintenance requirements [10]. However, the supply of these adjuvants, and hence adjuvanted vaccines may not be sufficient for a global supply, particularly the low-income countries [4, 11] suggesting the need to find efficient ways to manufacture these adjuvants. Although emulsions with similar composition are now tech-transferred to facilities [12, 13] and tested in clinical settings; newer and simpler methods of manufacturing these adjuvants may allow pandemic needs to be met and aid organizations such as WHO with pre-pandemic preparedness [14, 15]. It was known that α-tocopherol acts as an immunomodulator in AS03 and shows better immune response compared to squalene-only emulsions [16, 17]. This was also highlighted by recent work done in Pulendran lab [18, 19]. We have previously shown that an ex vivo self-emulsification process can be deployed as an alternative method to make emulsion adjuvants with composition similar to MF59 [20] and AS03 [14]. Although the α-tocopherol content was lower compared to AS03, SE-AS22 and SE-AS36 showed humoral and cell-mediated immune response similar to AS03 adjuvanted groups in BALB/c mice. This work was proof that the SE-AS with lower α-tocopherol

content could potentially be developed to show comparable immune responses to AS03 adjuvanted vaccines, despite difficulty in incorporating α-tocopherol in the self-emulsification process of squalene-based emulsions. Influenza antigens are usually the first choice for evaluating novel emulsion adjuvants for potency [6, 21, 22]; however a quadrivalent influenza vaccine (QIV) is a strong immunogen without an adjuvant [23] and would not allow for optimal appraisal of the novel adjuvants.

In this paper, we have optimized the SE-AS to allow for sterile filtration of the emulsion with minimal changes to CQAs; thus improving the feasibility to manufacture on a large scale [24]. We also compared AS03 and a well-characterized squalene-only emulsion i.e. SEA160 [20] with SE-AS44 in human PBMCs to evaluate induction of key antigen presenting cells. Finally, we have compared the potency of the optimized SE-AS with AS03 as well as SEA160 to confirm the role of α-tocopherol as an immunomodulator in these self-emulsified formulations using Cytomegalovirus (CMV) pentamer recombinant protein - a poorly immunogenic antigen as a model to critically appraise the adjuvant effect in improving the immunogenicity of the pentamer. We've reported the CMV pentamer neutralizing antibody responses, anti-CMV IgG antibody responses as well as CD4+ T cells and memory T cell responses in all vaccine groups.

Materials and Methods

Formulation Materials

Squalene oil was obtained from JX Nippon Oil Trading Company (Tokyo, Japan) and dl-α-tocopherol was obtained from DSM Nutritional Products (Heerlen, Netherlands). Polysorbate 80 (PS80) was obtained from JT Baker (Center Valley, PA). Hypure water for injection (WFI) like quality water and phosphate buffered saline (PBS) were obtained from HyClone Laboratories (Logan, UT). Tocopheryl poly-ethylene glycol sulphate (TPGS) was obtained from Antares Healthcare Products, Inc. (St. Charles, IL). CMV wild-type pentamer protein was provided by GSK Vaccines (Rockville, MD). AS03 (GSK, Rockville) is an Adjuvant System containing DL-α-tocopherol and squalene in an o/w emulsion.

Sterile filtration of emulsions

Formulation of SE-AS was carried out as previously described in chapter 4 section 4.2.2. Emulsions were filtered using a 33mm, 022μm pore size polyethersulfone (PES) membrane syringe filter from Millipore (Burlington, MA). The resultant filtered emulsions were diluted 500x

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to measure size and polydispersity index (PdI) using dynamic light scattering (DLS). % Content of squalene and α-tocopherol was evaluated using the method described below.

Percent content of squalene and α-tocopherol in emulsions

For percent content measurements, reverse phase ultra-high-performance liquid chromatography (RP-UPLC) was used. An Xterra C18 column from Waters (Milford, MA) was used to quantify the oils. Samples were prepared by disrupting emulsions using IPA such that the squalene concentration in each emulsion was 200µg/mL. 10µL in duplicate was injected in the UPLC. Mobile phase was 95:5 Methanol: Acetonitrile. The run time was 15 mins at 1mL/min flow rate. The column was heated at 37C during elution and PDA detector was used to record the eluting peaks. The retention time for tocopherol was ~ 4.3 mins and squalene was ~7.4 mins. A standard curve of squalene and tocopherol mixture was run before each run with concentrations ranging from 600 µg/mL to 2.34 µg/mL. Using the slope and intercept from this standard curve, concentration of squalene and tocopherol in emulsion samples was determined.

Optimization of the SE-AS

Keeping the α-tocopherol content constant at 15% v/v in oil: surfactant mixture, the surfactant content in SE-AS emulsions was increased to obtain an SE-AS with better PdI. The goal of this experiment was to obtain an SE-AS which maintains size and PdI post filtration. The emulsions were formulated similarly as previously described. pH measurement was performed using an Orion 3-star pH meter from Thermo Scientific (Waltham, MA) and osmolality was measured on advanced instruments osmometer model 2020 (Norwood, MA). Size, PdI and percent content of the oils were evaluated using previously described methods

Cryo-electron microscopy (Cryo-EM) imaging

To understand the morphology of the oil droplets in SE-AS44 compared to AS03, we collaborated with Creative Biostructure (Shirley, NY) to obtain cryo-EM images and size analysis for SE-AS44 and AS03. In summary, FEI Talos F200C Cryo-Transmission Electron Microscope was used for imaging and analysis purpose. Undiluted emulsion was placed on a thin copper grid that had been glow discharged and the sample was then loaded to the freezing chamber. The sample was rapidly frozen by plunging into a cryogen (liquid ethane cooled by liquid nitrogen). This frozen sample stored in liquid nitrogen was then used for imaging and size distribution analysis.

In vitro innate immune response using human whole blood

To compare the innate profile of the SEA S44 and SEA160 adjuvants with AS03 as standard, an in vitro assay setup was developed where venous whole blood was collected from three healthy volunteers into sodium heparin tubes (Becton Dickinson, San Jose, CA, USA) in accordance with relevant guidelines and regulations including obtaining an informed consent (ICF Pro00023228, Protocol GSK UM UP BDU). Whole blood was mixed at 1:1 ratio with RPMI 1640 media and stimulated for 18 hours with 1:100, 1:1000, and 1:3000 dilutions of AS03, SEA S44, and SEA160. TLR agonists R848 (1 µg/ml), LPS (2 µg/ml), and Poly I:C (20 µg/ml) were used as positive controls, and media and ex vivo stained samples were used as negative controls. The stimulated whole blood samples were stained with fluorescently labeled antibodies to identify leukocytes (anti-CD45-BB515), NKT cells (anti CD3-BV510, anti-CD8-BV786, and anti-CD56-BV650), NK cells (anti CD3-BV510 negative, anti-CD16-BUV496, and anti-CD56-BV650), monocytes (anti CD14-BUV737), mDC and pDC (anti CD11c-BV421 and anti CD123-PECy5), and neutrophils (anti CD66ace-APC) and acquired on BD Symphony A5 instrument (BD Biosciences). The activation status of the innate populations was characterized by measuring the change in the mean fluorescence intensity of scavenger receptor markers (anti CD68-PECy7), T-cell activation co-receptor markers (anti CD40-BV650, anti-CD86 BB700, and anti HLA-DR-PECF594), and Fcγ receptor markers (anti CD16-BUV496, anti CD32-BUV395, and anti CD64-BV605). All antibodies were sourced from BD Biosciences, San Jose CA except for anti CD66ace-APC (BioLegend, San Diego CA), anti CD123-PECy5 (BioLegend, San Diego CA), anti CD68-PECy7 (eBiosciences, Waltham MA), and anti CD159a-PE (Beckman Coulter, Brea CA).

In vivo evaluations using CMV wild-type pentamer antigen

Ethics statement: All animal studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) where the work was performed (Smithers Avanza, Gaithersburg; Approval # D012476). All studies were executed in compliance with provisions of the USDA Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the U.S. Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals.

In vivo study design and immunization regimen: Cell-line derived CMV pentamer was used to test the in vivo potency of the novel SE-AS in mice. The dose of antigen in each mouse was 0.05µg. The antigen content was determined by UV/visible spectrophotometry as well as

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reverse phase HPLC. Sample size of 10 animals per group was calculated such that it provided a power of 80% to detect a 3-fold difference between any 2 groups with 95% confidence interval. 6-8 weeks old female C57BL/6 mice from Charles River laboratories (Gaithersburg, MD) were used. The study design comprised of three immunizations three weeks apart, where 50µL of the vaccine was injected intramuscularly in the gastrocnemius muscle such that at each time-point alternate limb was used. CMV pentamer dose in each mouse for each immunization was 0.05µg. The groups in this study were: Physiological saline (as negative control), unadjuvanted CMV, CMV with SE-AS44, CMV with AS03 and CMV with SEA160.

