Systematic Review of the Non-specific Immunological Effects of Selected Routine Childhood Immunizations

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Abbreviations

BCG	Bacillus Calmette–Guérin
CD	cluster of differentiation
CI	Confidence interval
CSF	Colony-stimulating factor
CVL	Central Veterinary Laboratory
DTP	diptheria-tetanus-pertussis
EGF	Epidermal growth factor
E-Z	Edmonston Zagreb
FGF	fibroblast growth factor
Flt-3L	Flt3-ligand
GM	geometric mean
GMR	geometric mean ratio
GRO	GRO protein (cytokine)
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPP	isopentenyl pyrophosphate
IQR	Inter-quartile range
LPS	Lipopolysaccharides
MCP	Monocyte chemoattractant protein
MDC	Monocyte depleted mononuclear cells
MFI	Mean fluorescence index
MIP	Macrophage inflammatory protein
MMR	Measles mumps and rubella
NSIE	Non-specific immunological effects
PBMC	Peripheral blood mononucleated cell
PDGF	Platelet-derived growth factor
pg	Picograms
PHA	Phytohaemagglutinin
PMA	phorbol myristate acetate
PPD	Purified protein derivitive
RCT	Randomized controlled trial
RPMI	Roswell Park Memorial Institute medium
SAGE	Strategic advisory group of experts
SEB	Staphylococcal enterotoxin B
SK/SD	Streptokinase/Streptodornase
SSI	Staten Serum Institut
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TT	Tetanus toxoid
UCL	University College London
UK	United Kingdom
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Executive Summary

There is clear scientific evidence that exposure to infectious agents and vaccines results in non-specific inflammatory and innate immunological responses that subsequently direct "acquired" specific immunity through T cells and antibody, that recognise antigenic epitopes on the organism or vaccine. This concept underpins modern immunology and vaccinology. Agents, including infectious diseases, which modify these early non-specific signals might have important effects on the response that develop to a subsequent heterogenous stimulus. Despite widespread acceptance amongst immunologists that such non-specific immunological effects occur, there are no systematic studies which have summarised the literature in humans to provide a framework for understanding the circumstances under which such effects can be documented, when such responses occur, or for how long they are present or, most importantly, their biological significance.

In this review, conducted at the request of WHO, we have systematically searched the scientific literature to identify available data concerning non-specific effects which might be measured after immunisation with the main vaccine antigens that are including in the expanded programme of immunisation for children, namely, BCG, measles, diphtheria, tetanus and pertussis.

The review demonstrates that there are a substantial number of studies which contain data of relevance to the assessment, but the vast majority were not conducted to investigate this phenomenon, though they did report data which could be extracted. There were few studies with similar methodology or endpoints which could be formally meta-analysed and therefore data are presented in summary figures and tables for each vaccine. The reviewed studies were highly heterogeneous and the risk of bias was high or unclear in the majority. While some significant findings were present, the lack of replication of the findings and the low quality of the majority of studies, indicates that such findings should be interpreted with caution.

While these findings do not exclude the possibility of important non-specific immunological effects of vaccines, the published literature dose not provide confidence in the presence, quality, quantity, kinetics or impact of any non-specific immunological effects in young children after vaccination. It is, therefore, not currently possible to provide any guidance from the human data on expected effects or when/how to measure them.

Future studies using systems biology to capture the functional genomic, genetic, epigenetic and immunological effects of vaccines, might be applied to explore this biological phenomenon and to provide data on the timing, duration, quality and magnitude of such effects and to identify signals which might be used in large scale studies with relevant epidemiological endpoints.

