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RESEARCH PAPER

Evaluation of alum-based adjuvant on the immunogenicity of *salmonella enterica* serovar typhi conjugates vaccines

Erman Tritama[a,b], Catur Riani[a], Indra Rudiansyah[b], Arip Hidayat[b], Siti Azizah Kharisnaeni[b], and Debbie Sofie Retnoningrum[a]

[a]Laboratory of Pharmaceutical Biotechnology, Department of Pharmacy, School of Pharmacy, Institute of Technology Bandung, Bandung, West Java, Indonesia; [b]Research and Development Division, PT. Bio Farma, Bandung, West Java, Indonesia

ABSTRACT

The function of adjuvant in maintaining the long-term immune response to Typhoid conjugate vaccine (TCV) was evaluated in. Two TCV products, Vi-DT and Vi-TT, were formulated in either aluminum phosphate ($AlPO_4$) or aluminum hydroxide (AlOH) as adjuvants and TCV formulated in phosphate buffer saline were used as controls. In each case, a group of Balb/c mice was injected intramuscularly with two doses of the formulated vaccine at two-week intervals. The anti-Vi IgG responses were monitored by Enzyme-Linked Immunosorbent Assay and the levels of $CD4^+$ T-cells expressing cytokine were characterized using intracellular cytokine staining. All mice immunized by TCV formulated in adjuvant elicited anti-Vi response to a higher level than the group receiving TCV formulated in PBS. The extent of adsorption of TCV in AlOH was greater than that in $AlPO_4$, and this finding correlated well with the observation that the mice immunized with two doses of Vi-DT(AlOH) elicited anti-Vi IgG to a level higher than that seen with Vi-DT($AlPO_4$). The mice primed with Vi-TT(AlOH) produced lower anti-Vi IgG (25.901 GM) compared to those receiving Vi-TT($AlPO_4$) (49.219 GM). However, after the second injection, the former raised the antibody level significantly to 137.008 GM while the latter provided a value of only 104.966 GM. The groups of mice vaccinated by TCV formulated in AlOH expressed IL4 at higher levels than the other groups, which correlated positively with the high Anti-Vi IgG in these animals. In conclusion, AlOH could be recommended as an effective adjuvant for TCV to provide a long-term immune response.

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Introduction

Salmonella enterica serovar Typhi (*S. enterica* ser. Typhi) is a polysaccharide encapsulated bacteria, the causative organism of Typhoid fever, a foodborne and waterborne disease which in many developing countries spread broadly due to poor sanitation. This acute febrile illness with the symptoms of high fever, headache, abdominal pain, malaise, splenomegaly and relative bradycardia with high mortality among children.[1] Globally, it is estimated to cause over 21 million illnesses and 222,000 deaths per year.[2] In Indonesia, the annual incidences of Typhoid cases among group age 5–15 years-olds are 180.3 per 100,000 persons and 20% of the cases required hospitalization. Surveillance data show that 65%, 22% and 7% of clinical isolates from Pakistan, Vietnam and India, respectively, are multidrug resistant.[3] Therefore, Typhoid vaccination has emerged as a complementary strategy with improvement of water quality and sanitation to control, prevent and eliminate typhoid disease. Vaccination against Typhoid had been proven effective to control Typhoid infection in Bangladesh which decreased the number of Typhoid cases from 165 cases in 2008 to 67 cases in 2012.[4]

Two Typhoid vaccines, composed of purified Vi polysaccharide administered parenterally and a live attenuated oral Ty21a vaccine have been licensed and proven to be safe and effective against Typhoid infection. However, both vaccines are less effective for children below two year.[5] Children under two year cannot use this vaccine due to their poor responses to T-independent antigens such as polysaccharide.[6] Vi polysaccharide vaccine as other polysaccharide vaccines are T-independent antigen and lack of booster upon re-injection.[7] Typhoid conjugate vaccines (TCV) are subunit injectable vaccines in which the Vi polysaccharide (Vi) is coupled to a carrier protein.[7] TCV was developed to overcome the limitation of polysaccharide or oral Typhoid live attenuated vaccines.[8] This vaccine able to switch the immune response from T-independent antigen to T-dependant antigen and generate memory cell.[7] The similar principle of conjugate vaccines successfully applied to prevent *Haemophilus influenza* type b (Hib), meningococcal and pneumococcal diseases.[9-11] Currently, there are several TCV preparations under development using carrier proteins such as diphtheria toxoid (DT), recombinant DT-CRM 197, and recombinant exoprotein A from *Pseudomonas aeruginosa* (rEPA).[7] The TCV using Tetanus toxoid (TT) as carrier protein has already been licensed in the market.[12] However, none of these products contains an adjuvant. Vaccines made from killed whole organism or subunit vaccines require the addition of the adjuvant to be effective.[13]

