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Sex-differential non-vaccine specific immunological effects of diphtheria-tetanus-pertussis and measles vaccination

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Summary:
This study investigates the non-specific immunological effects of DTP and measles vaccination of healthy African infants. The results indicate sex-differential effects on both innate and adaptive immunity, providing the first mechanistic insights into how these vaccines alter infectious disease susceptibility.

ABSTRACT
Vaccines can have non-targeted heterologous effects which manifest as increased protection against non-vaccine infections, as described for measles vaccine (MV); or increased susceptibility to infections and death, as described following diphtheria-tetanus-whole cell pertussis (DTP) vaccination. The mechanisms are unknown and high quality immunological studies are lacking. This study was designed to investigate the heterologous effects of MV and DTP in 302 Gambian infants. The results support an immunosuppressive effect of DTP on innate pro-inflammatory responses and T cell immunity in females. Males but not females receiving MV had enhanced pro-inflammatory innate responses. The results point to modified signalling via Toll-like receptor (TLR4) as a possible mechanism for the effects on innate immunity. When both vaccines were administered together, PPD responses were enhanced in females but down-regulated in males. Collectively these data indicate immunological effects that could account for heterologous effects of MV and DTP, to take forward into prospective trials.
INTRODUCTION

Background

Multiple epidemiological studies have described heterologous effects of vaccines on infectious disease susceptibility and all-cause mortality [1-4]. Bacille Calmette-Guérin (BCG) vaccinated low-birth-weight neonates had a 45% lower all-cause mortality compared to unvaccinated controls in a randomized trial conducted in Guinea Bissau [5]; and an additional early measles vaccine (MV) at 4.5 months was associated with reduced mortality in the absence of measles infections in a randomized trial in Guinea-Bissau [6]. A recent population-level data analysis suggested that MV protects against all-cause mortality by preventing prolonged immunosuppression caused by wild type measles infection [7]. However, if true, then censoring for measles infection should prevent beneficial effects of MV, which is not the case in numerous epidemiological studies [8].

A review of all available studies where mortality was assessed in the context of diphtheria-tetanus-whole cell pertussis (DTP) vaccination suggests that DTP is associated with increased mortality, particularly in females [9]. Several observational studies suggest that receiving DTP with MV is associated with higher mortality compared to receiving MV only, with males generally more susceptible [10, 11].

A systematic review for non-specific effects of vaccines commissioned by WHO concluded that the epidemiological evidence supports beneficial heterologous effects of MV; but found insufficient evidence to confirm or refute deleterious heterologous effects of DTP [12], although the analytical principles used have been questioned.
The need to explore and understand the immunological mechanisms was emphasized in the WHO review.

We used state-of-the-art methodology to elucidate the immunological mechanisms that might account for beneficial effects of MV and deleterious effects of DTP in a prospective randomized trial, and further analysed the effect of administering both vaccines simultaneously.

METHODS

Study Subjects

Infants attending for routine vaccination were recruited at four months of age at Sukuta Health Centre, a peri-urban area 20 km from the coast of The Gambia. Eligibility criteria included being healthy, apyrexial (<37.5°C), normal weight-for-age, and all recommended vaccines received to date. The study was approved by the Joint Gambia Government/MRC Ethics Committee (project number SCC1085). Written informed consent was provided by the parent/guardian of the child.

Study Design

Four month old infants were block randomized to one of three vaccine groups in a prospective unblinded study. At 4 months of age group 1 received their third dose of diphtheria-tetanus-whole cell pertussis (DTP), hepatitis B vaccine (HBV) and oral polio vaccine (OPV); and groups 2 and 3 received HBV and OPV only. At nine months of age group 1 received a single standard intramuscular (i.m.) dose of measles vaccine (MV) (Edmonston Zagreb strain, Serum Institute of India Ltd., Pune, India); group 2 received MV and i.m. DTP (Serum Institute of India Ltd.); and group 3
received DTP alone. Outstanding vaccines were administered at 11 months of age (Supplementary Table 1). Venous blood was collected at nine months immediately before vaccination and 4 weeks later: 4.5mLs into a heparinized tube (7.5 units heparin/mL); 0.5mL into a Paxgene™ tube (Qiagen, Crawley, UK).

