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Short Communication

SNP may modify the effect of vitamin A supplementation at birth on cytokine production in a whole blood culture assay

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Abstract
Within a neonatal vitamin A supplementation (VAS) trial, we investigated the effect of VAS on TNF-α, IL-10, IL-5 and IL-13 production after lipopolysaccharide, purified protein derivative (PPD) of *Mycobacterium tuberculosis* and phytohaemagglutinin stimulation using a whole blood culture protocol. We found that VAS recipients had lower unstimulated TNF-α concentrations than placebo recipients. In the present paper, we investigated whether the SNP *TNF-α* −308, *TNF-α* −238, *IL-10* −592, *IL-10* −1082 and toll-like receptor 4 (*TLR4*) + 896 modified the effect of VAS on cytokine production. DNA and cytokine concentrations were available from 291 children. We found a significant interaction between *TNF-α* −308 genotype and VAS for the unstimulated TNF-α production ($P_{interaction} = 0·04$); among G homozygotes, TNF-α concentrations were significantly lower after VAS compared with placebo, whereas for A carriers, VAS did not appear to have any effect. For *TNF-α* −238, there was a tendency towards an increase in PPD-stimulated TNF-α production after VAS for the G homozygotes, but the opposite tendency for A allele carriers ($P_{interaction} = 0·07$). Stratification by sex revealed a significant VAS–genotype interaction for boys for *TNF-α* −238. There was a borderline-significant three-way interaction ($P = 0·05$) between sex, VAS and *TLR4* + 896 genotype. Although the present study had very limited representation of the genetic variation with potential for modification of the response to VAS, it adds to the efforts of untangling the diverse effects and impact of VAS.

Key words: SNP: Vitamin A supplementation: Immunology: Whole blood stimulation: Sex differences

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Vitamin A has a multitude of immunological effects, many of which are obvious in vitamin A deficiency, a state coined as 'a nutritionally acquired immunodeficiency disorder'[1]. Vitamin A supplementation (VAS) is a valued intervention in vitamin A-deficient populations; it has been reported to reduce overall child mortality by 23 %[2]. The underlying immunological mechanisms for this reduction remain elusive, but apparently, more is involved than mere prevention of deficiency[3]. We investigated the effect of high-dose VAS with Bacille Calmette-Guérin vaccine at birth in a randomised trial in Guinea-Bissau and surprisingly found that VAS did not improve survival[4]. However, as seen in other studies, VAS seemed to benefit boys more than girls[4]. Within the trial, we investigated the effect of VAS at birth on cytokine production in a whole blood culture assay and found that VAS was associated with decreased unstimulated TNF-α production (M. J. Jørgensen, unpublished results).

SNP in the promoter region of cytokine genes has the potential to modify the cytokine response to stimuli through their effect on transcription levels[5]. In the present study, we examined whether commonly investigated SNP in Toll-like receptor 4 (*TLR4*) and cytokine promoter regions modified the effect of VAS on the cytokine production in whole blood culture.

Abbreviations: GMR, geometric mean ratio; LLD, lower limit of detection; LPS, lipopolysaccharide; PPD, purified protein derivative of *Mycobacterium tuberculosis*; TLR4, Toll-like receptor 4; VAS, vitamin A supplementation.

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Methods

The present study was carried out as a substudy within a randomised, double-blind, placebo-controlled trial of the effect on mortality of VAS with Bacille Calmette-Guérin vaccine at birth. A total of 4345 infants were randomised to receive a single oral dose of 0·5 ml vegetable oil with 27·5 mg (50 000 IU) retinyl palmitate and 10 mg (10 IU) vitamin E added (VAS) or 0·5 ml vegetable oil with 10 IU vitamin E only (placebo)[4]. The present study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures were approved by the Ministry of Health in Guinea-Bissau and by the Danish Central Ethical Committee. For all subjects, verbal informed consent was obtained, witnessed and formally recorded.