An adjuvant can be defined as a product that increases or modulates the humoral or cellular immune response to the antigen.[14] The use of adjuvant can enhance the immune

CONTACT Erman Tritama ermantritama123@gmail.com Product Development Matrix, PT. Bio Farma (Persero), Jalan Pasteur No. 28, Bandung 40161, Indonesia.

response or immunogenicity, provide long-term protection against infection, and reduce the amount of antigen that is needed in the vaccine. The result will be a reduction in the cost of a dose, making the vaccine affordable, as a greater number of doses could be formulated from a fixed amount of Vi polysaccharide. Such a good quality and affordable Typhoid vaccine would be highly advantageous to national immunization programs, especially those of the developing countries such as Indonesia.

This report presents a study on the suitability of aluminum phosphate ($AlPO_4$) and aluminum hydroxide (AlOH) as effective adjuvants for TCV. Aluminum-containing adjuvant remains the first option for use in vaccine development due to their adjuvanticity with various of antigen, good track record for safety, easier production methods, low cost and being authorized for use in many countries. Although many novel alternatives such as bacterial-derived adjuvant, cytokine agonist, or polymeric microsphere adjuvants have become available, their use in vaccines presents regulatory challenges.[15,16] Aluminum-containing adjuvants are found in several conjugate vaccines such as Hib conjugate vaccine (Pedvax-Hib and Vaxem-Hib), *Pneumococcal* conjugate vaccine (Synflorix and Prevenar), and in combination vaccines such as DTP-HB-Hib-IPV, DTP-HB-Hib and DTP-Hib, (D, Diphtheria; T, Tetanus; P, Pertussis; HB, Hepatitis B; Hib, *Haemophilus influenza* type b; IPV, Inactivated Polio Vaccine).[17] The current study assessed the impact of adjuvant addition on the immunogenicity of TCV and characterized cytokine production stimulated by the vaccine and adjuvant combinations in mice. $AlPO_4$ and AlOH were selected as tested adjuvants because these compounds are commercially available in the market, already licensed for use in human[18] and the safety and efficacy of these adjuvants are well established.[19] Consequently, the licensing of TCV preparations containing these adjuvants will face less hurdle.

Results

Physical and chemical properties of vaccines

The vaccines were tested to assure that it met the requirements stipulated by European Pharmacopoeia and Technical Report Series (TRS) 987 which calls for a sterile formulation at pH 6.5-7.5 with a Vi polysaccharide concentration of 21.25-28.75 μg/doses providing a minimum of O-acetyl content of 0.085 μmol/dose and an osmolality value of 250–350 mOsmol/kg.[20] The results shown in Table 1 demonstrated that the vaccines formulated with the adjuvants or PBS met these requirements. TCV formulated in AlOH, $AlPO_4$, and phosphate buffer saline (PBS) shared similar physical and chemical characteristics, except the percentages adsorption of TCV in AlOH was relatively higher than that in $AlPO_4$. The observed extents of adsorption could have had an effect on the immunogenicity of the vaccines.