Whole blood cultures

Heparinized whole blood was cultured in 100μL aliquots in 96-well U-bottom plates with tetanus toxoid (TT) (10μg/ml, Sanofi Pasteur, France); purified protein derivative (PPD) (10μg/mL, Statens Serum Institute, Denmark); a measles peptide-pool of 122 15mer peptides overlapping by 10 amino acids spanning measles protein haemagglutinin (all 1μg/mL final concentration, Sigma-Genosys, UK); heat-killed listeria monocytogenes (HKLM) (10⁹ cells/mL) (TLR2 agonist); E. coli K12 lipopolysaccharide protein S (LPS) (1μg/mL) (TLR4 agonist); flagellin (10μg/mL) (TLR5 agonist); and CLO-75 (10μg/mL) (TLR7/8 agonist) (all from InvivoGen, San Diego, USA). Anti-CD3 (αCD3) (5μg/mL, Becton-Dickinson (BD), USA) and anti-CD28 (αCD28) (5μg/mL, eBiosciences, UK) were used as a general T cell stimulus, and medium alone was the negative control. Plates were incubated for 16 hours at 37°C, 5% CO₂, centrifuged at 2,000 rpm for 5 minutes and 50μL supernatant collected and stored at -20°C.

Multiplex cytokine assays for plasma and culture supernatants

The Bio-Plex 200 Suspension Array system (Bio-Rad, Hercules, California, USA) was used to analyze cytokines according to the manufacturer’s instructions (Bio-Rad, Belgium). A human 27-plex array (Bio-plex Pro Human Cytokine 27-plex Immunoassay for fibroblast growth factor basic [FGF basic], eotaxin, granulocyte-
colony-stimulating factor [G-CSF], granulocyte macrophage-CSF [GM-CSF], interferon-gamma [IFN-γ], interleukin-1beta [IL-1β], IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, interferon-gamma-inducible protein-10 [IP-10], melanocortin receptor-1 [MCR-1], macrophage inflammatory protein-1 alpha [MIP-1α], MIP-1β, platelet derived growth factor-BB [PDGF-BB], regulated on activation, normal T cell expressed and secreted [RANTES], tumour necrosis factor [TNF], vascular endothelial growth factor [VEGF]) or a customized 10-plex array (IL-1β, IL-4, IFN-γ, IL-10, IL-12(p70), eotaxin, GM-CSF, PDGF-BB, TNF, VEGF) was used to analyse cytokines in plasma samples (n=214 infants), and cytokines in cultures with vaccine related (TT, Measles Pool) and non-related (PPD, αCD3/αCD28) antigens (n=192 infants). For TLR agonist stimulations, a 5-plex array (IL-1β, IL-6, IL-10, IL-12(p70), TNF) was used (n=122 infants). Assays were not performed for all infants due to insufficient blood volume, clotted or contaminated blood samples. Medium background values were subtracted from antigen-stimulated values to establish net cytokine production. Cytokine ratios were analysed without background correction.

MV and DTP vaccine antibodies
A multiplex microsphere based fluorescent immunoassay for diphtheria toxoid (Dtx), tetanus toxoid (Ttx) and pertussis toxoid (Ptx) IgG antibodies was performed at the National Institute of Public Health and the Environment (RIVM), Netherlands using published protocols [14] (n=284 infants). The measles IgG haemagglutination inhibition assay (HAI) was performed using monkey red blood cells as previously described [15] (n=283 infants).
**Statistical analysis of antibody and cytokine data**

Cytokine and antibody variables were analysed by fitting a linear mixed model using restricted maximum likelihood. Bleed, group and sex, and their interactions were fitted as fixed effects and infant, and sample within infant, were fitted as random effects. The F-test was used to test for 2-way group-by-bleed and 3-way sex-by-group-by-bleed interactions. Comparisons between treatment groups, and sexes within treatment groups, at each time point, were based on t-tests utilizing the predicted means and standard errors of differences recovered from the fitted mixed model. Comparisons were conducted at 5% significance with no adjustments for multiple comparisons. Cytokine and antibody data required logarithmic transformation prior to analysis. Data were analysed using GenStat 17 (VSN International), R 3.1.2 (www.r-project.org) and Stata version 12.1 (StataCorp LP, USA).

**Whole blood RNA microarray analysis**

RNA was extracted from Paxgene™ tubes using PAXgene™ blood RNA extraction kits (Qiagen, Crawley, UK) according to manufacturer’s instructions. 100ng RNA was used to generate labeled cRNA using the Ambion Illumina TotalPrep-96 RNA Amplification Kit. cRNA was hybridized to Human HT-12 V3 microarrays (Illumina) comprising 47,293 features. 348 RNA samples from 183 infants (165 infants with paired samples) were deemed of sufficient quality for analysis using the array Quality Metrics package in Bioconductor [16]. Raw data were transformed using a variance stabilizing transformation (VST) prior to normalization across all arrays using the robust spline normalization (RSN) method. Eighteen manually chosen comparisons for differentially expressed genes were undertaken using an empirical Bayesian
approach (post- compared to pre- for each vaccine group, for all donors and by sex, for unpaired and paired data). The Benjamini-Hochberg p value adjustment for multiple testing was implemented in limma software package for R, to control for false discovery rate. The resulting gene list was fully annotated and sorted in order of decreasing significance. Functional enrichment analysis was performed using KEGG pathway (www.genome.jp/kegg/) membership (raw p<0.01, enrichment p<0.05), and Gene Ontology (GO) terms (www.geneontology.org) (raw p<0.01, enrichment p<0.001). Networks were laid out for all relationships exhibiting a Pearson correlation greater than 0.75. MCL clustering of nodes was undertaken (expansion value 1.7), and functional analysis of each cluster performed using public and proprietary databases. Expression networks were constructed employing the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com).