The vaccine was formulated to mimic bed-size immunizations, with 1:1 mixing of antigen and adjuvant, to reach the final antigen concentration. All the formulations were characterized for pH, Osmolality, size and PdI and endotoxin before immunization. Endotoxin was measured using the Endosafe NexGen – PTS from Charles River laboratories (Wilmington, MA) and limulus amoebocyte lysate (LAL) cartridges with test range 10-0.1 EU/mL. Additionally, protein integrity was confirmed via gel electrophoresis (SDS-PAGE). It was ensured that the vaccine dosed for each group had endotoxin lower than 1 EU per dose. Bleeds were collected 3 weeks post 1st (3wp1), 3 weeks post 2nd (3wp2) and 3 weeks post 3rd (3wp3) immunization and the processed sera was used to test the humoral immune responses by neutralizing antibody assay (nAb) and IgG titers by ELISA. Spleens from 5 animals per group were harvested 3wp3 and 4wp3 and used to measure T cell immune responses via intracellular cytokine staining.

Determination of neutralizing antibody (nAb) titers: Retinal pigment epithelial cell line (ARPE-19) was used since TB40 (a CMV virus strain) was known to infect these cells in this assay. On day 1, 100uL of ARPE-19 cells were plated in 96 well flat bottom plates in complete growth medium i.e., DMEM + 10% fetal bovine serum (FBS) + 1% Penicillin-Streptomycin. Plated were incubated in 37C overnight ~ 24 hours. On day 2, Tecan – liquid handling robot was used to perform serum dilutions. Different starting dilutions were used for different time-points depending on the expected titers. A positive control from Sera care known to neutralize TB40 virus was used in every plate at a constant 1:50 dilution. In each plate, 75uL of serum dilutions were prepared using Tecan and then 75uL of TB40 virus was added to each well to make a total of 150 in each plate. This was enough for duplicates of each sample. This virus-serum mixture was incubated at 37C, 5% CO_2 for 2 hours. The cells plates (duplicate for each group) were removed from the incubator. Media was taken out from each well and 50uL of virus-serum cocktail was

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added. These plates were incubated at 37C, 5% CO2 for at least 20 hours. On day 3, the cells were fixed using 4% paraformaldehyde and incubated at RT for 20 mins following by 1 wash using 1XPBS and then permeabilized using 0.1% TritonX-100 and incubated for another 10 mins. Primary antibody (anti-mouse anti-CMV IE monoclonal antibody) was added immediately and incubated for 1 hour in 37C, 5% CO2 incubator. Cells were washed twice and then secondary antibody (anti-mouse AlexaFlour488 antibody) was added and incubated for another 1 hour. Post incubation cells were washed 3 times and 1X PBS was added. These plates were then read using high content imaging – CX7 (by selecting to read 10-20 fields per well). Interpolated titers are then calculated at 50% fluorescence intensity.

IgG antibody ELISA: Antibody titers were determined in serum obtained from each animal at 3wp2 and 3wp3. To determine the CMV pentamer specific binding IgG antibody titers, sandwich ELISA was used. 96 well Nunc-immuno Maxisorp F96 plates were used to coat 100µL of 1µg/mL CMV pentamer antigen per well overnight at 4°C. Antigen coated plates were washed with 1X phosphate buffered saline (PBS) & 0.05% w/v Tween20 and blocked with 1% w/v bovine serum albumin (BSA) solution in PBS. Serum from immunized animals was added in the first row of the plate such that well A1 received positive control (serum from previous CMV study that showed consistently higher titers) and well A12 received sample buffer as negative control. The serum was prediluted before adding 10µL to row1. Serial dilution was the performed down the plate from row A to H. Serum incubation was allowed for one hour before washing the plates and adding horse radish peroxidase (HRP) conjugated goat anti-mouse IgG from Jackson Immunoresearch (West Grove, PA) for another one-hour incubation at room temperature. Substrate was added quickly after washing plates again, for 15 mins and then immediately stop solution was added. Plates were read using EnVision 2105 Multimode plate reader from Perkin Elmer (Waltham, MA). Titers were calculated at 50% interpolated optical density (OD) value obtained from the plate reader.

Intracellular Cytokine Staining Assay: T-cell responses were analyzed 4wp3 by intracellular cytokine staining of in vitro antigen-stimulated splenocytes. Spleens from individual animals were processed to single-cell suspensions, followed by treatment with RBC lysis buffer (Ebioscience, Thermo Fisher Waltham, MA). CMV pentamer peptides gH, gL, UL128, UL130 and UL131 from GeneScript (were used for stimulation of splenocytes. These splenocytes were stimulated at one million cells per well density with anti-CD3 from BD Biosciences (San Jose CA)

used as positive control, media was used as negative control, and peptide pool was prepared for antigen stimulation condition. Anti-CD28 antibody from BD Biosciences was added to each well as a co-stimulant and brefeldin A (BFA) from BD Biosciences was added two hours after stimulation at 1 μg/ml concentration for blocking cytokine secretion. The cells were stimulated overnight and stained with live/dead reagent (Near IR, EX 633/EM 750). Before the cells were fixed and permeabilized using Cytofix/Cytoperm reagent, Fc block was added to avoid extracellular non-specific binding, followed by memory marker staining using CD62L conjugated with BV510 and CD127 conjugated with BV421 from BD Biosciences. Fc block was again added to avoid intracellular non-specific binding before single-step staining with CD3 conjugated with BV711, IL-17F conjugated with AF647 from BioLegend (San Diego, CA), CD4 conjugated with BUV395, CD8 conjugated with BB700, CD44 conjugated with PEFC594, Interleukin 2 (IL-2) conjugated with APCR700, Interferon γ (IFN-γ) conjugated with BV786, tissue necrotic factor α (TNF-α) conjugated with BV650, IL-17A conjugated with BV421 from BD Biosciences, and IL-13 and IL-4 conjugated with AF488 obtained from Thermo fisher Scientific (Waltham, MA). Since most of the anti-mouse antibodies used are rat or hamster derived; anti-rat anti-hamster Ig, κ/Negative control compensation particles from BD Biosciences stained with all the above fluorochrome conjugated antibodies including an unstained control for preparing compensation controls. The samples were acquired on a BD FortessaX20 SORP flow cytometer from BD Biosciences (San Jose, CA) followed by analysis with FlowJo software (Ashland, OR). We defined our gating strategy (Supplementary Figure 1a) in FlowJo where, the live cells were first differentiated from dead and were then used to differentiate singlets. From the singlets, we identified CD3+ T cells and used them to gate for CD4 and CD8 T cells. Antigen specific cells were identified by gating on upregulated CD44 cells. Individual cytokine gates were then established on these antigen specific CD4 and CD8 T cells. Memory markers were used to identify antigen-specific transitional, central memory, effector memory, and effector populations. Individual cytokine gates were established on these memory populations (Supplementary Figure 1b).

Statistics and Data analysis: GraphPad Prism software (San Diego, CA) was used to analyze and plot data from the in vivo immune responses. For humoral responses, one-way analysis of variance (ANOVA) followed by Tukey's Multiple comparisons test was used to evaluate differences in immune responses from individual animals in the dosing groups. For nAb

titers, Dunnett's test post one-way ANOVA was used to compare SEA160 with SE-AS 44. For ICS, a nonparametric Kruskal-Wallis test was run followed by Dunn's multiple comparisons test for comparison within different dosing groups.

Results

Optimization of the SE-AS emulsions

Emulsions formulated via microfluidics offer increased reproducibility and capability for sterile filtration [25] and hence it was essential to manifest this for the SE-AS emulsions for comparability and to avoid using other methods of terminal sterilization [24]. Our previous work provided a proof of concept that an emulsion adjuvant containing α-tocopherol can be formulated using self-emulsification, albeit with lower α-tocopherol content, and show statistically non-inferior immune responses to clinically available AS03 adjuvant [14]. As a next step in development of these SE-AS emulsions, it was essential to evaluate the feasibility to sterile filter these emulsions. Both the exploratory emulsions, SE-AS 22 and SE-AS 36, showed comparable immune responses to AS03 and had size distribution with PdI in the range of 0.2-0.4 [14]. For sterile filtration of a nanoparticle less than 200nm, it is essential to have low PdI for efficient filtration process using a 0.22μ filter [24, 26]. We used a PES membrane syringe filter from Millipore to filter 2 mLs of the emulsion. Using a method developed on UPLC to quantify squalene and tocopherol in these emulsions, we noticed that both SE-AS 22 and SE-AS 36 showed losses in content of the oils post filtration (Supplementary Table 1). Not only was there a loss in content of the oils in the emulsion, but also an increase in PdI with multiple mode of size distribution (Supplementary Figure 2) further suggesting that these exploratory emulsions were not compatible with sterile filtration under these conditions. Thus, further optimizations would improve the droplet homogeneity in these emulsions to consequently demonstrate feasibility for sterile filtration supporting scale-up and development of these emulsion adjuvants.