Blood sampling

At 6 weeks after receiving VAS or placebo, a subgroup of children was visited at home. The visits were conducted between 1 June and 9 December 2004. Blood samples were obtained by finger prick into a heparinised tube and a tube containing 500 μl of Nuclisens Lysis Buffer® (bioMérieux, Boxtel, The Netherlands).

Whole blood culture

A whole blood culture was performed as a 1:10 dilution of heparinised blood in a final volume of 200 μl using Roswell Park Memorial Institute-1640 medium. Stimulation was performed at 37°C/5 % CO_2 in the presence of phytohaemagglutinin (2 μg/ml; Wellcome Diagnostics, Dartford, UK), lipopolysaccharide (LPS, 100 ng/ml; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and purified protein derivative (PPD) of *Mycobacterium tuberculosis* (10 μg/ml; Statens Serum Institut, Copenhagen, Denmark). Supernatants were collected on days 1 (LPS) and 3 (PPD, phytohaemagglutinin). On both days, medium-only control samples were collected (control days 1 and 3).

Cytokine determinations

Supernatant concentrations of IL-5, IL-10, IL-13 and TNF-α were measured simultaneously using a commercial Luminex kit (BioSource, Camarillo, CA, USA) on a Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA). The lower limit of detection (LLD) was 3 pg/ml (IL-5), 5 pg/ml (IL-10), 10 pg/ml (IL-13) and 10 pg/ml (TNF-α).

SNP analysis

DNA was manually purified using a Nuclisens Isolation kit according to the directions of the manufacturer (bioMérieux). Flourescence-based real-time PCR analysis (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Minneapolis, MN, USA) was performed in uniplicate on alleles: *TLR4* +896A/G (rs4986790); *TLR4* + 1196C/T (rs4986791); *IL-10* − 1082T/C (rs1800896); *IL-10* − 592T/C (rs1800872); *TNF-α* − 238A/G (rs361525); *TNF-α* − 308A/G (rs1800629). Results were analysed using SDS 2.2 software (Applied Biosystems).

Statistical methods

All analyses were done using Stata/SE 10 (Statacorp LP, College Station, TX, USA).

Hardy–Weinberg equilibrium was assessed for each SNP, and no deviations were seen overall or within treatment groups. Distribution of genotypes by treatment group and sex was evaluated using χ^2 tests.

We chose to examine both TNF-α and IL-10 for all SNP, as the production of the two cytokines could be interdependent. For *TLR4* + 896, we investigated TNF-α, IL-10, IL-5 and IL-13 production for controls on days 1 and 3 and after LPS and PPD stimulation. We included PPD as TLR4 signalling is reportedly involved in the response to Bacille Calmette-Guérin vaccination[6].

For cytokines in which less than one-third had non-detectable concentrations, samples with concentrations below LLD were assigned half the LLD. As visual assessments indicated log-normality, we compared log-transformed cytokine responses in linear regression models. Regression coefficients and 95 % CI were back-transformed, providing geometric mean ratios (GMR (95 % CI)) of: (1) carriers of the rare allele relative to homozygotes for the major allele; (2) VAS relative to placebo by genotype. We adjusted stimulated responses for the cytokine concentration in the control. For cytokines in which more than one-third had non-detectable concentrations, responses were dichotomised as detectable ($>$ LLD) or non-detectable ($<$LLD). The proportions of detectable responses were compared in Poisson regression models with robust variance estimation, providing relative risks with 95 % CI[7].

We observed systematic differences in the cytokine production, coinciding with the use of two different batches of Luminex reagents. After assuring that no interactions were present between batch number and cytokine production, we adjusted all analyses for batch by including this as a categorical covariate in the models. Besides this, no further adjustments were made.

We explored the potential modifying effect of genotype on the response to VAS by inserting an interaction term. As sex may modify the effect of VAS[4], we also conducted analyses stratified by sex. If we observed significant interactions between genotype and VAS, we investigated potential three-way interactions between genotype, VAS and sex. A significance level of 0·05 was used for all analyses.