RESULTS

Cohort characteristics

302 children were randomized at four months of age into vaccine groups (Fig. 1). Ethnic mix was comparable in the 3 vaccine groups (Table 1). Weight-for-age z scores, haemoglobin and platelet counts were comparable in all groups and sexes (Table 1). MV+DTP males had significantly higher total white cell counts and significantly lower lymphocyte counts after vaccination compared to other groups, although 3-way interactions were not significant. Local HIV seroprevalence [17] and helminth infection rates were low [18] and unlikely to be significant confounders.
Vaccine IgG levels

The expected induction of IgG antibodies to measles occurred in the MV and MV+DTP groups and not the DTP group (Fig. 2A and B). Similarly, diphtheria, tetanus and pertussis toxoid responses were boosted in the DTP and MV+DTP groups, but not the MV group (Fig. 2C-H). Females had higher Ptx antibody levels than males post-vaccination (p=0.01) (Figs. 2G and H), but there were no other sex differences.

Plasma cytokine profiles post-vaccination

Plasma cytokine group differences were found for TNF:IL-10, IFN-γ:IL-10, IL-7, PDGF and IP-10 (Fig. 3). The DTP group had significantly lower IL-7 than the MV group (p=0.029) (Fig. 4A). The DTP group had lower TNF:IL-10 than the other vaccine groups whether for all donors combined, males or females (Fig. 4B). IFN-γ:IL-10 ratios were also lower in DTP males and females, with high levels in MV+DTP females (Fig. 4C). MV males had higher TNF:IL-10 and IFN-γ:IL-10 (p=0.039) than MV females (p=0.011). DTP females had lower IP-10 than DTP males (p=0.037) and MV females (p=0.021) (Fig. 4D). PDGF levels were generally higher in the DTP vaccinated group, and MV females (Fig. 4E).

Toll-like receptor agonist responses

Cytokine responses were tested to TLR2, 4, 5 and 7/8 in vitro stimulation, but only TLR4 (LPS) responses changed following vaccination. The DTP group had lower post-vaccination TNF production following LPS stimulation than the MV group (p=0.049) (Figs. 5A). The MV males but not females had increased TNF (p=0.003) and increased TNF:IL-10 (p=0.008) in LPS cultures post- compared to pre-vaccination (Fig. 5B and 5C). In addition, LPS stimulated TNF:IL-10 was
significantly higher in MV males compared to both DTP and MV+DTP males at 10 months (p=0.013 and 0.021, respectively) (Fig. 5C), but this was not observed in females.

Vaccine-specific cellular responses

Priming of measles-specific cellular responses occurred in the MV and MV+DTP but not DTP groups, with upregulation of IFN-\(\gamma\):IL-10 and IFN-\(\gamma\):IL-4 to the measles peptide pool post-vaccination (Fig. 6A). TT reactivity was low and not boosted in the DTP vaccinated infants for any of the cytokines tested (not shown). There was no evidence of any sex interaction in measles or tetanus specific in vitro reactivity.

Cytokine responses to T cell stimulation

Broad T cell reactivity was assessed by \(\alpha\)CD3/\(\alpha\)CD28 stimulation. Levels of \(\alpha\)CD3/\(\alpha\)CD28 stimulated eotaxin declined significantly in the MV+DTP group post-vaccination (p<0.001); and post-vaccination levels of eotaxin were lower in this group than the MV (p=0.001) and the DTP groups (p=0.009) (Fig. 6B).

DTP vaccinated females had significantly lower IL-12(p70) levels than males post-vaccination (p=0.022) but not pre-vaccination (p=0.626) (Fig. 6C). Anti-CD3/\(\alpha\)CD28 stimulated IFN-\(\gamma\):IL-4 declined significantly in the DTP group post-vaccination (p=0.029), and post-vaccination levels were lower in the DTP group compared to the MV (p=0.008) and MV+DTP groups (p=0.005) (Fig. 6D). The MV+DTP males had significantly increased \(\alpha\)CD3/\(\alpha\)CD28 stimulated IFN-\(\gamma\):IL-4 post-vaccination (p=0.042) (Fig. 6E); and DTP vaccinated females had significantly lower IFN-\(\gamma\):IL-4 than males at 10 months (p=0.002) but not baseline (p=0.451) (Fig. 6F).
Reactivity to the unrelated antigen PPD

Significant sex-by-bleed-by-group interactions were found for TNF (p=0.001), eotaxin (p=0.004) and IL-10 (p=0.032) following PPD stimulation. For all 3 cytokines the PPD responses were equivalent in males and females at baseline, but increased significantly in MV+DTP females from pre to post-vaccination (p=0.048, 0.02 and 0.043, respectively) (Figs. 7A, C, E), while decreasing in MV+DTP males (p=0.002, 0.024 and 0.016, respectively) (Figs. 7B, D, F). MV males had significantly increased TNF following PPD stimulation post compared to pre-vaccination (p=0.019) (Fig. 7B) whereas females did not (Fig. 7A).