We have previously reported that the amount and type of surfactant is key to ex-vivo self-emulsification [14, 15]. Very high amounts of surfactants showed PdI less than 0.15 but with lesser oil content and lower size (80-100nm). However, our intent was to optimize the SE-AS maintaining the size close to AS03 i.e., 155nm for comparison in vivo. Keeping this in mind, a series of new emulsion combinations (Table 1) were evaluated with varying polysorbate 80 concentrations, keeping tocopherol content between 10-18%, as comparability with AS03 for SE-

AS with lower tocopherol was established in our previous work [14]. SE-AS14 was use as a starting point owing to its low PdI and tocopherol content in the desired range of evaluation. The trend in general showed that as the surfactant content decreased, the size increased. The increase in PdI was seen in emulsions with higher than 15% v/v α-tocopherol.

SE-AS	Tween80	Squalene	α-Tocopherol	Avg (Z.ave)	PdI
14	35	55	10	79.76	0.113
41	33	55	12	94.13	0.166
42	30	55	15	120.73	0.219
43	30	52	18	134.20	0.360
44	25	60	15	131.73	0.192
45	20	65	15	139.10	0.215
22	15	70	15	129.07	0.212

Table 1: % v/v in oil: surfactant mixture for novel SE-AS combinations and size and PdI using DLS. SE-AS 22 is shown for comparison.

Both SE-AS 44 and 45 showed size closest to AS03 and PdI less than 0.3, which fit our criteria. These emulsions were then filtered as described previously to evaluate the size post filtration. Interestingly, SE-AS 45 showed a bimodal size distribution (graph not shown) and increased PdI post filtration (Table 2); however, SE-AS44 maintained size and showed similar PdI post filtration (Table 2, supplementary figure 3). Further optimization of SE-AS 44 was performed using excipients such as TPGS and poloxamer 188 however, the size and PdI did not further improve (data not shown). In summary, SE-AS 44 was the most optimized emulsion with 60:15:25 Squalene:α-tocopoherol:Polysorbate80 showed comparable size pre and post filtration and maintained the content of the oils within the range of 100 ± 20%. This emulsion was further tested for its short-term physicochemical stability at different temperatures for 2 weeks.

SE-AS	Before filtration		After filtration		% Content loss	
	Size	PdI	Size	PdI	Squalene	α-tocopherol
44	133.7	0.184	134.9	0.203	16.3	12.64

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45	141.5	0.201	177.1667	0.329	-	-

Table 2: Size, PdI and percent content loss pre and post filtration for SE-AS44 and 45.

For comparison, SE-AS 22 was also subjected to the same stability conditions. Both the emulsions were stored at three different temperatures i.e., 5°C, 25°C and 50°C. pH, osmolality, size, PDI were measured for timepoints up to 10 weeks. Percent oil content, however, was measured at different time-points up to 2 weeks. Osmolality remained in the range for both emulsions (Supplementary figure 4); however, pH for emulsions stored at 50 °C seemed to drop ~0.3 units. SE-AS 44 showed no change in size and PdI at all temperatures (Figure 1a) unlike SE-AS 22 which showed an increase in size and PdI at 10 weeks. Similarly, SE-AS44 showed both squalene and α-tocopherol content within the 80-120% and comparable to at all time points up to 2 weeks (Figure 1b), making it the final emulsion adjuvant candidate for further evaluation. Before in vitro and in vivo evaluations, SE-AS44 was compared to AS03 using Cryo-EM to compare the droplet morphology and size. The droplet morphology of SE-AS44 was similar at a 500nm scale to AS03. The size distribution and analysis for SE-AS44 showed that majority of the droplets (~87% considered in the analysis) were less than 150nm in size (Figure 1c).

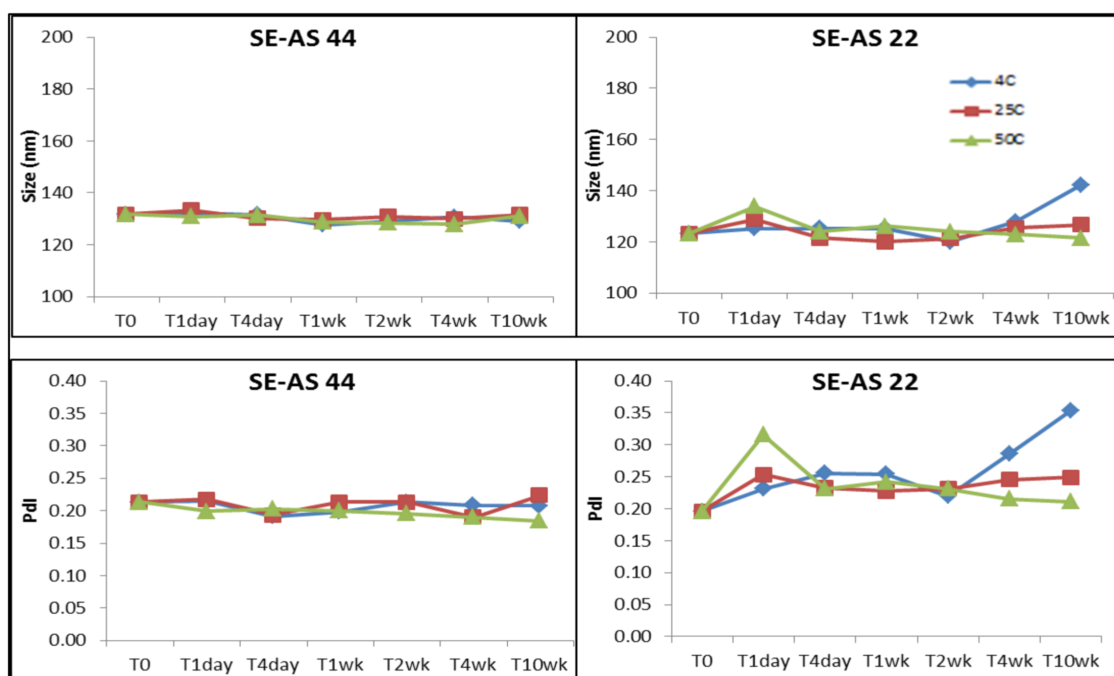


Figure 1a: Size and PdI for emulsions up to 10 weeks stored at 4C (blue), 25C (red) and 50C (green)

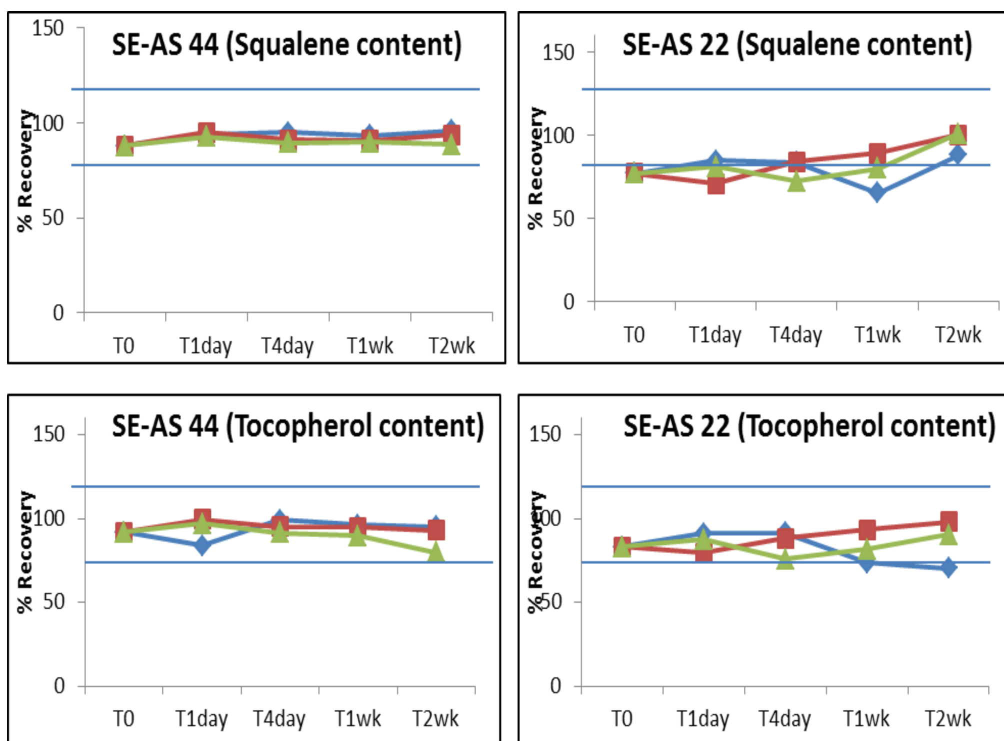


Figure 1b: % Squalene and Tocopherol content for emulsions stored at 4C (blue), 25C (red) and 50C (green) up to 2 weeks. The blue lines represent the acceptable limits of % content from an i.e., 100% ± 20%.