Anti Vi IgG responses

The groups of mice receiving the first injection with TCV formulated in either $AlPO_4$ or AlOH, elicited anti-Vi IgG response higher than those receiving TCV formulated in PBS (Table 2). Vi-DT ($AlPO_4$) and Vi-DT (AlOH), respectively, produced anti-Vi IgG levels of 1.6 and 2.0 times higher than Vi-DT (PBS), whereas the groups of mice primed with Vi-TT ($AlPO_4$) and Vi-TT (AlOH), respectively, elicited anti-Vi IgG levels 2.9 and 1.5 times higher than that with Vi-TT (PBS). The high level of Anti-Vi IgG in mice receiving adjuvanted TCV compare to the group immunize by unadjuvanted TCV in the first administration was statistically significant (Kruskal-Wallis; $P = 0.001$). After the second injection, the vaccines boosted the production of anti-Vi IgG in all group. Vi-TT (AlOH) provided the highest increment, a 5.29-fold (25.901 ± 1.765 GM to 137.008 ± 35.674 GM). Although the level of anti-Vi IgG in the group of mice vaccinated with Vi-DT (AlOH) increased only 1.53 times after the second dose, this formulation induced the highest anti-Vi IgG level (43.953 ± 20.362 GM) among Vi-DT vaccines. In the second dose, the observed increase in the level of anti-Vi IgG in the adjuvanted TCV groups compared to the unadjuvanted TCV groups was statistically significant (Kruskal-Wallis; $P = 0.001$).

CD4 T-lymphocyte response

To evaluate the impact of TCV formulated in adjuvants on cytokine expression, the levels of $CD4^+$ cells from immunized mice expressing various cytokines were assessed via intracellular cytokine staining and flow cytometry. Four differents cytokines, namely, Interleukin (IL) 4, IL 10, and

Table 1. Characterization of vaccines based on WHO TRS 987.

		Vi-DT			Vi-TT		
Item	Requirement	AlPO	AlOH	PBS	AlPO	AlOH	PBS
Sterility	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile
O-acetyl content (μmol/dose)	Min 0.085	69.67 ± 1.37	67.83 ± 1.13	69.00 ± 2.24	67.00 ± 0.45	66.83 ± 0.52	67.67 ± 0.93
Molecular Weight (kDa)	No requirement	1830.67 ± 18.25	1830.67 ± 18.25	1830.67 ± 18.25	1917.33 ± 36.40	1917.33 ± 36.40	1917.33 ± 36.40
pH	6.5 – 7.5	7.26 ± 0.03	7.17 ± 0.03	7.03 ± 0.02	7.23 ± 0.04	7.19 ± 0.01	7.04 ± 0.05
Endotoxin (EU/μg Vi)	No requirement	8.52 ± 0.52	8.39 ± 0.62	8.33 ± 0.54	9.49 ± 0.51	9.04 ± 1.10	9.49 ± 0.51
Vi content (μg/dose)	21.25 – 28.75	25.16 ± 0.91	25.56 ± 0.53	25.72 ± 0.90	25.10 ± 0.50	25.58 ± 0.20	25.23 ± 0.90
Free Vi (%)	No requirement	21.72 ± 2.11	21.40 ± 2.35	21.26 ± 2.14	21.80 ± 2.41	21.41 ± 2.70	21.67 ± 2.16
Osmolality (mosmol/Kg)	250 – 350	343.03 ± 2.64	345 ± 3.22	344.01 ± 2.35	341.33 ± 5.96	337.67 ± 4.93	340.33 ± 4.59
Identity	Polysaccharide +	Polysaccharide +	Polysaccharide +	Polysaccharide +	Polysaccharide +	Polysaccharide +	Polysaccharide +
Adsorption (%)	No Requirement	75.00 ± 1.79	84.33 ± 1.37	NP	11.00 ± 0.89	26.33 ± 1.37	NP

Vi-DT : Vi conjugated with Diphtheria Toxoid; Vi-TT : Vi conjugated with Tetanus Toxoid; AlPO : Adjuvant Aluminum phosphate; AlOH : Adjuvant Aluminum hydroxide; PBS : Phosphate Buffer Saline; NP : Not performed.