Differential whole human genome RNA expression profiles

Ex-vivo gene expression profiles were analysed to gain further insights into mechanisms of heterologous effects of MV and DTP. While a series of significantly differentially expressed genes were identified (Fig. 8A), after multiple test adjustment only 2 showed statistically significant genes (MV females paired and unpaired - 1 probe of unknown function). Therefore, a less stringent explorative approach accepting a high rate of false discovery was taken. Genes were filtered by fold-change expression post-vaccination compared to pre-vaccination for each group for paired and unpaired data for all donors and males and females separately identifying 70 differentially expressed probes with ≥1.5 fold (up- or down-regulated) in one or more groups (Supplementary Table 2). There was no differential expression ≥1.5 fold unless vaccine treatment groups were separated by sex. Heat maps of the 70 differentially expressed probes were generated, and clustered by genes and conditions using Pearson’s dissimilarity (Fig. 8B).
There were 29 probes ≥1.5 fold down-regulated in DTP vaccinated females, the majority being type 1 interferon stimulated genes involved in pattern recognition of pathogens and interferon signalling (Table 2). Ingenuity Pathway Analysis suggested down-regulated interferon signalling and dendritic cell (DC) maturation pathways (Fig. 9A), and the gene ontology terms implicated were recognition of viruses and bacteria. By contrast, DTP vaccinated males had 18 probes ≥1.5 fold up-regulated (Table 2). One major network was produced consisting of up-regulated functions associated with developmental pathways, RNA transcription complex and post-transcriptional modification pathways (Fig. 9B). The top canonical pathway functions were up-regulated assembly of RNA polymerase II complex, T-cell receptor signalling and protein kinase A signalling; the major associated functions being genes involved in developmental and repair functions (Table 2).

While DTP vaccinated males had exclusively up-regulated genes, males vaccinated with MV+DTP had only down-regulated genes (Table 2, Fig. 9C). The top canonical pathway associated functions were for down-regulated amino acid metabolism and signalling processes. There were insufficient differentially expressed genes among MV males and females and MV+DTP females to perform pathway analysis.

**DISCUSSION**

This is the first longitudinal randomized trial designed specifically to study for heterologous effects of vaccination with MV, DTP or both together. Lower post-vaccination pro-inflammatory responses to TLR4 stimulation in DTP vaccinated infants implies impaired innate immunity to gram-negative bacteria, whereas responses
to TLR2, TLR5 and TLR7/8 were unaffected. BCG vaccination enhances TLR4 reactivity via epigenetic upregulation of NOD2 activity on macrophages, which in turn increases pro-inflammatory responses to unrelated pathogens [19]. Therefore a plausible mechanism for DTP-induced TLR4 inhibitory effects is via epigenetic modulation. The tentative down-regulation of predominantly type 1 interferon genes in DTP vaccinated females but not males further supports this theory, since the STAT1 pathway, which is strongly dependent on type 1 interferons, is a pivotal mechanism for ‘trained immunity’ leading to altered innate memory [20].

DTP vaccinated females also had suppressed T cell reactivity, as evidenced by decreased αCD3/αCD28 stimulated type 1 cytokines following vaccination. Vaccines may influence T cell responses to unrelated pathogens via induction of cross-reactive T cells that can either be protective or harmful [21]; but suppression of reactivity to anti-CD3/anti-CD28 stimulation suggests a more generalized suppression of T cell immunity. Lower plasma IL-7 in DTP vaccinated infants, a haematopoietic growth factor that plays an important role in T and B cell development and survival [22], could further cause impaired cellular immunity.

These broad immunosuppressive effects of DTP vaccination, particularly in females, could account for increased susceptibility to infections and all-cause mortality following DTP [9, 13]. That these effects were not significant when MV was given with DTP suggests co-administration of the live measles vaccine modifies the immunosuppressive effects of DTP.
Measles vaccination provides beneficial heterologous effects, particularly in females, via unknown mechanisms [6, 8]. In our study, the major MV effect was in males who had more pro-inflammatory plasma and enhanced TLR4 reactivity following vaccination. Therefore modified TLR4 reactivity is major candidate mechanism for heterologous effects of both MV and DTP. Plasma IP-10 was higher in MV females, a Th1 biasing chemokine with a role in effector T cell trafficking [24], but also known to predict severity of a number of inflammatory diseases [25].