Background

A growing number of published reports have suggested that several vaccines routinely administered to infants around the world may have "heterologous" or "non-specific" effects on mortality unrelated to prevention of illness and deaths caused by the specific diseases against which the vaccines have been formulated. For example, studies have suggested that receipt of both the Bacillus Calmette–Guérin (BCG) and measles vaccine are associated with a reduced risk of death (i.e. all cause mortality), while receipt of diphtheria-tetanus-pertussis (DTP) vaccine is associated with an increased risk of death, at least among female infants.[1,2] The vast majority of the studies demonstrating these effects have been observational in nature, rather than randomised controlled trials with non-specific effects as the primary outcome, and as a result, poorly-controlled or uncontrolled confounding and various types of selection and information bias have been suggested as alternative explanations for these findings.[3,4]

The biological plausibility of one or more vaccines having heterologous effects, either detrimental or beneficial, is supported by a number of studies in animals (for example mice) and observations in humans.[5-8] Nevertheless, the biological mechanisms and immune pathways that would underlie and explain such effects remain largely unspecified and open to question. At the same time, the possible implications of any such heterologous vaccine effects for the formulation or re-formulation of the infant immunization schedule remain unclear, but it has been suggested that if such effects can be established beyond a reasonable doubt, the infant immunization schedule might need to be re-configured.[9] However, prior reviews of this subject, including periodic assessments by the World Health Organization (WHO) Global Advisory Committee on Vaccine Safety, have concluded that any such effects remain unproven and are therefore not a justification for altering the current schedule.[10]

The WHO Strategic Advisory Group of Experts (SAGE) has requested the WHO Secretariat to review the evidence surrounding the possible non-specific/heterologous effects of vaccines included in the routine infant immunization schedule.[9] Overall, our aim is to determine whether the current evidence is sufficiently sound to warrant further scientific investigation; and if so, to define the path towards obtaining unequivocal evidence on these issues that would support future robust, evidence-based adjustments in immunization policies, if warranted. Preparatory to such a review of the evidence by SAGE at its April, 2013 meeting, it is necessary to assemble the available evidence, both published and unpublished, and subject that evidence to a systematic review.

Thus the objective of this review is to systematically identify, assemble, review and critically appraise all available studies with immunological endpoints describing the possible non-specific or heterologous effects of BCG, diphtheria, pertussis, tetanus and measles containing vaccines.

Methods

Definitions

Specific immunological effects:- The effect on an immunological parameter in response to an antigen derived from the vaccines target pathogen.

Non-specific immunological effects: - The effect on an immunological parameter that is not in response to an antigen derived from the vaccines target pathogen.

General Approach

All available evidence (published and unpublished) that addressed possible non-specific effects of vaccines when given was identified and critically appraised, with a focus on the effects of vaccines on the child's immune system and the development of the immune system. Included in the review are randomized controlled trials (RCTs), quasi-randomized control trials, clinical trials, cohort studies, case-control studies, case series and case reports. The vaccines examined included live attenuated vaccines (BCG and measles containing vaccines), inactivated vaccines and toxoids (all diphtheria and tetanus toxoids, and *Bordetella pertussis* containing vaccines). The target population was infants under five years of age, however inclusion of studies was not limited to this age group. Gender, age at vaccination, and co-administration of vitamin A were examined as possible effect measure modifiers.

Search Strategy

Embase.com, which includes all records from MEDLINE, was searched from 1947 onwards, through to December 2012. Complementary, less extensive searches of the PubMed library, the Cochrane library, and the trip database, were performed in order to detect any articles missed by the search on Embase.com. A list of search entries used is displayed in Appendix A. In addition, the reference lists of all included articles found and all relevant review articles were manually searched to identify studies not included in the previously described search. Experts in the field were asked if they are aware of any unpublished reports of studies possibly meeting the inclusion criteria. Full text of all articles identified were sought, using internet downloads, interlibrary loans, and contacting of authors. Articles in any language were sought. A further limited search from December 2012 to January 2014 was performed in the PubMed library using the same search terms to provide an update. Experts in the field were also asked to review the initial search results and identify any further studies that should also be included. Fourteen additional papers were identified that had been missed in the search.

Selection of Eligible Studies

Each full text article was examined by two independent reviewers and a list of studies considered eligible for inclusion was made. Studies identified by both reviewers as being eligible for inclusion and having adequate data for extraction were included in the review. For studies where non-specific immunological data were generated but not reported, a request for provision of the data was sent to the authors. Where there were discrepancies, the reasons for these were discussed and a decision about inclusion was reached by consensus. If there was no agreement, a further independent reviewer adjudicated to make a final decision about eligibility.