Table 2. Anti-Vi IgG immune responses in mice.[a]

Products[b]	Level of Anti Vi IgG After 1st Injection[c]			Level of Anti Vi IgG After 2nd Injection[c]		
	Geometric Mean Anti-Vi IgG (95% confidence interval)	Median[†]	Average Rank[†] (Overall 15.5)	Geometric Mean Anti-Vi IgG (95% confidence interval)	Median[†]	Average Rank[†] (Overall 15.5)
Vi-DT (AlPO)	23.851 ± 4.933	23.85	16.20	39.105 ± 18.266	39.10	13.80
Vi-DT (AlOH)	28.697 ± 3.995	28.70	20.60	43.953 ± 20.362	43.95	16.80
Vi-DT (PBS)	14.339 ± 1.190	14.34	3.00	22.526 ± 1.302	22.53	10.8
Vi-TT (AlPO)	49.219 ± 11.523	49.22	28.00	104.966 ± 80.648	104.97	23.00
Vi-TT (AlOH)	25.901 ± 1.765	25.90	17.20	137.008 ± 35.674	137.01	25.60
Vi-TT (PBS)	16.961 ± 0.350	16.96	8.00	17.337 ± 0.617	17.34	3.00
AlPO	0.034 ± 0.035	NA	NA	0.035 ± 0.039	NA	NA
AlOH	0.020 ± 0.000	NA	NA	0.020 ± 0.000	NA	NA

[†]Test was done using Kruskal-Wallis test.
[a]Six week old male and female mice were injected intramuscularly with two dose products, dose were given 0 and 2 weeks.
[b]Vi-DT (AlPO) : Vi conjugated with Diphtheria Toxoid and added adjuvant Aluminum phosphate in the final product; Vi-DT (AlOH) : Vi conjugated with Diphtheria Toxoid and added adjuvant Aluminum hydroxide in the final product; Vi-DT (PBS): Vi conjugated with Diphtheria Toxoid and added Phosphate Buffer Saline in the final product; Vi-TT (AlPO): Vi conjugated with Tetanus Toxoid and added adjuvant Aluminum phosphate in the final product; Vi-TT (AlOH): Vi conjugated with Tetanus Toxoid and added adjuvant Aluminum hydroxide in the final product; Vi-TT (PBS) : Vi conjugated with Tetanus Toxoid and added Phosphate Buffer Saline in the final product; AlPO: Adjuvant Aluminum phosphate; AlOH: Adjuvant Aluminum hydroxide.
[c]Bleeds were taken at 2 (1st) and 4 (2nd) weeks and sera assayed for Vi antibodies.

Interferon (IFN)-γ were targeted for measurement due to their roles in the maturation of plasma cell and production of antibody; IL2 was use as a control as the expression of this cytokine was expected to remain unaltered. Table 3 presents data on the proportions of the $CD4^+$ cells from mice vaccinated with TCV formulated in adjuvants or PBS expressing all four cytokines. The group of mice receiving Vi-DT and Vi-TT formulated in adjuvant AlOH expressed IL4 at levels higher than the groups receiving TCV formulated in adjuvant $AlPO_4$ or PBS and resulting in the highest anti-Vi IgG production in the former group. The production of IL10 and IFNγ were influenced differently The mice group receiving adjuvanted TCV expressed IFNγ at levels higher than that with unadjuvanted antigen. However, in term of the expression of IL10, only adjuvanted Vi-DT induced higher expression level for this cytokine compared to the control (Vi-DT in PBS), while for Vi-TT the mice group receiving unadjuvanted antigen expressed higher level of IL10 than those with adjuvanted Vi-TT. These results indicated that the expression of a macrophage attracting cytokine (IFNγ) but not a Treg cytokine (IL10) was enhanced by the adjuvant addition to TCV and increased the effectiveness of this conjugated antigen. All groups elicited IL2 poorly, indicating that the vaccines induced Th2 cytokine production and suppressed the production of Th1 cytokine. The increase in IL4 and IFN-γ expression in mice vaccinated with adjuvanted TCV compared to those receiving unadjuvanted antigens was statistically significant (Kruskal-Wallis; $P = 0.002$ and $P = 0.004$ respectively).

Discussion

Although Polysaccharide Typhoid conjugate vaccine has been proven to provide protection for children under age two, the roles of adjuvant in enhancing the stability of the vaccine and in maintaining the long-term immunogenicity and reducing the cost per dose remain to be considered. The essential factor impacting the effectiveness of vaccines formulated with the adjuvant is the extent of adsorption of the cognate antigen into the adjuvant.[21] It has been shown for model vaccines such as ovalbumin, alpha casein or dephosphorylated alpha casein that a high degree of adsorption an antigen into adjuvant increases respective immunogenicity. Also, such positive effects have been reported for the use of montanide oil-based adjuvants in clinical trials against HIV and malaria.[22,23] Similarly, immunostimulating complexes (ISCOMs) are known to induce strong

Table 3. %CD4+ expressing IL4, IL2, IL10 and IFN-γafter receiving 2 doses of vaccines.