Reactivity to the mycobacterial antigen PPD can be due to BCG vaccination and priming by environmental mycobacteria [26]. Enhanced PPD responses in MV+DTP females, and down-regulated in MV+DTP males, suggest that when the vaccines are given together, females immunity becomes more reactive, while males become less so. In keeping with this was down-regulation of several cellular proliferation and growth genes in MV+DTP vaccinated males but not females. MV+DTP males also had higher WBC and lower lymphocytes than other groups post-vaccination, suggesting a bone marrow effect. Negative effects in males concurs with epidemiological data suggesting males may be more susceptible than females to adverse heterologous effects following administration of MV with DTP [10, 11].

Our results highlight the importance of analysing for sex interactions. Males and females are immunologically skewed in opposite directions due to sex hormones, most evident during puberty, but also in infancy [27, 28]. The X-chromosome contains numerous immune response genes and micro-RNAs contributing to immunological sex differences [29, 30].
Several limitations should be noted. Samples were taken at a single time point post-vaccination, and immune dynamics may differ in the sexes. Plasma was only analysed at 10 months but not baseline. The MV group received an additional DTP at four months of age compared to the other two groups. Differentially expressed genes did not reach statistical significance after correction for multiple testing and a less stringent analysis approach was taken.

Our data provide further evidence that vaccines educate both innate and adaptive immunity in previously unsuspected ways [2, 21]. We have identified plausible immunological effects at the protein level underscoring the heterologous effects of MV and DTP vaccines and their sex-differential nature, and differentially expressed genes that might be explored as targets of these vaccine effects. The epigenetic effects of DTP and MV on TLR4 signalling should be explored to investigate for negative and positive innate ‘immune training’ effects. It will also be crucial to investigate the mechanism of suppressed T cell signalling in DTP vaccinated females. The effects of hormones and X-linked immune response genes could also be explored. The public health implications of understanding these vaccine effects are enormous since they could be exploited in future vaccine design. Furthermore, minor changes to vaccine schedules could maximize beneficial and minimize deleterious effects of vaccines [32].

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Potential conflicts of interest
None
References


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Figure Legends

Figure 1 Participant numbers and drop outs
302 infants were randomized at 4 months of age to vaccine groups, 16 of whom dropped out of the study before the first bleed at 9 months of age. Adequate blood samples were obtained from 273 of the 286 vaccinated infants (95.5%) prior to vaccination at 9 months of age, and 254 (93%) at 10 months of age. Numbers tested in each assay in the different vaccine groups are indicated. Blood volumes were often limited and not all assays could be performed on each sample.

Figure 2 Measles and DTP antibody titres before and after vaccination in females and males
Measles (in log₂ HAI titres), diphtheria toxoid (Dtx, IU / mL), tetanus toxoid (Ttx, IU / mL), and pertussis toxoid (Ptx, EU / mL) IgG levels for males (left panels A, C, E, G) and females (right panels B, D, F, H). Mean values are indicated by the horizontal bar. *** indicates p<0.001 from the fitted linear mixed model of log transformed mean antibody values for Dtx, Ttx and Ptx and the logit for measles HAI. HAI = haemagglutination inhibition assay. The horizontal dotted line indicates protective Ab titre cutoff, but there is no accepted protective level for Ptx titres (results are shown for n=283 infants for MV Abs and n=284 infants for DTP Abs).
Figure 3  Summary of vaccine group differences in post-vaccination plasma cytokine levels

The figure summarizes where significant differences were found in post-vaccination plasma cytokine levels between vaccine groups for all donors combined (All), males (M) and females (F) showing that for IL-7, TNF:IL-10, IFN-γ:IL-10 and IP-10 the DTP group generally had lower levels than other vaccine groups, whereas for PDGF the DTP group generally had higher levels.

Figure 4 Plasma cytokine levels at 10 months of age

Plasma cytokine levels were measured at 10 months of age, 4 weeks after the study vaccines were administered. No baseline analysis was performed. The figure shows results for IL-7 (A) and TNF:IL-10 ratios (B), IFN-γ:IL-10 ratios (C), IP-10 (D), and PDGF (E). A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group and sex. * p<0.05, ** p<0.01. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=214 infants).