Exclusion criteria

Ecological, animal and *in vitro* studies were excluded. Studies utilising recombinant vaccines or no vaccine at all were excluded. Those studies only reporting/generating study vaccine specific immunological endpoints were excluded.

Data Extraction

Acquisition of consistent data from studies, such as participants, methodology, potential confounders and background data was performed by the utilisation of specifically created data extraction forms using DistillerSR software. All relevant data were extracted from articles meeting inclusion criteria and entered into a database.

Data Analysis

Descriptive tables summarizing information about study design, study quality, and results of all included studies were generated. Data on non-specific immunological effects were extracted from papers which reported summary statistics in tabular form. Where results were presented in figures, data were extracted wherever possible using GetData Graph Digitizer version 2.26.0.20. Due to the heterogeneity of the study methodology, data presented and analysed using non-parametric statistics and substantial differences in reporting of outcomes it was not possible to meta-analyse any outcomes from different studies. Where at least two papers reported results from the same assay, descriptive figures demonstrating non-specific immunological outcomes were generated, with comparable assays clustered according to vaccine type.

Description of included studies

Completion of the search process resulted in a total of 77 studies meeting the eligibility criteria for the review (Figure 1). The composition of the studies is summarily described in Table 1 and extensively described in Appendix B. Relatively equal proportions of RCTs, cohort and case-controls studies were identified. There was a wide range (3-2345) of total study participants involved across the studies. The majority of studies (48%) utilised BCG as the study vaccine intervention, whilst 68% were exclusively conducted in a paediatric population. The final time-point of outcome measurement was primarily performed (70%) between one and 12 months after vaccination.