Results

DNA and cytokine responses were available from 291 children. All SNP were distributed evenly between the two treatment arms and by sex. Homozygote frequency for the major allele was 0·67 for *TNF-α* − 308 (G), 0·87 for *TNF-α* − 238 (G), 0·25 for *IL-10* − 592 (A), 0·47 for *IL-10* − 1082 (A), 0·83 for *TLR4* + 896 (A) and 0·99 for *TLR4* + 1196 (C).

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Effect of vitamin A supplementation on cytokine production

As reported elsewhere, VAS recipients had lower unstimulated TNF-α concentrations than placebo recipients (GMR 0·82 (0·68, 0·99); $P=0·04$).

Effect of genotype on cytokine production

We found only moderate effects of the SNP on the cytokine production (Table 1).

No effect was seen for *IL-10* −592 and *TNF-α* −308. For *TNF-α* −238, A allele carriers displayed higher LPS-stimulated IL-10 responses than G homozygotes (GMR 1·16 (1·01, 1·34); $P=0·04$). For *IL-10* −1082, carriers of the G allele had lower TNF-α concentrations on days 1 and 3 in control wells (GMR 0·82 (0·68, 0·99); $P=0·04$ and 0·76 (0·60, 0·96); $P=0·02$, respectively). *TLR4* + 896 displayed an increased PPD-stimulated TNF-α response for G allele carriers in comparison with A homozygotes (GMR 1·25 (1·00, 1·55); $P=0·05$).

Interaction between vitamin A supplementation and genotype

There was significant interaction between *TNF-α* −308 genotype and VAS for the unstimulated TNF-α production on day 3 ($P_{interaction}$ between *TNF-α* −308 genotype and VAS 0·04). Among G homozygotes, TNF-α concentrations were significantly lower after VAS compared with placebo (GMR 0·73 (0·56, 0·96); $P=0·02$). However, for A carriers, VAS appeared to have no or the opposite effect (GMR 1·23 (0·79, 1·92); $P=0·37$).

For *TNF-α* −238, there was a tendency towards an increase in the PPD-stimulated TNF-α production after VAS for G homozygotes (GMR 1·12 (0·94, 1·34); $P=0·22$), but the opposite tendency for A carriers (GMR 0·71 (0·43, 1·19); $P=0·19$; $P_{interaction}$ between *TNF-α* −238 genotype and VAS 0·07; Table 1).

Among boys, for *TNF-α* −308, VAS tended to decrease unstimulated TNF-α production on day 3 for G homozygotes and increase it for A allele carriers (GMR 0·75 (0·51, 1·11); $P=0·15$ and 1·45 (0·80, 2·62); $P=0·22$, respectively; $P_{interaction}=0·08$). For *TNF-α* −238, a significant decrease in the unstimulated TNF-α production on day 1 was seen among G homozygotes, whereas there was no effect for A carriers (GMR 0·70 (0·54, 0·92); $P=0·01$ and 1·32 (0·53, 3·24); $P=0·53$, respectively), resulting in a tendency towards interaction between VAS and genotype ($P_{interaction}=0·08$). For the TNF-α response to PPD, there was no effect of VAS among A allele carriers, but a significant increase among homozygotes for the G allele (GMR 0·53 (0·23, 1·26); $P=0·139$ and 1·31 (1·02, 1·67); $P=0·03$, respectively; $P_{interaction}=0·007$). No significant three-way interactions between genotype, sex and VAS were present in the three instances mentioned above.

Among girls, there was a borderline significant interaction between *TLR4* + 896 genotype and VAS. There was a tendency towards a decrease in the unstimulated TNF-α production on day 3 among A homozygotes and no effect or a tendency towards the opposite for G carriers (GMR 0·72 (0·49, 1·04); $P=0·08$ and 1·75 (0·70, 4·38); $P=0·22$, respectively; $P_{interaction}=0·09$). For boys, there was no effect of VAS among A homozygotes (GMR 1·03 (0·72, 1·47); $P=0·86$) and a tendency towards decrease for the G allele carriers (GMR 0·57 (0·25, 1·28); $P=0·16$), resulting in a borderline significant three-way interaction ($P=0·05$) between sex, *TLR4* + 896 genotype and VAS.