Products[a]	%CD4+ (95% confidence interval)						
	IL2	IL4[b]	Average rank[†] (Overall 15.5)	IL10	Average rank[†] (Overall 15.5)	IFN-γ	Average rank[†] (Overall 15.5)
Vi-DT (AlPO)	ND	53.4 ± 7.86	17.6	74.5 ± 19.7	17.4	91.4 ± 8.66	23.2
Vi-DT (AlOH)	0.68 ± 1.00	56.1 ± 7.86	20.2	88.5 ± 19.7	24.0	88.0 ± 8.66	18,8
Vi-DT (PBS)	1.39 ± 2.77	44,5 ± 10.4	10.4	71.6 ± 12.9	17.6	62,3 ± 18.9	7.4
Vi-TT (AlPO)	0.93 ± 1.00	49.1 ± 6.79	14.2	56.2 ± 11.2	8.3	90.6 ± 9.84	21.8
Vi-TT (AlOH)	1.43 ± 1.00	65.6 ± 9.25	26.4	56.2 ± 11.2	8.3	82.6 ± 7.23	15.8
Vi-TT (PBS)	0.38 ± 2.72	34.5 ± 2.83	4.2	71.2 ± 10.9	17.4	66.8 ± 0.50	6.0

[†]Test was done using Kruskal-Wallis test.
[a]Vi-DT (AlPO) : Vi conjugated with Diphtheria Toxoid and added adjuvant Aluminum phosphate in the final product; Vi-DT (AlOH) : Vi conjugated with Diphtheria Toxoid and added adjuvant Aluminum hydroxide in the final product; Vi-DT (PBS) : Vi conjugated with Diphtheria Toxoid and added Phosphate Buffer Saline in the final product; Vi-TT (AlPO) : Vi conjugated with Tetanus Toxoid and added adjuvant Aluminum phosphate in the final product; Vi-TT (AlOH) : Vi conjugated with Tetanus Toxoid and added adjuvant Aluminum hydroxide in the final product; Vi-TT (PBS) : Vi conjugated with Tetanus Toxoid and added Phosphate Buffer Saline in the final product; ND : Not Determined.
[b]IL-4 : Interleukin 4; IL-2 : Interleukin 2; IL-10 : Interleukin 10; IFN-γ : Interferon Gamma.

mucosal responses (IgG and IgA) against influenza,[24] and CpG oligodeoxynucleotides are effective as molecular adjuvants in DNA vaccines.[25]

In our study, TCV formulated in AlOH showed greater adsorption than a preparation formulated in $AlPO_4$. The values of the Isoelectric Points (IEP) of Vi-DT and Vi-TT are close to 2, giving negative charges to these conjugates under a formulation process which is carried out at pH 7. AlOH had Potential Zero Charge (PZC) in the alkaline range (pH 11,4)[26] while this value for $AlPO_4$ is in the acidic range (pH 4)[27] resulting a positive charge for AlOH and negative charge for $AlPO_4$ under the pH of formulation.[28] Negative charge in TCV and positive charge in AlOH facilitated greater adsorption of the antigen in this system compared to that offered by the reaction of TCV with $AlPO_4$. According to the guidelines from WHO TRS 987, the use of TCV requires two injections giving a booster effect. The immunogenicity test in mice showed that TCV formulated with adjuvant not only showed a booster effect but also yielded a higher anti-Vi IgG level compared to TCV formulated in PBS.