Figure 5 Cytokine responses in vitro to TLR agonist stimulation

Infant whole blood was cultured overnight at 9 and 10 months with TLR2, 4, 5 and 7/8 agonists. Only pro-inflammatory responses to LPS (TLR4 agonist) were altered following vaccination: the TNF response to LPS in all donors combined (A); and the TNF (B) and TNF:IL-10 ratios (C) in males only. A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group, bleed and sex. * p<0.05, ** p<0.01. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=122 infants).
Figure 6 Cytokine responses to measles peptides and αCD3/αCD28 stimulation in vitro

Infant whole blood was cultured overnight at 9 and 10 months with a measles peptide pool or the general T cell stimulus αCD3/αCD28 and cytokines measured in culture supernatants. Measles-specific responses were stimulated in the MV and MV+DTP groups, but not the DTP alone group (A). A significant group-by-bleed interaction was found for eotaxin responses to αCD3/αCD28 (B); and there were significant sex-by-group-by-bleed interactions in αCD3/αCD28 cultures for IL-12(p70) (C) and IFN-γ:IL-4 ratios (D-F). A fitted linear mixed model of log transformed cytokine values was used to determine significant differences by vaccine group, bleed and sex. *p<0.05, **p<0.01, ***p<0.001. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=192 infants).

Figure 7 Cytokine responses to PPD stimulation in vitro

Infant whole blood was cultured overnight at 9 and 10 months with the recall antigen PPD and cytokines measured in culture supernatants. There was a significant group-by-sex-by-bleed interaction for raw TNF (A, B), IL-10 (C, D), and eotaxin (E, F). Female responses are shown in the right hand panels and male responses on the left. Asterisks indicate a significant interaction from the fitted linear mixed model of log transformed cytokine values, *p<0.05, **p<0.01. The height of the bar indicates the mean value, the error bars indicate SEM (results are shown for n=192 infants).
Figure 8 Analysis for differentially expressed probes in the vaccine groups

(A) A significance landscape was plotted for the 5,915 non-redundant significant genes (x-axis) by comparisons (y-axis). The number of significantly altered genes in each comparison are shown in parentheses after the comparison description. Comparisons with the highest number of significant changes are shown at the bottom. F = females, M = males, P = paired analysis, where no M or F are shown the analysis is for males and females combined, where no P is shown the analysis is for unpaired data. (B) Hierarchical clustering of the 70 probes with ≥1.5-fold differential expression post-vaccination compared to pre-vaccination organized by vaccine group (left hand y-axis) where purple indicates females and males combined, pink indicates females, and pale blue indicates males. Pairs of data are shown for each group – the upper set representing paired data (P) and the lower set shows unpaired (U) data from all donors. Genes are clustered by Pearson dissimilarity as shown above, values are displayed as log2 fold change in expression. For both panels red = up-regulated, blue = down-regulated, grey = non-significant or no change. Results shown are for n=183 infants.

Figure 9 Networks of differentially expressed genes in males and females in the DTP and MV+DTP groups

Differentially expressed genes and their associated pathways following DTP vaccination of infant females (A) or males (B), and MV+DTP vaccination of males (C). Networks were generated using Ingenuity Pathway Analysis software. Green nodes indicate down-regulated genes, and red nodes indicate up-regulated genes. There were insufficient differentially expressed genes to generate pathways for the MV males and females, and the MV+DTP females.
Assessed for eligibility (n=368)

Excluded (n=66)
- Not meeting inclusion criteria (n=21)
- Declined to participate (n=4)
- Other reasons (n=41)

Randomized (n=302)

Allocated to MV at 4m (n=106)
- Received allocated intervention at 9m (n=101)
  - Did not receive allocated intervention (n=5)
    - Protocol deviation (n=0)
    - Declined to participate (n=3)
    - Died (n=2)
  - Other (n=2)

Allocated to MV+DTP at 4m (n=115)
- Received allocated intervention at 9m (n=111)
  - Did not receive allocated intervention (n=4)
    - Protocol deviation (n=0)
    - Declined to participate (n=0)
    - Died (n=1)
    - Other (n=3)
  - Other (n=3)

Allocated to DTP at 4m (n=81)
- Received allocated intervention at 9m (n=74)
  - Did not receive allocated intervention (n=7)
    - Protocol deviation (n=0)
    - Declined to participate (n=1)
    - Died (n=0)
    - Other (n=6)

Lost to follow-up at 10m (n=12)
- Protocol deviation (n=0)
- Declined to participate (n=4)
- Died (n=0)
- Other (n=8)

Analysed for vaccine antibodies (n=100); Plasma cytokines (n=77); 10-plex cultures (n=65); 5-plex cultures (n=44); Transcriptome (n=64)
- Excluded from analysis if insufficient blood available, clotted specimen, culture contamination, poor quality RNA

Analysed for vaccine antibodies (n=111); Plasma cytokines (n=77); 10-plex cultures (n=69); 5-plex cultures (n=47); Transcriptome (n=82)
- Excluded from analysis if insufficient blood available, clotted specimen, culture contamination, poor quality RNA

Analysed for vaccine antibodies (n=74); Plasma cytokines (n=53); 10-plex cultures (n=58); 5-plex cultures (n=31); Transcriptome (n=57)
- Excluded from analysis if insufficient blood available, clotted specimen, culture contamination, poor quality RNA
A) Measles Abs Males