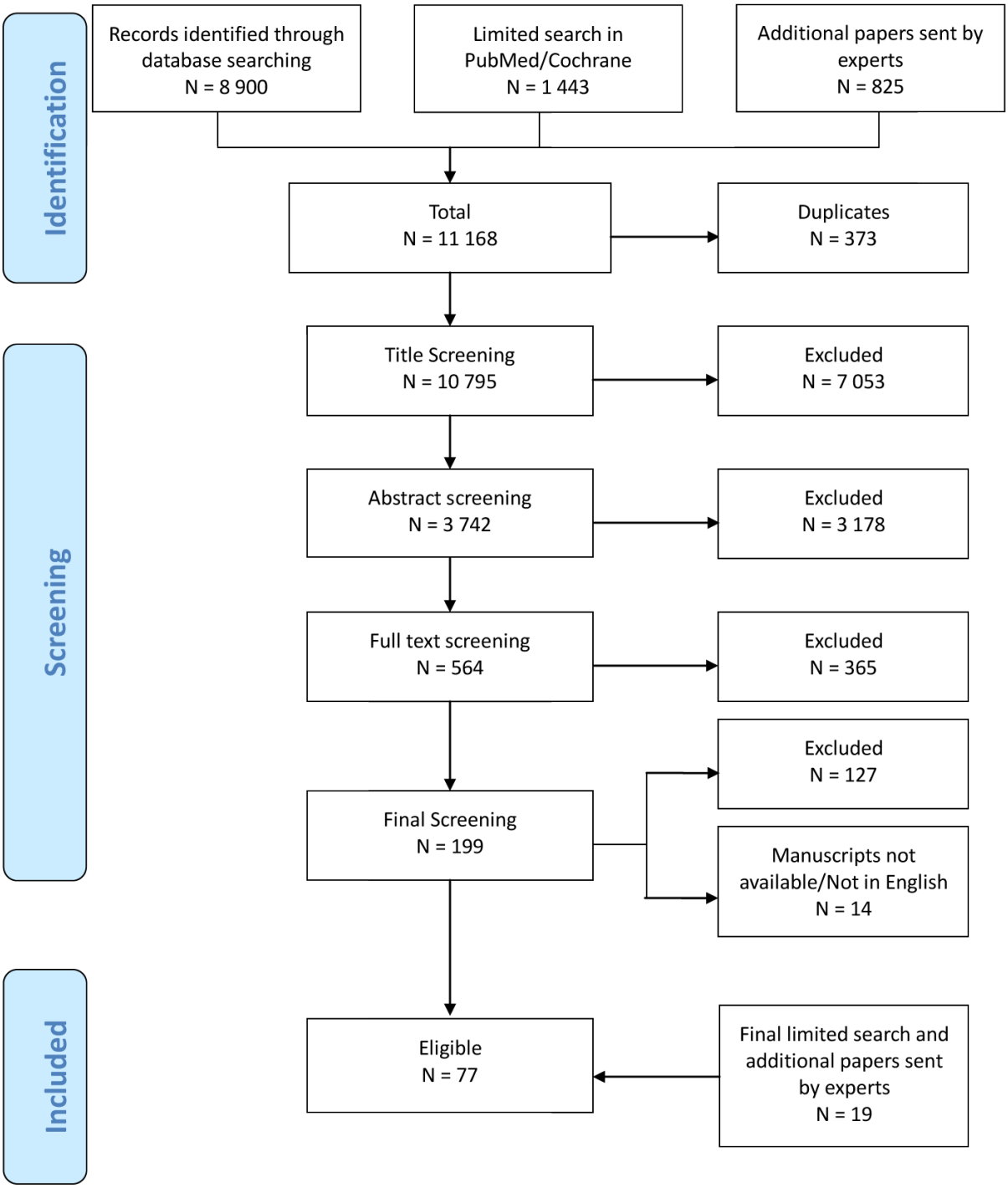


Figure 1. Overview of identification process for eligible studies.

Table 1. Summary description of included studies

Study vaccine	N
BCG	37
Measles	14
MMR	3
DTP	7
Pertussis	1
DT	4
TT	11
Other Vaccine/s used in study?	
Yes	24
No	31
Not described or not applicable	22
Age	
Neonate	15
Infant	18
Children	14
Adults	19
Elderly	0
Combination	11
Gender of study population	
Male and Female	39
Male	2
Female	1
Not reported	35
Geographic location	
Africa	19
Europe	22
Asia	8
Americas	20
Oceania	4
Combination	4
Co-administration with Vitamin A?	
Yes	3
No/Not reported	74
Presence of attribute that may affect response?	
Yes	22
No	55
Interval between vaccine administration and final outcome measure	
< 1 month	11
1 - < 6 months	29
6 - ≤12 months	25
>12months	10
Not reported	2
Number of participants	
Mean	206
Median	77
Range	3-2345

Study design	
RCT	25
Prospective Cohort	23
Prospective Case-control	23
Other	6

Methodological attributes of included studies

Included studies[5,11-87] had their methodological attributes analysed and tabulated according to the study vaccine used (Appendix C). No one study was rated as having low risk of bias for all criteria (Table 2). This is likely to be in part due to the heterogeneous spread of study designs. In addition the outcome of non-specific immunological effects does not feature as a primary outcome parameter in any of the RCTs. Only 55% of the included studies actually reported data in a usable format for this review (Table 3). A diverse array of immunological assays were utilised to report non-specific effects in the included studies, which taken in conjunction with the differences in measurement parameters and statistical analysis creates a high number of possible combinations in outcome reporting. For this reason no meta analysis of the data was possible. Data from the included papers were not presented in such a form (that is data sets were not sub-classified according to sex) that the affect of sex on non-specific immunological effects could be analysed. Overall review of the methodological attributes demonstrates a consistently low level of evidence and exemplifies the lack of any high quality (low risk of bias) randomised controlled trial with focussed primary endpoints designed around non-specific immunological outcomes.