S. enterica ser. Typhi is a specific human pathogen, and therefore, challenging immunized mice using this pathogen produces results of limited value.[29] *Salmonella enterica* serovar Typhimurium engineered to express the Vi polysaccharide[30] or the use of Balb/c mice Rag2-/-γc/ are viable alternatives.[29] However, the infection of *Salmonella enterica* serovar Typhimurium produces clinical outcomes that are different from human NTS gastroenterities.[31] Therefore, the capability of TCV to offer sterile protection was evaluated via the analysis of $CD4^+$ cytokine production and not by the animal challenge. The mice vaccinated with TCV formulated in adjuvant AlOH expressed higher levels of IL4 than the groups receiving another vaccine. This is consistent with the ELISA result that demonstrated the highest Anti-Vi IgG level with the former group. Since IL4 is a Th2 cytokine that promotes the maturation and differentiation of B-cells into Plasma Cells and memory B-cells, our results indicated that the addition of adjuvant AlOH to TCV could improve the effectiveness of vaccine in terms of producing long-term immunity. Therefore, TCV with AlOH as the adjuvant is recommended as a preferred vaccine against Typhoid.

Material and methods

Vaccines

TCV was prepared by conjugation of Vi into DT or TT following a standard protocol.[32] To prepare TCV formulated in $AlPO_4$, 50 μg of each conjugate was mixed with 3 mg $AlPO_4$. These vaccines were designated Vi-DT ($AlPO_4$) and Vi-TT ($AlPO_4$). AlOH was used as another adjuvant, and for this study 1.2 mg of AlOH was mixed with 50 μg of each TCV, producing Vi-DT (AlOH) and Vi-TT (AlOH). A 50 μg of each conjugate TCV formulated in PBS was used as a control. The adsorption percentage of TCV in each adjuvant was measured by Hestrin method.[33]

All TCV vaccines were characterized according to Europe Pharmacopoeia and WHO Technical Report Series (TRS) 987 Annex 3 2014.[20] The conjugated and free-Vi contents were assayed using High-Performance Anion Exchange Chromatography with Pulse Amperometric Detection (HPAEC-PAD).[34] The identities of the vaccines were confirmed by ELISA. The O-acetyl contents of Vi in the preparations were measured using the Hestrin method with acetylcholine chloride as a standard.[33] The molecular weight of the vaccines were estimated by High-Performance Liquid Chromatography (HPLC) and the osmolality was determined by use 2020 Multi-Sample Micro-Osmometer (Advanced Instruments). The vaccines were tested for sterility by filtration on a membrane and examining whether a fluid thioglycollate medium (FTM) and soyabean casein digest medium (SCDM) inoculated with the membrane exhibit bacterial growth.[35]

Immunization and sample collection

Outbreed male and female Balb/c less than six weeks old were housed in the animal facility in Bio Farma. The mice weighing 20–25 gram were acclimated in a room (temperature, 20–26°C) with a humidity 40–85% and a 12 hours light/dark cycle for five days prior to vaccination. Each animal was provided with a 5–9 gram of food and water *ad libitum*. Per cage five mice of the same sex, male or female, were housed. Groups of 10 mice were vaccinated by intramuscular injections of a 200 μL solution containing 25 μg of either Vi-DT or Vi-TT formulated in $ALPO_4$, ALOH or PBS; two doses were applied at 2-week intervals to mimic the route administration in the clinical stage. Balb/c mice were chosen due to their known usefulness in a variety immunological studies. Even though *S. enterica* ser. Typhi is an obligate human pathogen, Vi conjugated to the protein are well tolerated in animal model and found to elicit antibody response whereas Vi alone does not.[36]

Blood samples were collected from the mice two weeks after each immunization via submandibular bleeding. At the endpoint the mice were sacrificed and the blood was collected via cardiac puncture. All samples were analyzed for antibody level using ELISA. The blood from endpoint was analyzed for IL2, IL4, IL10 and IFN-γ expression induced by the vaccines using flow cytometry. All procedures performed on mice were reviewed and approved by PT. Bio Farma's Institutional Animal Care and Use Committee (IACUC) No. Ref. 01/IACUC-BF/VIII/16.