B) Measles Abs Females

C) Dtx Abs Males

D) Dtx Abs Females

E) Ttx Abs Males

F) Ttx Abs Females

G) Ptx Abs Males

H) Ptx Abs Females
### Table 1

Cohort Characteristics and Infant Numbers

<table>
<thead>
<tr>
<th></th>
<th>MV Group</th>
<th>MV+DTwP Group</th>
<th>DTwP Group</th>
<th>Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>No. randomised at 4 months</td>
<td>48</td>
<td>58</td>
<td>56</td>
<td>59</td>
</tr>
<tr>
<td>No. infants bled at 9 months</td>
<td>42</td>
<td>55</td>
<td>51</td>
<td>53</td>
</tr>
<tr>
<td>No. infants bled at 10 months</td>
<td>36</td>
<td>53</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>Weight-for-age z-score at 4 mths</td>
<td>(-0.56-0.72)</td>
<td>(-0.46-1.00)</td>
<td>(-0.71-0.92)</td>
<td>(-0.63-0.8)</td>
</tr>
<tr>
<td>Ethnicity of mother</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandinka</td>
<td>38 (79.2%)</td>
<td>42 (72.4%)</td>
<td>47 (83.9%)</td>
<td>41 (69.5%)</td>
</tr>
<tr>
<td>Wolof</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Fula</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Jola</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>10.2 (9.3-10.8)</td>
<td>10.2 (9.2-11.1)</td>
<td>9.9 (8.7-10.9)</td>
<td>9.8 (8.9-10.9)</td>
</tr>
<tr>
<td>10 months</td>
<td>10.3 (8.9-10.8)</td>
<td>9.6 (8.6-10.7)</td>
<td>9.5 (8.5-10.2)</td>
<td>9.4 (8.5-10.1)</td>
</tr>
<tr>
<td>White blood cells (x10^3/μL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>8.4</td>
<td>7.8</td>
<td>8.8</td>
<td>8.7</td>
</tr>
<tr>
<td>10 months</td>
<td>8.0</td>
<td>9.3</td>
<td>9.0</td>
<td>11.3</td>
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<tr>
<td>Lymphocytes (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>63.4</td>
<td>61.1</td>
<td>64.8</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td>66.2</td>
<td>63.7</td>
<td>65.5</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>10 months</td>
<td>9 months</td>
<td>10 months</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>----------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td><strong>Platelets (x10⁹/L)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 months</td>
<td>(61.7-73.9)</td>
<td>(59.2-70.5)</td>
<td>(46.8-66.4)*</td>
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</tr>
<tr>
<td></td>
<td>165 (104-265)</td>
<td>141 (103-251)</td>
<td>197 (135-272)</td>
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<tr>
<td>10 months</td>
<td>(56.8-67.7)</td>
<td>(56.8-67.7)</td>
<td>(51.6-71.2)</td>
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</tr>
<tr>
<td></td>
<td>163 (108-279)</td>
<td>162 (106-234)</td>
<td>153 (123-206)</td>
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</tr>
<tr>
<td></td>
<td>(59.2-70.5)</td>
<td>(59.2-70.5)</td>
<td>(61.9-68.4)</td>
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<tr>
<td></td>
<td>178 (144-265)</td>
<td>208 (122-263)</td>
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<tr>
<td>No. infants tested in the study assays</td>
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<tr>
<td>Measles antibodies</td>
<td>44</td>
<td>56</td>
<td>55</td>
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<tr>
<td>DTP antibodies</td>
<td>45</td>
<td>55</td>
<td>56</td>
<td></td>
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<tr>
<td>Plasma cytokines</td>
<td>32</td>
<td>44</td>
<td>42</td>
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<tr>
<td>10-plex culture supernatants</td>
<td>30</td>
<td>35</td>
<td>35</td>
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<tr>
<td>5-plex culture supernatants</td>
<td>23</td>
<td>21</td>
<td>22</td>
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<tr>
<td>Transcriptome</td>
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</table>