Discussion

The present study examined genetic influences on the immunological effects of VAS. The investigated panel of SNP did not appear as a major determinant for cytokine production. However, we found indications that SNP related to TNF-α modified the effect of VAS on cytokine production.

The study has obvious limitations. It was explorative, we conducted many analyses and some results may be statistically significant by chance. Hence, the results should be interpreted with great caution. The chosen panel of SNP is a very small representation of the genetic variation, with potential for modifying the effects of VAS. It would also be relevant to investigate the impact of known SNP in genes related to vitamin A transport (*RBP4*) and metabolism such as retinaldehyde dehydrogenase (*ALDH1A1*/*Raldh1*) and alcohol dehydrogenase (*ADH1*).

Given the substantial volume of literature reporting effects of the chosen SNP, we found surprisingly modest effects. However, the infant and the adult immune system differ, and cytokine promoter SNP could have age-differential effects. This was shown for the *IL-6* −174 SNP, which affected neonatal IL-6 production but had no effect among adults[8]. On the other hand, a recent study reported no effects of genotype on plasma cytokine concentrations of healthy young men, neither at baseline nor after *in vivo* LPS treatment, investigating a similar panel of SNP[9]. Potentially, the investigated SNP has little effect after stimulation, perhaps due to redundancy in regulatory mechanisms, but publication bias may also inflate the perceived effects. In addition, it should be considered that *in vitro* results, at least regarding the effects of *TNF-α* −308 and *IL-10* −592, have been inconsistent[10,11], stressing the need for clarification of the exact mechanism and impact of SNP *in vivo*.

We had found a lowered unstimulated TNF-α production after VAS, in line with previous reports of anti-inflammatory capacities of vitamin A. Interestingly, in the present study, it was the two investigated *TNF-α* SNP that interacted with VAS. Overall, the response to VAS was lower among *TNF-α* −308 G homozygotes, whereas there was no effect among A carriers. The interaction reached statistical significance for the unstimulated TNF-α production on day 3, but the same tendencies were seen for unstimulated IL-10 production on days 1 and 3 and unstimulated TNF-α production on day 1.

In parallel with the present finding, an interaction between *TNF-α* −308 genotype and effect of vitamin E supplementation on cytokine production was recently reported[12]. However, in that study, vitamin E supplementation was associated with decreased TNF-α response to LPS among A carriers.

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Table 1. Effect of genotype and vitamin A supplementation (VAS) on *in vitro* cytokine production† ‡
(Geometric mean ratios (GMR), risk ratios (RR) and 95 % confidence intervals)