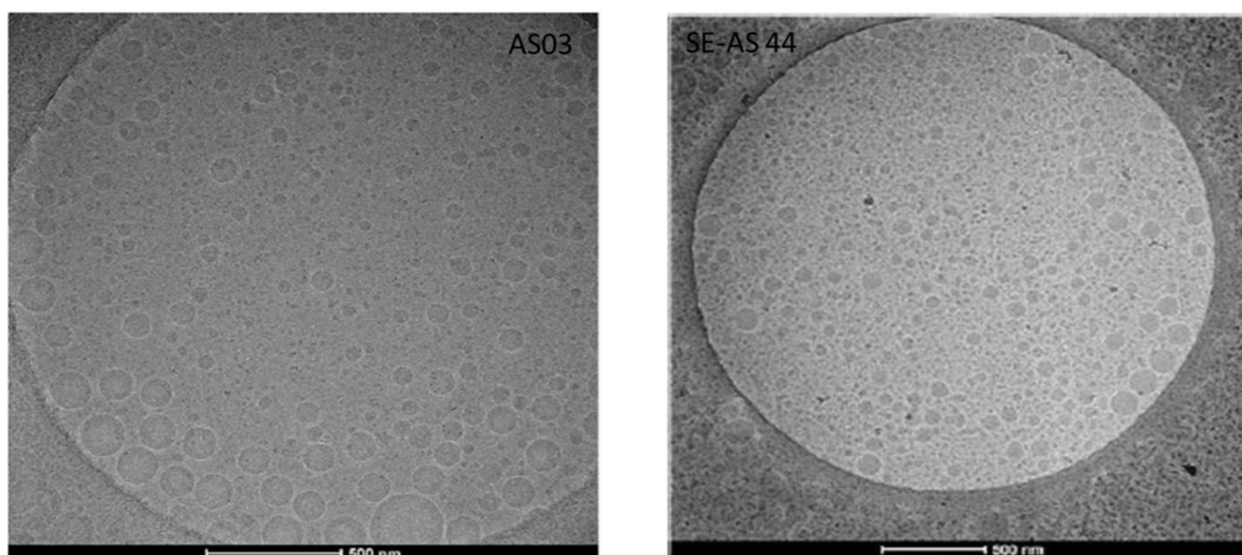


Figure 1c: Cryo-EM images of AS03 and SE-AS44 showing comparable droplet morphology. The oil droplets in AS03 are slightly bigger than SE-AS44 as expected.

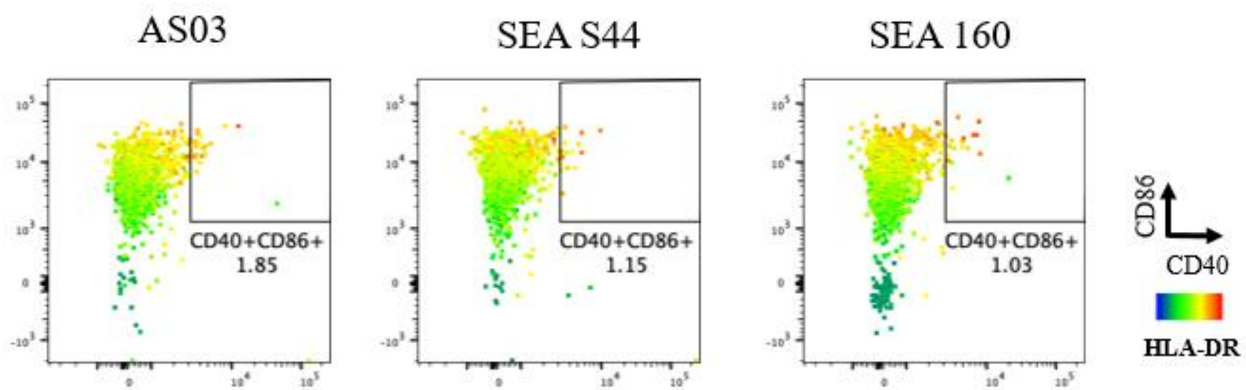
In vitro evaluation of novel emulsion adjuvants using human innate signaling in whole blood cells

Prior to evaluating SE-AS44 in animals and compare with AS03, the role of α-tocopherol was investigated by comparing in vitro readouts with SEA160, an optimized squalene-only emulsion adjuvant made using self-emulsification process [20]. Among different innate populations, T-cell co-receptor markers CD40, CD86, and HLA-DR were upregulated primarily in monocytes, mDC, and neutrophils (Figure 2) in whole blood samples stimulated with media (not shown), AS03, SEA S44, and SEA160. These receptor markers have been previously shown to induce antigen presentation capability [27]. SEA160 showed similar levels of monocytes and mDCs compared to AS03 and SE-AS44. Although, overall levels of Neutrophils were low for all adjuvants; SE-AS44 and AS03 showed 4 times higher levels compared to SEA160. Similar observation between AS03 with and without tocopherol has been reported previously [16]. All these emulsions were evaluated in mice using CMV pentamer as model antigen.
vaccines.

In vivo potency of emulsion adjuvants in mice using CMV pentamer antigen

The goal of this *in vivo* study was to compare the optimized SE-AS with a well characterized squalene only emulsion SEA160 using a soluble subunit protein with inherently low immunogenicity. AS03 was used as a control. The groups are as described in the materials and methods section.

(A)



(B)

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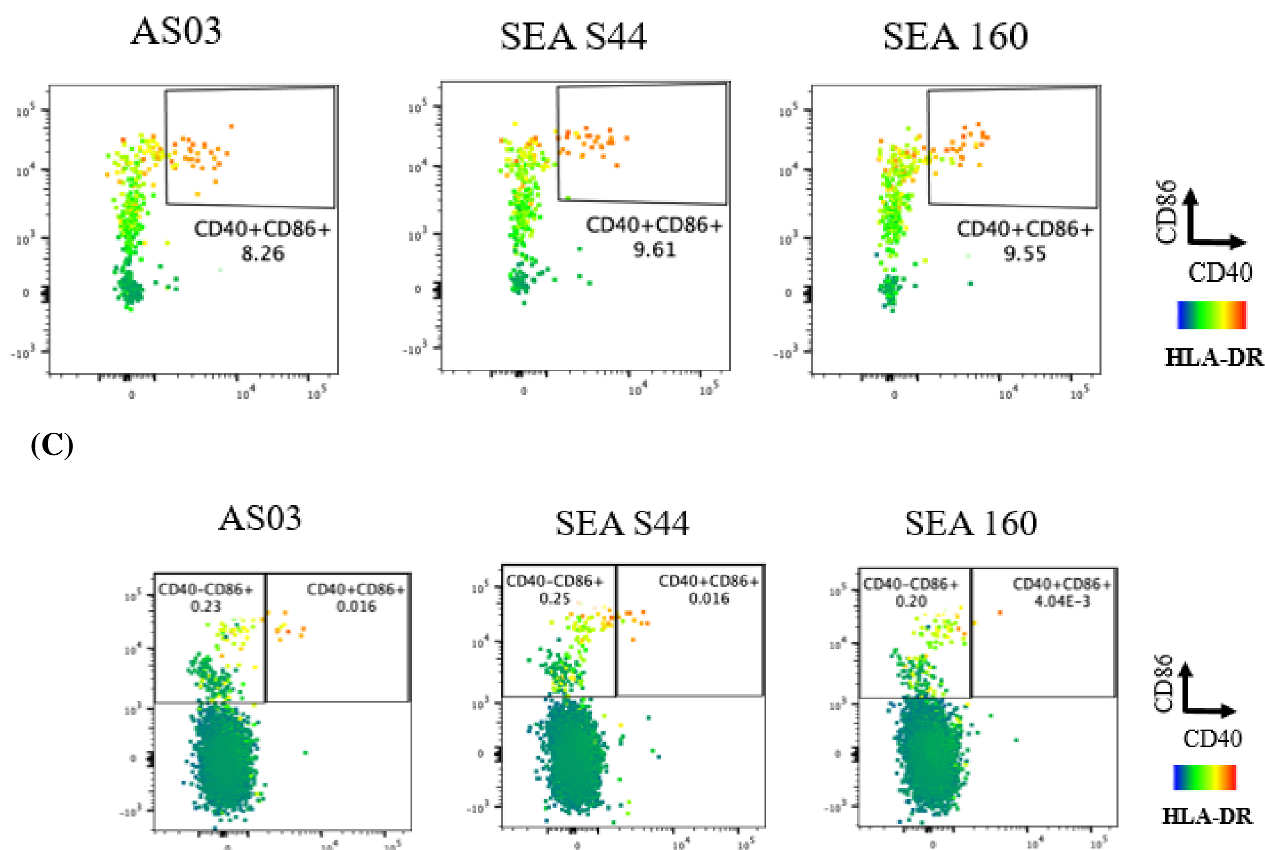

Figure 2: Representative pseudo color plots showing the tri-variate analysis of CD40 vs CD86 markers with characterization of HLA-DR expression using heatmap analysis human whole blood samples stimulated with adjuvants (AS03, SEA S44, and SEA160) with media treated and ex vivo stained samples as negative controls. AS03, SEA S44, and SEA160 stimulated whole blood samples showed increase in CD40, CD86, and HLA-DR markers when compared to media treated samples in monocytes (A), mDC (B), and neutrophils (C) indicating induction of antigen-presentation role.