Table 2. Risk of Bias Summary of Included Studies

Study Author	Vaccine	Random sequence generation	Allocation concealment	Blinding, All outcomes	Incomplete outcome data, All outcomes	Selective reporting	Other bias	Overall
Akkoc *et al* 2010	BCG	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Anderson *et al* 2013	BCG	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High
Black *et al* 2001	BCG	Low risk	Unclear risk	Unclear risk	Unclear risk	High risk	Unclear risk	High
Black *et al* 2002	BCG	Low risk	Low risk	Low risk	Low risk	Unclear risk		Unclear
Burl *et al* 2010	BCG	Low risk	Low risk	Unclear risk	Low risk	Low risk		Unclear
Burl *et al* (Aug.)	BCG	Low risk	Low risk	Unclear risk	Low risk	Unclear risk		Unclear
Djuardi *et al* 2010	BCG	High risk	High risk	Unclear risk	Unclear risk	Unclear risk		High
Elliot *et al* 2011	BCG	High risk	High risk	Unclear risk	High risk	Low risk	High risk	High
Faustman *et al* 2012	BCG	Low risk	Low risk	Low risk	Low risk	Unclear risk		Unclear
Fjallbrant *et al* 2007	BCG	Unclear risk	High risk	High risk	Low risk	Unclear risk		High
Gruber *et al* 2000	BCG	Unclear risk	High risk	Unclear risk	High risk	Low risk		High
Hoft *et al* 1998	BCG	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk		Unclear
Hoft *et al* 1999	BCG	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Hussey *et al* 2002	BCG	High risk	High risk	Unclear risk	Unclear risk	Unclear risk		High
Kagina *et al* 2009)	BCG	Low risk	Low risk	Unclear risk	Low risk	Unclear risk		Unclear
Kleinnijenhuis *et al* 2012	BCG	High risk	High risk	Unclear risk	Low risk	Low risk		High
Lalor *et al* 2009	BCG	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Lalor *et al* 2010	BCG	High risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk	High
Lalor *et al* 2011	BCG	High risk	High risk	Unclear risk	Unclear risk	Low risk	High risk	High
Libraty *et al* 2014	BCG	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk		Unclear
Lowry *et al* 1998	BCG	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk	Unclear risk	High
Marchant *et al* 1999	BCG	Low risk	Low risk	Unclear risk	High risk	Unclear risk		High
Marks *et al* 2003	BCG	High risk	High risk	High risk	Unclear risk	Low risk		High
Miles *et al* 2008	BCG	High risk	Unclear risk	Unclear risk	High risk	High risk	High risk	High
Miles *et al* 2009	BCG	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Ota *et al* 2002	BCG	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Smith *et al* 2012	BCG	High risk	Unclear risk	Unclear risk	Low risk	Low risk		High
Soares *et al* 2013	BCG	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk		Unclear
Steenhuis *et al* 2007	BCG	High risk	Unclear risk	Low risk	Low risk	Unclear risk	High risk	High
Tastan *et al* 2005	BCG	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear
van den Biggelaar *et al* 2009	BCG	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Vargas *et al* 2004	BCG	Low risk	Unclear risk	Unclear risk	Low risk	Low risk		Unclear
Vekemans *et al* 2004	BCG	Unclear risk	Low risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Vijaya Lakshmi V, *et al* 2005	BCG	High risk	High risk	High risk	Unclear risk	Unclear risk	High risk	High
Weir *et al* 2004	BCG	Low risk	Low risk	Low risk	Low risk	Unclear risk		Unclear
Weir *et al* 2008	BCG	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Weir *et al* 2008	BCG	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear
Armitage *et al* 1993	TT	High risk	High risk	High risk	Unclear risk	Unclear risk		High