	Effect of genotype		Effect of VAS major allele homozygote		Effect of VAS carriers of minor allele		
	GMR/RR	95 % CI	GMR/RR	95 % CI	GMR/RR	95 % CI	$P_{interaction}$ VAS × SNP
TNF-α −308 G → A (rs1800629) allele frequency − A 0·19							
TNF-α							
Control day 1	1·05	0·86, 1·28	0·76*	0·61, 0·95	0·95	0·67, 1·34	0·24
LPS	1·02	0·82, 1·26	0·79	0·62, 1·00	0·89	0·60, 1·32	0·43
Control day 3	0·97	0·76, 1·25	0·73*	0·56, 0·96	1·23	0·79, 1·92	0·04*
PHA	1·04	0·90, 1·20	1·07	0·90, 1·26	1·07	0·84, 1·38	0·89
PPD	0·94	0·79, 1·12	1·06	0·87, 1·29	1·09	0·78, 1·53	0·95
IL-10							
Control day 1	0·96	0·78, 1·20	0·77*	0·60, 0·99	0·99	0·68, 1·44	0·30
LPS	0·98	0·88, 1·09	0·98	0·86, 1·10	0·99	0·82, 1·19	0·80
Control day 3	0·92	0·72, 1·18	0·73*	0·56, 0·95	1·02	0·67, 1·56	0·20
PHA	1·06	0·95, 1·17	1·06	0·94, 1·19	0·99	0·83, 1·19	0·43
PPD	0·96	0·88, 1·05	0·96	0·87, 1·06	1·06	0·90, 1·26	0·22
TNF-α −238 G → A (rs361525) allele frequency − A 0·07							
TNF-α							
Control day 1	0·96	0·73, 1·26	0·80*	0·65, 0·97	1·01	0·59, 1·73	0·40
LPS	0·94	0·70, 1·25	0·85	0·68, 1·05	0·72	0·39, 1·31	0·57
Control day 3	0·89	0·64, 1·24	0·83	0·65, 1·07	1·01	0·51, 2·03	0·57
PHA	0·93	0·76, 1·14	1·01	0·87, 1·17	1·23	0·82, 1·86	0·30
PPD	0·93	0·74, 1·18	1·12	0·94, 1·34	0·71	0·43, 1·19	0·07
IL-10							
Control day 1	1·07	0·81, 1·42	0·82	0·65, 1·02	0·97	0·57, 1·63	0·56
LPS	1·16*	1·01, 1·34	0·99	0·89, 1·11	1·01	0·80, 1·27	0·93
Control day 3	1·11	0·83, 1·49	0·79	0·62, 1·02	0·92	0·53, 1·60	0·60
PHA	0·95	0·82, 1·09	1·01	0·91, 1·12	1·13	0·82, 1·56	0·46
PPD	1·13	1·00, 1·27	1·02	0·93, 1·11	0·94	0·75, 1·19	0·54
IL-10 −592 A → C (rs1800872) allele frequency − C 0·50							
TNF-α							
Control day 1	0·82	0·66, 1·02	0·79	0·54, 1·14	0·84	0·67, 1·04	0·79
LPS	1·05	0·83, 1·33	0·73	0·47, 1·15	0·87	0·69, 1·09	0·56
Control day 3	0·83	0·64, 1·08	0·89	0·54, 1·45	0·85	0·66, 1·11	0·80
PHA	1·13	0·96, 1·32	0·91	0·61, 1·35	1·10	0·97, 1·25	0·31
PPD	1·12	0·93, 1·36	0·99	0·65, 1·51	1·08	0·91, 1·28	0·83
IL-10							
Control day 1	0·90	0·72, 1·13	0·77	0·52, 1·15	0·86	0·67, 1·09	0·68
LPS	1·11	0·99, 1·25	1·05	0·88, 1·26	0·96	0·85, 1·08	0·41
Control day 3	0·88	0·69, 1·11	0·74	0·49, 1·12	0·84	0·64, 1·10	0·64
PHA	1·07	0·96, 1·20	0·94	0·74, 1·20	1·06	0·95, 1·17	0·32
PPD	1·08	0·98, 1·19	0·99	0·86, 1·15	1·00	0·90, 1·11	0·91