Although the primary readout to meet the objective of this study was to evaluate neutralizing antibodies (nAb) post immunization; anti-CMV IgG antibodies as well as CD4+ T-cell population were also looked at to compare overall humoral immune response and cell-mediated immune response, respectively. nAb titers (Figure 3a) three weeks after 1st immunization (3wp1) were low, owing to inherently low immunogenicity of the antigen even though adjuvanted groups showed slightly higher titers. At 3wp2, however, there was a considerable increase in titers for adjuvanted groups compared to unadjuvanted or negative control. SE-AS44 showed significantly higher titers compared to unadjuvanted protein but not significantly different compared titers to AS03 indicating comparability with AS03. Most importantly, significant difference was observed between SE-AS44 and SEA160 titers at 3wp2. At 3wp3, the trend

remained similar to titers from 3wp2 with little to no increase in titers for unadjuvanted protein, but SE-AS44 showing significantly higher titers compared to the unadjuvanted group. Mean titers for SE-AS44 were higher compared to SEA160 and not very different compared to AS03.

Binding antibody titers in sera obtained from 3wp2 and 3wp3 time-points were measured by IgG ELISA using CMV pentamer protein. The assay was a secondary read-out to evaluate the humoral immune response from the adjuvanted vaccine. Mean titers from individual animals in each group show an overall trend between the groups similar to the nAb titers (Figure 3b). All adjuvants showed significantly higher titers compared to CMV alone. AS03 gave the highest titers post 3rd immunization. However, like nAb titers at 3wp2, significant difference in titers from SE-AS44 adjuvanted group were obtained compared to SEA160. Thus, overall humoral response favored adjuvanted groups with SE-AS44 showing evidence of higher titers post 2nd immunization and overall higher mean IgG and nAb titers compared to SEA160. This data suggests that α-tocopherol may be playing a role in improving the humoral immune response compared to squalene only emulsion adjuvants.

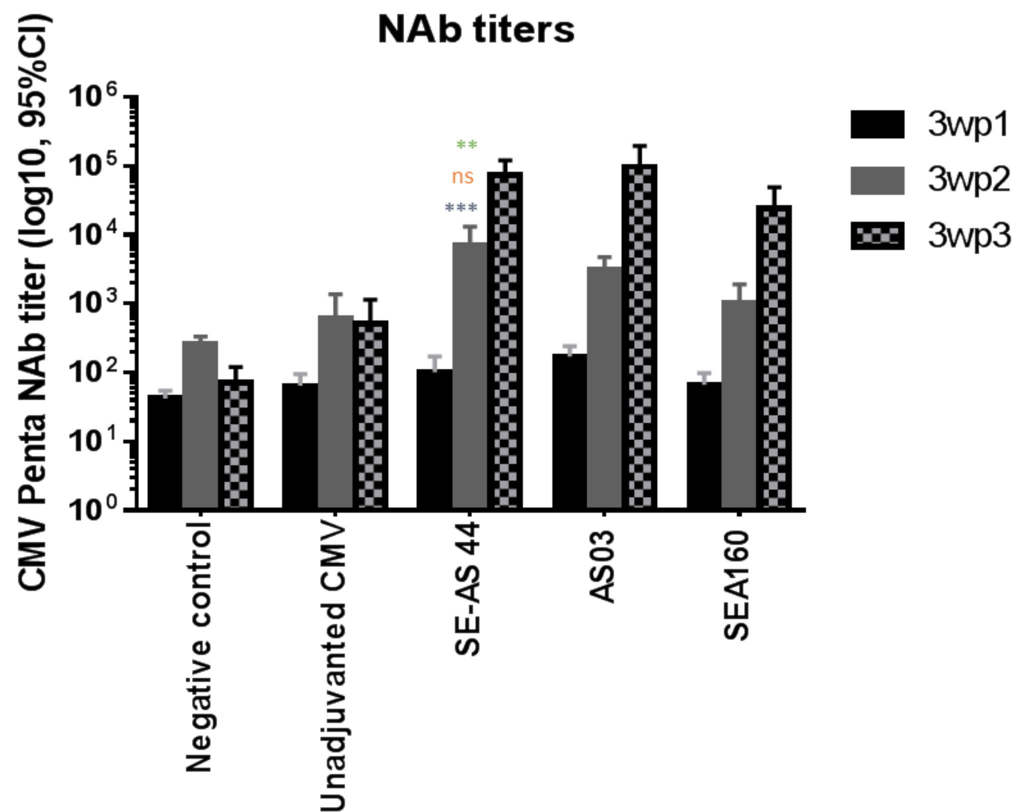

Figure 3a: Neutralizing antibody titers in serum obtained three weeks post 1st, 2nd and 3rd immunization, against CMV TB40 strain of virus. Each bar represents geometric mean titers

(GMT) with 95% confidence interval (CI) from n=10 animals per group. Statistics was performed using one-way ANOVA followed by Tukeys test to compare all groups with each other followed by Dunnett's multiple comparisons test to compare each group with SE-AS44. Significant differences are marked on the graph. Comparison with CMV alone is shown in blue, with AS03 is shown in red and with SEA160 is shown in green; where, ns = not significant, *= p<0.05, **= p<0.005, ***= p<0.0005 and ****= p<0.00005.

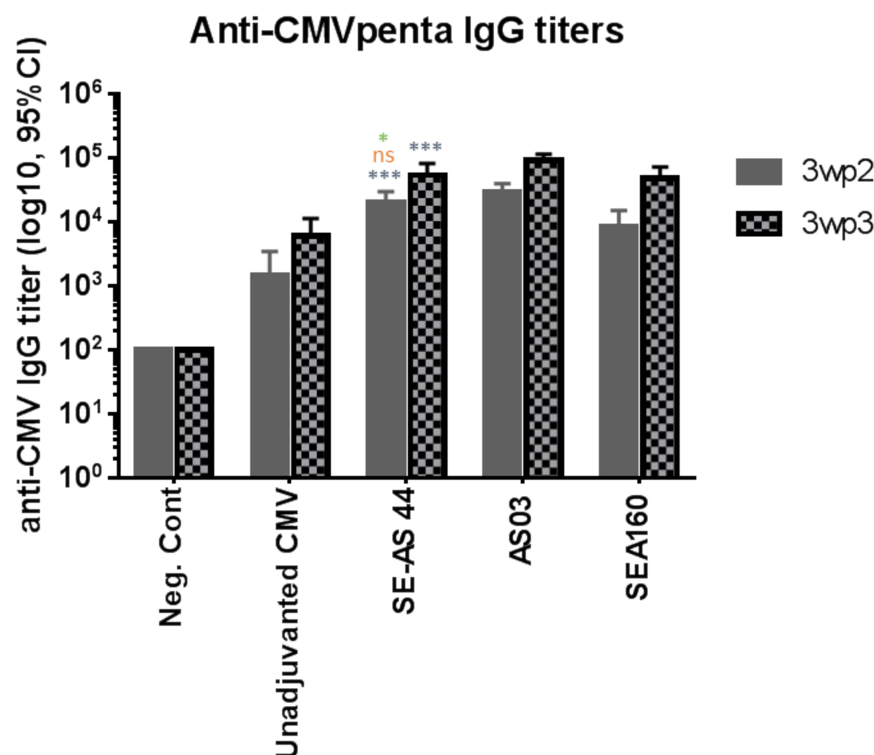

Figure 3b: Anti-CMV Penta IgG antibody titers in serum obtained three weeks post 1st, 2nd and 3rd immunization, against CMV TB40 strain of virus. Each bar represents geometric mean titers (GMT) with 95% confidence interval (CI) from n=10 animals per group. Statistics was performed using one-way ANOVA followed by Dunnett's multiple comparisons test to compare difference between each group with SE-AS44. Significant differences are marked on the graph. Comparison with CMV alone is shown in blue, with AS03 is shown in red and with SEA160 is shown in green; where, ns = not significant, *= p<0.05, **= p<0.005, ***= p<0.0005 and ****= p<0.00005.

Finally, to evaluate cell-mediated immune response, spleens from immunized mice 4 weeks post 3rd immunization were used. To evaluate the cellular memory immune response, we used the memory markers CD62L and CD127 to distinguish the CD4+ and CD8+ T cells into transitional, central memory, Effector memory and Effector cells. Prophylactic vaccines aim at developing efficient memory T cell response against any pathogen that can eventually help the effector response upon infection with that pathogen [28, 29]. Splenocytes were stained for both

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CD4+ and CD8+ T cells; however, frequency of CD8+ T cells were little to negligent and hence only CD4+ T cell data was characterized in detail. There were little to no transitional cells, as we expected most frequent T cells as either effector or effector memory cells. Effector memory cells prevailed as the majority CD4+ T cells when gated to look at the memory response (Figure 4a). There were no significant differences between different dosing groups, however SE-AS44 showed higher total frequency of CD4+ T cells. As expected, when the CD4+ cells were gated into subtypes, Th2-subtype prevailed over others (data not shown). Th2-type response is a characteristic of most emulsion adjuvants alone without an immune potentiator [10, 14, 30, 31] and within this population SE-AS44 showed highest frequency of cells. Among the different memory populations of the CD4+T cells, effector memory cells prevailed as the majority population (Figure 4a). Upon further gating of these effector memory CD4+ T cells into subtypes, we observed a Th0/Th2 dominant immune response with an overall high frequency but no significant difference among different groups (Figure 4c). Although SE-AS44 shows slightly lower effector memory CD4+ T cells, our hypothesis is that this decrease in effector memory cells may have translated into higher frequency of effector cells (Figure 4d). Since the overall frequency of T cells was low, this effect of conversion from effector memory to effector cells may not be prominent. Overall, the trend for ICS remained like humoral immune response.