Median and IQ range shown for weight and blood parameters. The donor numbers tested for some assays are higher than the numbers at each bleed because some infants attended for 1 bleed and not the other. The bold text indicates a significant difference in the indicated group compared to other groups.
<table>
<thead>
<tr>
<th>Abbrev</th>
<th>Gene Name</th>
<th>Pathway Description</th>
<th>Function</th>
<th>Fold Change</th>
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<tr>
<td></td>
<td>DTP Vaccinated Females</td>
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<tr>
<td>IFIT2</td>
<td>Interferon-induced protein with tetratricopeptide repeats 2</td>
<td>Interferon signaling</td>
<td>Cell-mediated immune response, Antiviral activity</td>
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<td>RSAD2</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
<td>Pattern Recognition Receptors in Recognition of Bacteria and Viruses</td>
<td>Cell-mediated immune response, Response to virus</td>
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<td>IFIT1</td>
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<td>Interferon Signaling</td>
<td>Antiviral defense, Innate immunity</td>
<td>-2</td>
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<tr>
<td>IFIT3</td>
<td>Interferon-induced protein with tetratricopeptide repeats 3</td>
<td>Interferon signaling</td>
<td>Antiviral defense, Innate immunity</td>
<td>-1.986</td>
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<tr>
<td>HERC5</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase 5</td>
<td>Protein modification; protein ubiquitination</td>
<td>Post-translational modification</td>
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<td>IFI44</td>
<td>Interferon-induced protein 44</td>
<td>Activation of IRF by Cytosolic Pattern Recognition Receptors RIG-I-like receptor signaling pathway; Interferon signaling</td>
<td>Response to virus</td>
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<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
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<td>Antiviral response</td>
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<td>OAS3</td>
<td>2'-5'-oligoadenylate synthetase 3</td>
<td>Interferon signaling</td>
<td>Antiviral response</td>
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<td>IFITM3</td>
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<td>IFI44L</td>
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<td>Interferon signaling</td>
<td>Immune response; defense response to virus</td>
<td>-1.828</td>
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<tr>
<td></td>
<td>DTP Vaccinated males</td>
<td></td>
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<td>ADD3</td>
<td>Adducin 3 (gamma)</td>
<td>Activation of cAMP-Dependent PKA</td>
<td>Hematological System Development and Function; Tissue Morphology</td>
<td>1.625</td>
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<td>Gene</td>
<td>Function/Pathway</td>
<td>Regulation</td>
<td></td>
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<td>JMJD1C</td>
<td>Jumonji domain containing 1C</td>
<td>Leukocyte Extravasation Signaling</td>
<td>Hematopoiesis 1.58</td>
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<td>MST4</td>
<td>Serine/threonine protein kinase MST4</td>
<td>Apoptotic pathway</td>
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<td>ANKRD10</td>
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<td>ZFAND5</td>
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<td>TNF-alpha/NF-kB Signaling Pathway; FoxO family signaling</td>
<td>N/A 1.558</td>
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<tr>
<td>DR1</td>
<td>Down-regulator of transcription 1, TBP-binding (negative cofactor 2)</td>
<td>Chromatin Regulation / Acetylation</td>
<td>Assembly of RNA Polymerase II Complex 1.548</td>
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<td>OSBPL8</td>
<td>Oxysterol binding protein-like 8</td>
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<tr>
<td>RASGRP1</td>
<td>RAS guanyl releasing protein 1 (calcium and DAG-regulated)</td>
<td>Gas Signaling, T cell receptor signaling pathway</td>
<td>Hematological System Development and Function; Cellular Development 1.548</td>
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<tr>
<td>PRNP</td>
<td>Prion protein</td>
<td>Prion diseases</td>
<td>Cellular Development; Cell Death and Survival 1.537</td>
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<tr>
<td>BNIP3L</td>
<td>BCL2/adenovirus E1B 19kDa interacting protein 3-like</td>
<td>Apoptosis</td>
<td>Cancer; Induces apoptosis 1.526</td>
<td></td>
</tr>
</tbody>
</table>

**SMOX**
- Spermine oxidase
- Amine and polyamine degradation; spermine degradation
- Regulation of polyamine intracellular concentration -1.647

**DPYS7L**
- Dihydropyrimidinase-like 5
- Axon guidance
- Neural development -1.636

**DVL1**
- Dishevelled, dsh homolog 1 (Drosophila)
- Wnt signaling pathway,
- Notch signaling pathway,
- Pathways in cancer
- Regulation of cell proliferation, Cancer, Dermatological conditions -1.613

**RNF213**
- Ring finger protein 213
- Protein modification; protein ubiquitination.
- Ubiquitin-protein ligase activity -1.58

**SPRYD3**
- SPRY domain containing 3
- N/A
- N/A -1.548

**TPRA1**
- Transmembrane protein, adipocyte associated 1
- G-protein coupled receptor signaling pathway
- Small Molecule Biochemistry -1.537
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Function</th>
<th>Score</th>
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<tbody>
<tr>
<td>MUC6</td>
<td>Mucin 6, oligomeric mucus/gel-forming</td>
<td>N/A</td>
<td>-1.526</td>
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<td>ARTN</td>
<td>Artemin</td>
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<tr>
<td>ADRA2C</td>
<td>Adrenergic, alpha-2C, receptor</td>
<td>Neuroactive ligand-receptor interaction</td>
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<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>Insulin receptor signaling pathway</td>
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</table>

*The top 10 differentially expressed genes are shown for each group*