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Table 1. *Continued*

	Effect of genotype		Effect of VAS major allele homozygote		Effect of VAS carriers of minor allele		
	GMR/RR	95 % CI	GMR/RR	95 % CI	GMR/RR	95 % CI	$P_{interaction}$ VAS × SNP
IL-10 − 1082 A → G (rs1800896) allele frequency − G 0·33							
TNF-α							
Control day 1	0·82*	0·68, 0·99	0·86	0·66, 1·11	0·78	0·60, 1·01	0·61
LPS	1·15	0·94, 1·41	0·74	0·52, 1·04	0·95	0·76, 1·19	0·29
Control day 3	0·76*	0·60, 0·96	0·92	0·65, 1·31	0·79	0·58, 1·07	0·48
PHA	1·00	0·87, 1·15	1·06	0·84, 1·34	1·05	0·89, 1·22	0·77
PPD	1·03	0·87, 1·22	1·01	0·76, 1·35	1·10	0·91, 1·34	0·84
IL-10							
Control day 1	0·92	0·75, 1·13	0·86	0·64, 1·15	0·80	0·60, 1·09	0·75
LPS	1·03	0·93, 1·14	0·99	0·87, 1·13	0·98	0·85, 1·14	0·99
Control day 3	0·89	0·71, 1·11	0·84	0·62, 1·15	0·76	0·54, 1·07	0·65
PHA	1·04	0·95, 1·15	1·00	0·84, 1·17	1·06	0·94, 1·18	0·55
PPD	1·03	0·94, 1·12	1·00	0·89, 1·11	1·01	0·89, 1·14	0·84
TLR4 + 896 A → G (rs4986790) allele frequency − G 0·09							
TNF-α							
Control day 1	0·95	0·74, 1·22	0·86	0·70, 1·05	0·64	0·38, 1·10	0·33
LPS	1·07	0·82, 1·39	0·83	0·66, 1·03	0·81	0·51, 1·28	0·87
Control day 3	1·10	0·82, 1·49	0·85	0·66, 1·10	0·93	0·50, 1·71	0·78
PPD	1·25*	1·00, 1·55	1·06	0·87, 1·28	1·02	0·73, 1·43	0·91
IL-10							
Control day 1	0·94	0·71, 1·24	0·86	0·69, 1·08	0·66	0·37, 1·20	0·49
LPS	0·88	0·78, 1·01	0·97	0·87, 1·08	1·06	0·81, 1·39	0·63
Control day 3	0·97	0·72, 1·29	0·84	0·66, 1·08	0·61	0·33, 1·16	0·42
PPD	0·92	0·83, 1·03	1·01	0·92, 1·11	0·94	0·74, 1·20	0·57
IL-5							
Control day 3	1·62	0·83, 3·15	0·87	0·42, 1·79	2·33	0·77, 6·98	0·14
PPD	1·18	0·97, 1·43	1·05	0·89, 1·24	1·20	0·82, 1·76	0·56
IL-13							
Control day 3	1·16	0·84, 1·60	0·99	0·73, 1·36	1·50	0·86, 2·63	0·21
PPD	1·16	1·00, 1·35	1·06	0·93, 1·21	1·15	0·85, 1·55	0·57

LPS, lipopolysaccharide; PHA, phytohaemagglutinin; PPD, purified protein derivative of *Mycobacterium tuberculosis*; TLR, toll-like receptor.
* Findings were statistically significant (P=0·05).
† Cytokine responses were compared in linear regression models of the log-transformed values for cytokines, for which less than one-third had non-detectable concentrations. Regression coefficients and CI were back-transformed providing GMR of: (1) minor allele carriers relative to major allelle homozygotes; (2) VAS relative to placebo. For stimulants in which cytokine responses had less than one-third detectable samples, responses were compared in Poisson regression models with robust variance estimation.
‡ Allele frequency for *TLR4* + 1196 was 0·003 for the T allele. This did not leave enough carriers of this allele for meaningful interpretation, and analysis was thus abandoned for this genotype.

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The authors speculated whether an anti-inflammatory effect of vitamin E is only visible among people who are predisposed to increased inflammation. A similar speculation from the present study could be that VAS primarily exerts anti-inflammatory effects in children who are not predisposed to inflammation. The high carrier frequency for *TNF*-α −308 A of 33 % makes the present results noteworthy, as a genotype differential response to VAS could give rise to measurable differential morbidity and mortality effects of VAS within the genotypes.

In the present study, the interactions were most pronounced for boys and there was a borderline-significant three-way interaction between sex, *TLR4* + 896 genotype and VAS for unstimulated TNF-α production. We speculate whether differences in the steady-state inflammatory level are a key to the observed sex differences in the mortality response to VAS. We hope it will encourage others to conduct analyses stratified by sex.

Conclusions

VAS at birth reduced unstimulated TNF-α production. The investigated SNP *per se* had only moderate effects on cytokine production in a whole blood culture assay. However, the present results indicate that sex and SNP genotype together modified the effect of VAS on cytokine production.

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