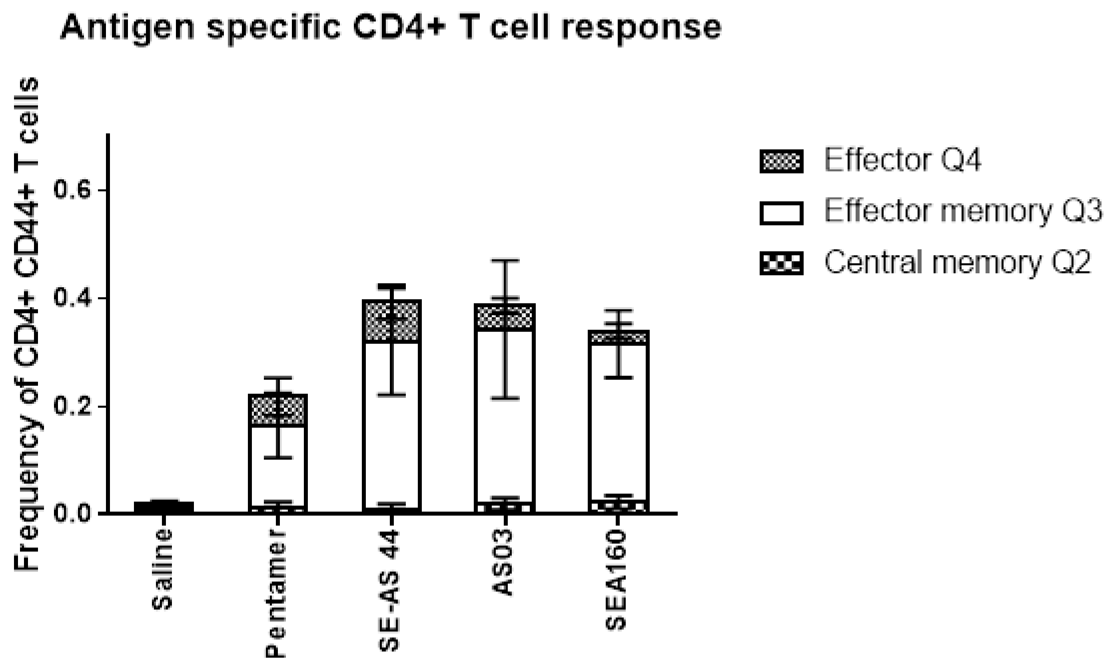

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Figure 4a: Frequencies of overall antigen-specific CD4+ T cells classified as either Central memory, effector memory or effector cells. Each bar represents stacked mean frequency data from n=5 animals with standard deviation.

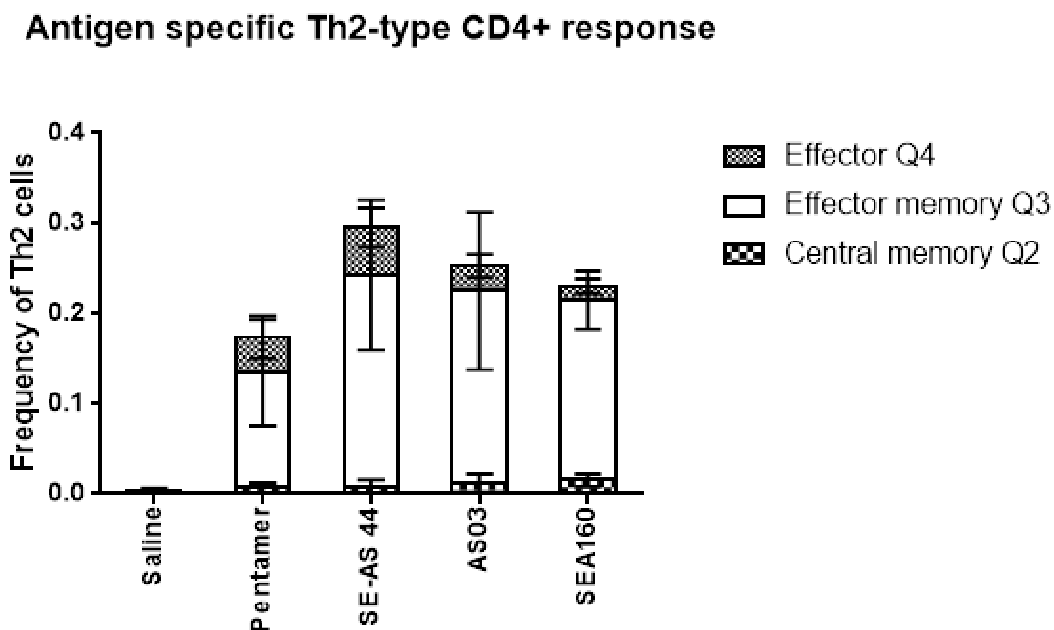

Figure 4b: Frequencies of antigen-specific Th2-type CD4+ T cells classified as either Central memory, effector memory or effector Th2 cells. Each bar represents stacked mean frequency data from n=5 animals with standard deviation.

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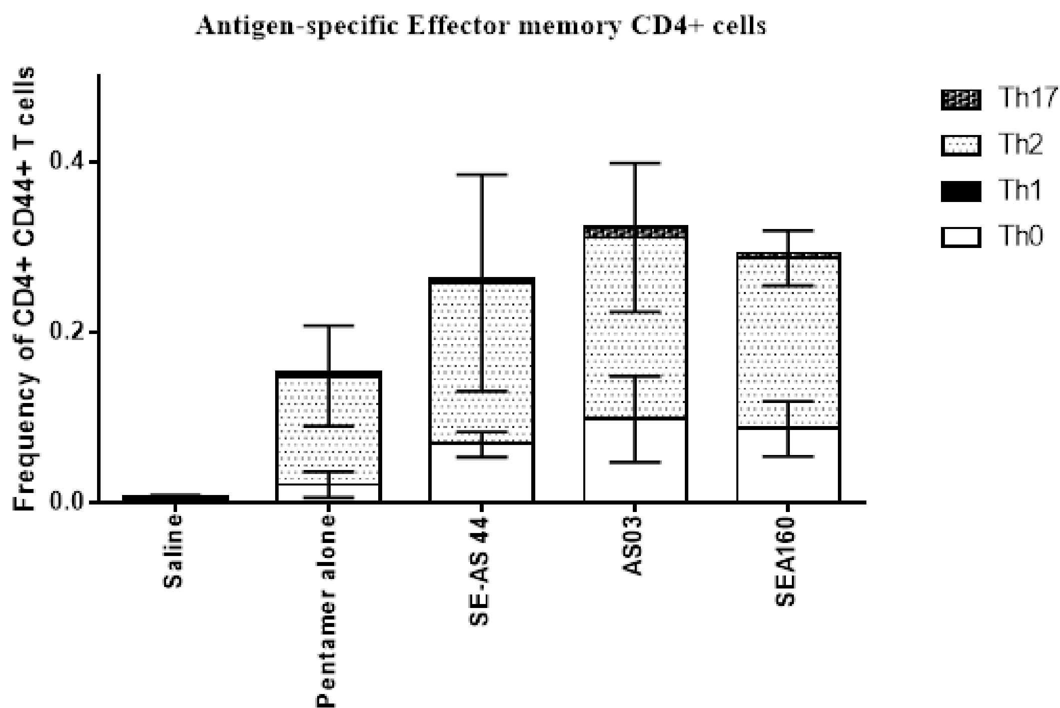

Figure 4c: Frequencies of antigen-specific effector memory cells gated from Quadrant 3 after gating for memory markers. Each bar represents stacked mean frequency data from n=5 animals with standard deviation.