Serological analysis

Vi antibody levels in mice sera were evaluated by ELISA as described previously[37] using Microtitre plates (Nunc 439454). Briefly, the well plate was coated with 100 μL (per well) of diluted Vi and incubated at room temperature overnight. Each well was washed four times using 300 μL washing buffer (0.01% Tween 20 in PBS). Then 100 μL of blocking buffer (1% BSA in PBS) was added to each well and the plate was left at room temperature for 4 hours. At this stage, solution was removed from the plate, wells were washed four times with washing buffer and to each well 100 μL dilution buffer (0.1% BSA in PBS with 0.01% Tween 20) and 100 μL diluted sample were added. The plate was incubated at room temperature overnight. After the plate was washed four times with washing buffer, a 100 μL of the secondary antibody (Jackson

Laboratories, Cat. no 415-055-166) was added to each well and the plate was incubated at 37°C for 4 hours. The unbound secondary antibody was removed by using washing buffer. Then to each well the detection reagents, 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, 71768) was added and the plate was incubated at room temperature for 25 minutes. The resultant colored was measured at 405 nm using an ELISA plate reader. The data were processed using Program ELISA for Windows (CDC). Anti-Vi Hyperimmune serum from International Vaccine Institute (IVI) was used as a reference and IgG anti-Vi level was expressed in ELISA Unit (EU) and the results expressed as the geometric mean (GM).

Isolation of peripheral blood mononuclear cells (PBMCs)

To characterize the cytokine production, blood samples from endpoint were analyzed. PBMCs were isolated from whole blood via density gradient centrifugation, 400g for 30 minutes at 18–20°C using Ficoll-Paque Premium (GE Healthcare Life Science), washed with PBS and then resuspended in RPMI-1640 media (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco).[32]

Intracellular cytokine staining

Cytokine productions in mice receiving immunization with TCV (formulated in adjuvant or PBS) were characterized by intracellular cytokine staining as described previously.[32] Briefly, 10^6 PBMCs from immunized mice were cultured in microtubes and stimulated with the vaccine that was used for immunization or medium alone. The co-stimulatory molecules, anti-CD28 (BioLegend, Cat. No. 102101, Clone 37.51) and anti-CD49d (BioLegend, Cat. No. 103701, Clone 9C10), were added to the culture and the mixture was incubated for 2 hours at 37°C under an atmosphere containing 5% CO_2. Then monensin (BioLegend, Cat No. 420701) was added to a final concentration of 1X and the culture was incubated for additional 4 hours. The cells were washed twice using PBS and 5% FBS and then stained for cell surface markers using anti-CD3-PerCp (BioLegend, Cat. No. 100328, Clone 145-2C11), anti CD4-FITC (BioLegend, Cat. No. 100406, Clone GK1,5), anti-CD8-PE (BioLegend, Cat.No. 100708, Cat. No. 100708), anti CD69-PerCP (BioLegend, Cat. No. 104522, Clone H1.2F3) monoclonal antibodies. Red blood cells were lysed and PBMCs were fixed using 1x RBC Lysis/Fixation solution (BioLegend, Cat. No. B222815) for 15 minutes. The culture was washed with PBS and permeabilized using 1x intracellular staining permeabilization wash buffer (BioLegend, Cat. No. 421002). Then the intracellular cytokines were stained using anti-IL2-APC (BioLegend, Cat. No. 503810, Clone JES6-5H4), anti-IL10-PE (BioLegend, Cat. No. 505008, Clone JES5-16E3), anti-IL4-PE (BioLegend, Cat. No. 504104, Clone 11B11) and anti-IFN-y (BioLegend, Cat. No. 506822, Clone XMG1.2) monoclonal antibodies for 20 minutes. The cells were washed and stored at 4°C until flow cytometry was performed. Lymphocyte population was identified on forward vs. side scatters plot, then $CD3^+$ subpopulations were gated. Flow cytometry data analysis was performed using Flow-jo software (Treestar).

Statistical analysis

A comparison of immune response and expression of cytokines between the different animal groups was performed. A univariate, nonparametric Kruskal-Wallis test was used to assess the statistical significance of the differences between outcomes of the immune response. The test asses primary predictor variables namely, Vi-DT (AlOH), Vi-DT ($AlPO_4$), Vi-DT (PBS), Vi-TT (AlOH), Vi-TT ($AlPO_4$), Vi-TT (PBS) versus 2 immune response parameter (antibody and cytokine production). To analyze the data, the threshold of significance $P < 0.05$ and 95% confidence intervals were calculated.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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