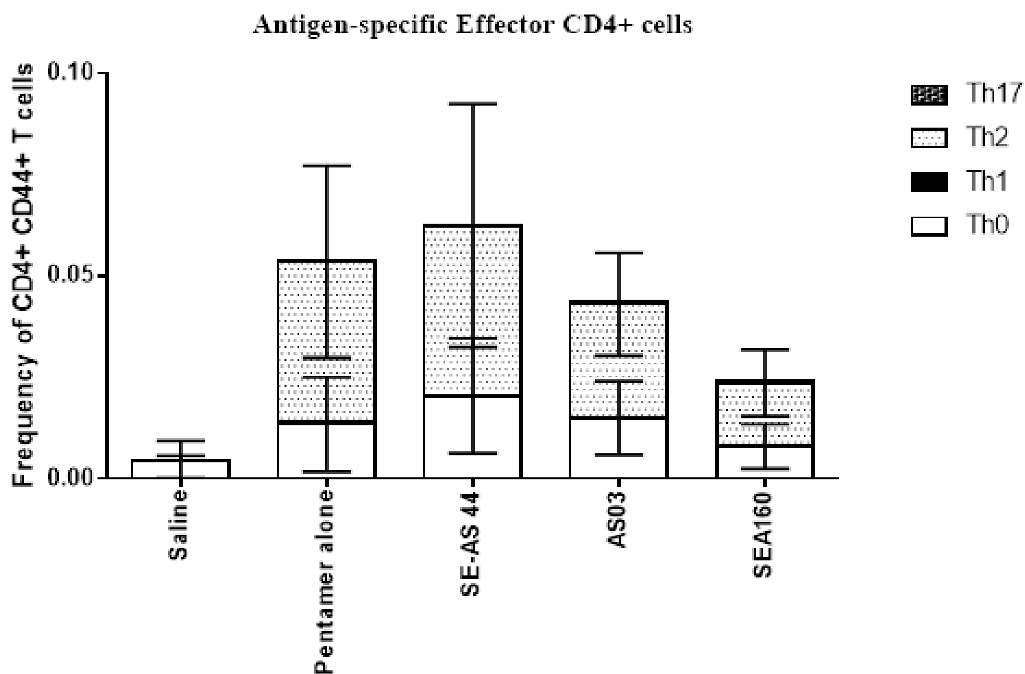

Figure 4d: Frequencies of antigen-specific effector cells gated from Quadrant 4 after gating for memory markers. Each bar represents stacked mean frequency data from n=5 animals with standard deviation.

Discussion

Previously we published proof-of-concept work using Quadrivalent influenza vaccine as a model antigen to show that SE-AS adjuvanted groups had non-inferior immune responses compared to AS03 in BALB/c mice. Although incorporating α-tocopherol in these SE-AS to obtain a stable emulsion was a challenge, this study proved the possibility of formulating emulsion adjuvants with the same components as AS03, using self-emulsification albeit the total amount of α-tocopherol in these was reduced to 15% v/v compared to 42%v/v in AS03. To advance these findings, we optimized the SE-AS formulations, maintaining 15% v/v concentration of α-tocopherol but optimizing the surfactant content, to improve the filterability of the emulsion adjuvants and to demonstrate short term stability at accelerated storage conditions. The optimized candidate SE-AS44 was stable in terms of squalene and α-tocopherol content even at 50°C for up to two weeks, and even longer in terms of size and PdI of oil droplets. Additionally, the morphology of these droplets was similar to AS03, suggesting that there was no impact of the process of formulation on overall droplet morphology of the emulsions with same composition but slightly different oil content. SE-AS44 also showed induction of antigen presenting cells particularly neutrophils, when tested in vitro in human PBMC cells compared to the SEA160 which was the squalene-only emulsion control in this experiment, which was consistent with previous findings [16]. SE-AS44 was hence the optimized candidate for evaluation of potency with a model antigen and in this case, it was CMV pentamer, which was a soluble recombinant protein, expressed in CHO cells [32]. CMV pentamer alone, being a subunit protein had very poor immunogenicity [32] and hence was an ideal candidate to evaluate the role of adjuvant in improving this immunogenicity. C57BL/6 mice. The in vivo study showed that all adjuvants significantly improved the humoral immune response after the second immunization. Particularly, the SE-AS44 adjuvanted groups showed significantly higher nAbs as well as anti-CMV IgG antibodies compared to SEA160 groups. This data confirmed our hypothesis and the role of α-tocopherol as an immunomodulator which is also shown previously [16] as well as recently [19]. For a typical prophylactic response, it is essential to get a sufficient memory response and hence cell-mediated immunity was tested in terms of CD4+ T cells which were further characterized based on the memory responses using CD62L and CD127 markers. As expected, the emulsion adjuvants overall showed a Th0/Th2 biased T cell response. Within the Th2 population, a higher

frequency of effector cells was observed for SE-AS44 compared to other emulsions. On the contrary, the frequency of effector memory cells was low. This may suggest that SE-AS44 was quick to convert effector memory cells to effector CD4+ T cells upon re-stimulation with the peptide indicating quick on-set of action. However, since the overall frequency of CD4+ T cells was low, more studies will be needed to confirm this hypothesis.

Summary

Emulsion adjuvanted vaccines have been in the forefront of the vaccine development landscape in response to a pandemic [4, 7, 10, 33]. This work provides a proof of concept towards developing the emulsion adjuvants in a simpler way to maximize and expedite its production in all, particularly low-income, countries. In addition to this, its shown that α-tocopherol acts as an immunomodulator in these squalene-based emulsion adjuvants and can be formulated using a self-emulsification process albeit at lower concentrations. This work along with recently published work [18, 19] bolsters the role of alpha tocopherol in emulsion adjuvants and can provide a perspective to researchers to explore the specific molecular mechanism of immune-potentiation further.

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Conflict of Interest Statement

RNL, APK, AA, DB and DTO are employees of the GSK group of companies.

Authors contributions

RNL, MMA and DTO were involved in the conception and design of the study and/or the development of the study protocol. RNL executed the study design and conducted experiments and analysis. RNL, AA, DB and AK participated to the acquisition of data. All authors participated

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in interpretation of the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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SUPPLEMENTARY FIGURES AND TABLES

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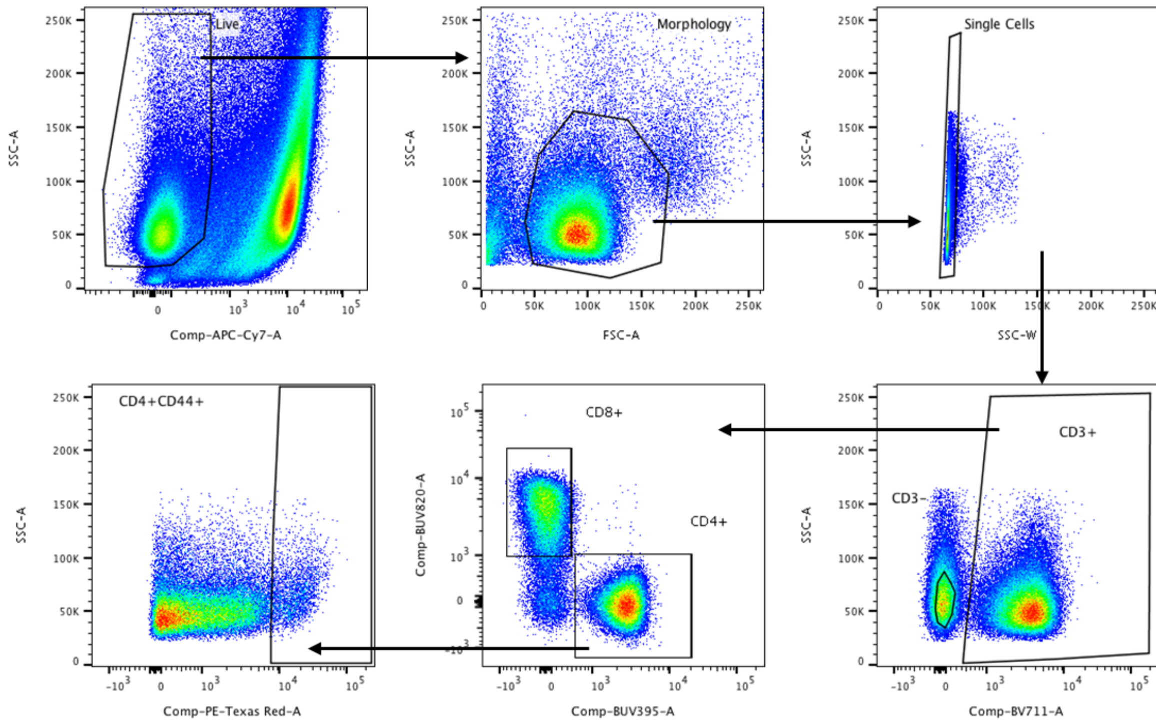
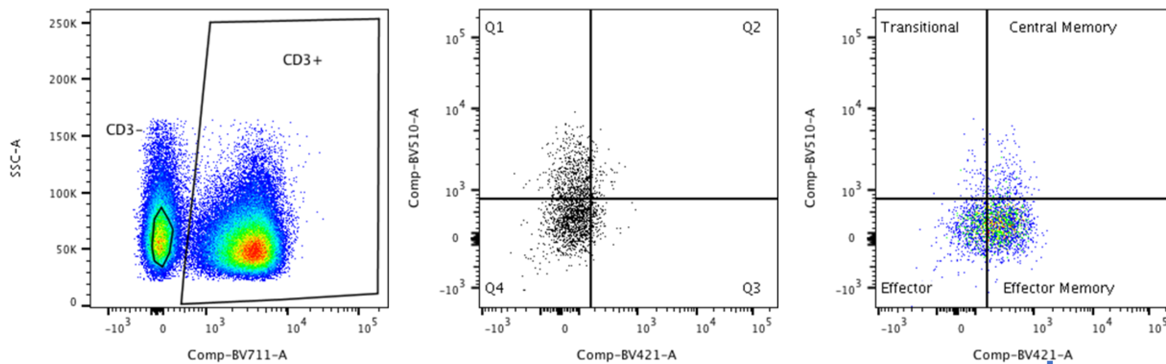


Figure 1a: Example of gating strategy for ICS. First live cells are differentiated from dead, and then separated based on morphology to get single cell lymphocytes, differentiated based on CD3 marker. CD4+ and CD8+ T cells are then gated based on CD3 gates followed by identifying antigen specific CD4+ or CD8+ T cells.



Cells from each quadrant are then gated for CD4+CD44+cytokine+ cells or CD8+CD44+cytokine+ cells as explained in Figure 2a

Figure 1b: Example of gating strategy for Memory markers. CD3- cells are used to gate four quadrants of T lymphocyte cells based on CD62L and CD127 markers. Q1 represents transitional

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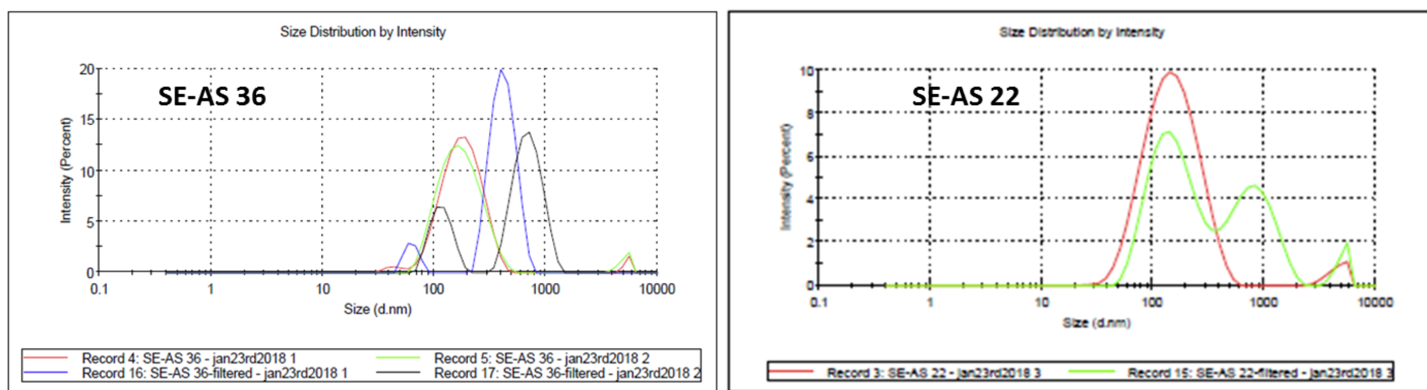
T cells, Q2 represents Central Memory T cells, Q3 represents Effector memory and Q4 represents Effector cells.

SE-AS	Before filtration		After filtration		% content loss after filtration	
	Size	PdI	Size	PdI	Squalene	Tocopherol
22	135.2	0.241	230.1	0.484	31.12	32.38
36	173.3	0.283	371.3	0.619	45.12	61.02

Supplementary Table 1: Size of the emulsions before and after filtration and % content loss after filtration for SE-AS 22 and 36.



Supplementary Figure 2: Bimodal size distribution after filtration through 0.22µ PES filter. Graphs in red & green for SE-AS 36 are before filtration, and those in blue and black are post filtration. For SE-AS 22, red is before and green is after filtration.

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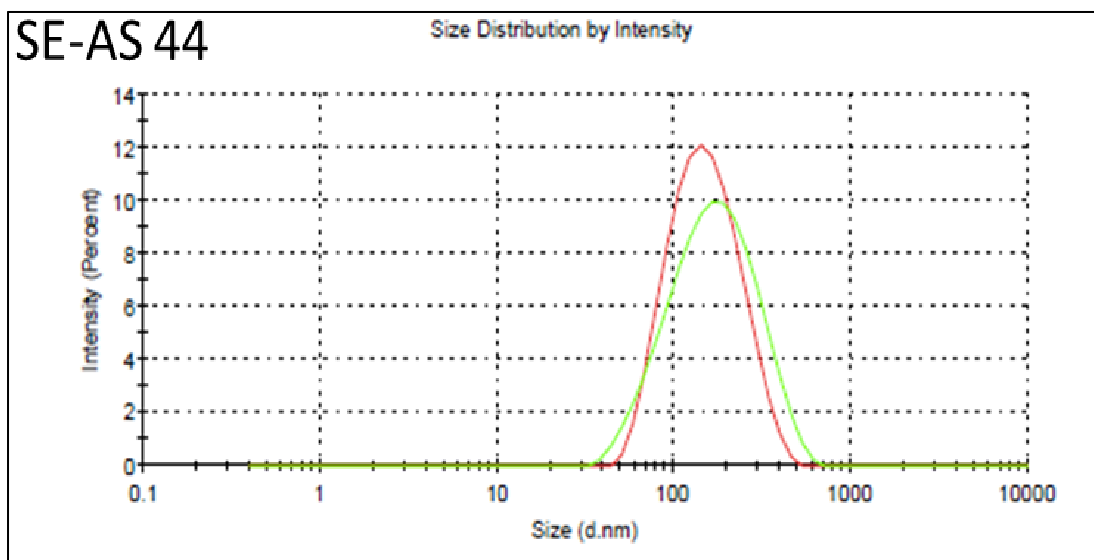
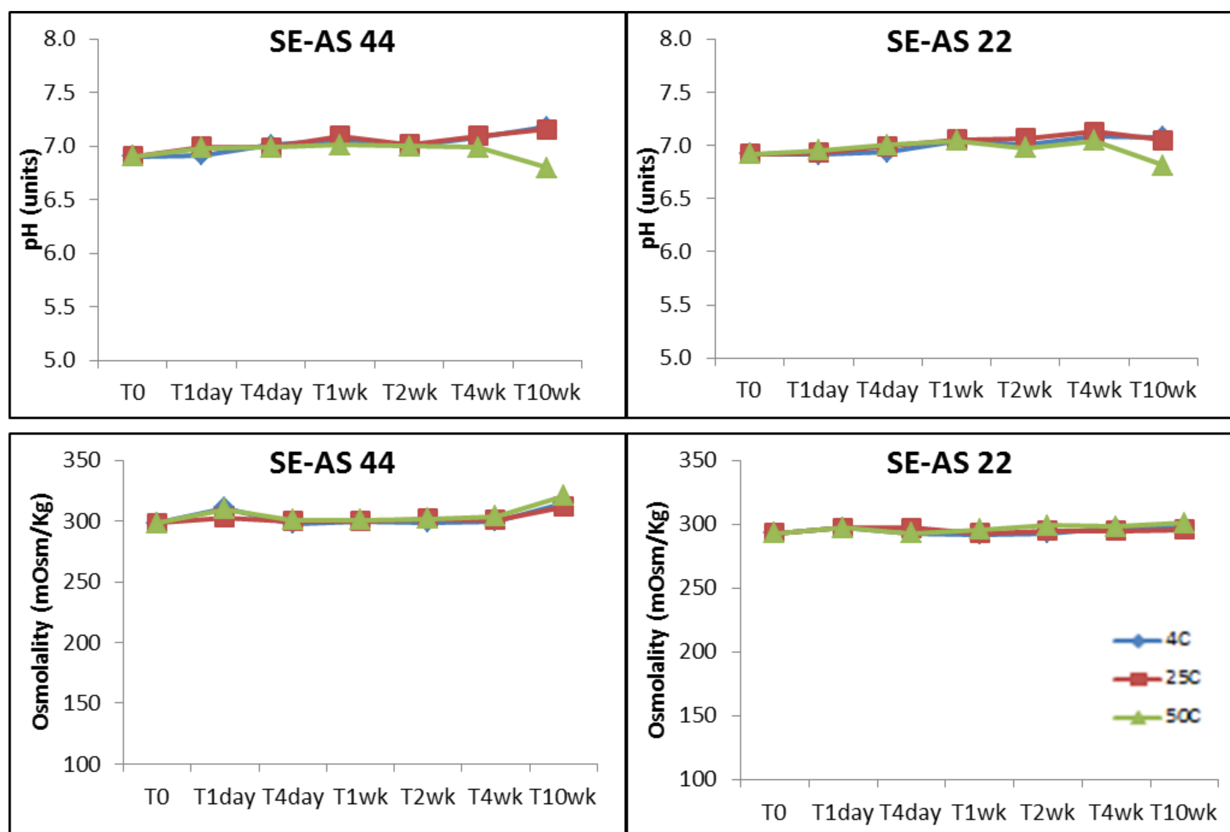


Supplementary Figure 3: Size distribution by intensity for SE-AS 44, showing distribution pre- (red curve) and post- (green curve) filtration.



Supplementary Figure 4: pH and Osmolality for emulsions stored at 4C (blue), 25C (red) and 50C (green) for up to 10 weeks.

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