Borut *et al* 1980	TT	High risk	High risk	High risk	Unclear risk	Unclear risk		High
Chollet *et al* 1979	TT	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Chui *et al* 2004	TT	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Cooper *et al* 1998	TT	High risk	High risk	Unclear risk	Unclear risk	Unclear risk		High
Di Genova *et al* 2006	TT	High risk	High risk	Unclear risk	Unclear risk	High risk		High
Fernandez *et al* 1994	TT	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Fevrier *et al* 1977	TT	High risk	High risk	Unclear risk	Unclear risk	High risk	High risk	High
Gentile *et al* 2006	TT	High risk	High risk	Unclear risk	Low risk	Unclear risk	Unclear risk	High
Livingston *et al* 2013	TT	High risk	High risk	Unclear risk	Low risk	Unclear risk		High
Mahalingham *et al* 2010	TT	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Bertley *et al* 2004	Measles	Unclear risk	Unclear risk	Unclear risk	High risk	High risk		High
Gans *et al* 1999	Measles	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk	Unclear
Gans *et al* 2004	Measles	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk		High
Hennino *et al* 2007	Measles	Low risk	Low risk	Low risk	High risk	Unclear risk	High risk	High
Hussey *et al* 1996	Measles	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Jaye *et al* 2014	Measles	High risk	Unclear risk	Unclear risk	High risk	Unclear risk		High
Liguori *et al* 1998	Measles	Unclear risk	Unclear risk	Unclear risk	High risk	Low risk	High risk	High
Lisse *et al* 1994	Measles	Unclear risk	Unclear risk	Unclear risk	High risk	Low risk		High
Nakayama *et al* 1990	MMR	Unclear risk	Unclear risk	Unclear risk	Low risk	High risk	Unclear risk	High
Njie-Jobe *et al* 2012	Measles	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear
Okada *et al* 2001	Measles	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk	High risk	High
Ovsyannikova *et al* 2003	Measles	Low risk	Unclear risk	Unclear risk	High risk	High risk	High risk	High
Pabst *et al* 1997	MMR	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk		Unclear
Pabst *et al* 1999	Measles	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	High risk	Unclear
Rager-Zisman *et al* 2003	MMR	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk	Unclear
Samb *et al* 1995	Measles	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk		Unclear
Schnorr *et al* 2001	Measles	Unclear risk	High risk	Unclear risk	Unclear risk	High risk		High
Dirix *et al* 2009	DTP/DT	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High
Fernandes *et al* 2010	DTP/DT	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk	Unclear
Fryauff *et al* 1998	DTP/DT	High risk	High risk	Unclear risk	Unclear risk	Unclear risk	High risk	High
Halasa *et al* 2008	DTP/DT	Low risk	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear
He *et al* 1998	DTP/DT	Unclear risk	Unclear risk	Unclear risk	Unclear risk	High risk	High risk	High
Heine *et al* 2011	DTP/DT	High risk	Unclear risk	Low risk	Unclear risk	High risk	High risk	High
Jorgensen *et al* 2013	DTP/DT	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear
Lin *et al* 1997	DTP/DT	Unclear risk	Unclear risk	Unclear risk	High risk	Unclear risk		High
Rowe *et al* 2000	DTP/DT	Unclear risk	Unclear risk	Unclear risk	Low risk	High risk	Unclear risk	High
Yousfi *et al* 2005	DTP/DT	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear
Zorzeto *et al* 2009	DTP/DT	Low risk	Unclear risk	Low risk	Unclear risk	High risk		High
Di Tommaso *et al* 1997	Pertussis	Unclear risk	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear

Table 3. Immunological assays and the combination of reporting parameters used within the included studies

	Vaccine				
Counts	**BCG**	**TT**	**Measles**	**MMR**	**DTP**
N. Studies reporting data in usable format or supplying raw data	20	10	8	3	1
N. Immunological parameters (Cytokines/Chemokines)	88	21	23	10	1
N. Stimulants	20	14	6	5	7
Cytokine/Stimulant combinations	**167**	**36**	**35**	**13**	**7**
N. different units (pg/mL, SI, %, mm2, cpm)	16	11	9	3	1
N. different statistics report (Geometric mean, raw mean, median, % etc)	17	8	7	3	1
N. Total number of combinations of the above	**223**	**37**	**33**	**13**	**7**

Data from included studies

BCG

Overall 37 studies were found which measured non-specific immunological effects of BCG vaccination. In 11 of these papers the results of assays conducted were not reported as they were not the main focus of the paper. Of the included studies, 24 included children under the age of 5 years.

There were 20 papers reporting non-specific immunological effects with data reported in tables or a graphical format which could be extracted using a digitizer program, and one study which supplied raw data (Lalor *et al*). These papers reported 89 different immunological parameters the main ones being CD4, CD8, EGF, Eosinophils, Eotaxin, FGF-2, Flt-2L, Fractalkine, (FoxP3)+ regulatory CD4+ T cells, G-CSF, GM-CSF, GRO, γδ T cells, IFNα2, IFN-γ+/TNF-α+ CD4+ T cells, IFN-γ, IgE, IL-10, IL-12p40, IL-12p70, IL-13 IL-15, IL-17, IL-1α, IL1-β, IL-1Rα IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, Leukocytes, MCP-1, MCP-3, MDC, MFI TLR4, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, Proliferation (delta cpm), Proliferation (Stimulation Index) , RANTES, sCD40L, sIL-2Ra, TGF-α, TNF-α, TNF-β, Total lymphocytes , VEGF, MFI CD11b, CD11b in CD14, CXCR1 in CD14, TLR4 in CD14, MFI CD14, MFI CXCR1, MFI dectin-1, TLR2 in CD14, MFI TLR2, MR in CD14 and MFI MR.

There were 20 types of stimulants used in the above assays (*C. albicans*, ConA, Diphtheria toxin, *E. coli*, House dust mite, HBsAg, IPP plus IL-2, LPS, *M. avium* PPD (CVL), *M. avium* PPD (SSI), *M. intracellulare* PPD (SSI), *M. intracellulare* PPD-B, *M. leprae*, *M. scrofulaceum* PPD (SSI), PHA, *S. aureus*, SK/SD, Tetanus toxoid and unstimulated assays) resulting in 167 unique combinations of the above. Immunological responses to PHA stimulated and unstimulated cultures were most frequently reported.

There were 6 papers from which data could not be extracted;

- Black *et al* 2001 report IFN-γ responses to control antigens (*M. avium* (SSI), *M. avium* (CVL), *M. intracellulare* (PPD–B), *M. intracellulare* (SSI), *M. scrofulaceum* (SSI), M. *marinum*, *M. kansasii* (SSI)), *M. kansasii* (UCL), *M. fortuitum*, and *M. vaccae*.) in lymphocyte cultures from 616 young adults in Malawi however the analyses are correlation coefficients between IFN-γ responses for all possible pairs of antigens used in this study.
- Faustman *et al* 2012 report on 6 diabetic and 6 healthy non-diabetic subjects randomised to BCG vaccination or control. T-cells, auto-antibodies and C-peptide are reported on 3 placebo and 3 BCG vaccinated subjects from each group. No group summary statistics are presented.
- Burl *et al* 2010 present a scatter plot of activated T cells (CD4+CD25+) in 48 children (aged 4 ½ months) who were vaccinated at birth and 39 unvaccinated control children. There were no

statistically significant differences between vaccinated and unvaccinated children (p=0.9388). Other NSIE were not reported.

- Ota *et al* 2002 reported PBMC proliferation, cytokine responses and antibody responses to TT and HBsAg in newborns according to timing of BCG vaccination (birth, 2.5 months and 4 months). Significant differences were identified for at least one comparison between study groups at 2 and 4.5 months for all NSIE assays.
- Miles *et al* 2009 reported on newborns of HIV positive (n=16) and negative (n=21) Malawian women. Maternal HIV status resulted in differential expression of T cells in children vaccinated at birth. No unvaccinated control group was included in the study.
- Kagina *et al* 2009 reported SEB-induced cytokine expressing CD4+ T cell responses (IFN-γ, TNF-α, IL-2) in 25 children vaccinated at birth and 21control children (Vaccinated at 10 weeks of age). Comparisons at 10 weeks were non-significant.

Data for the overall immunological outcomes of the included studies were summarized for all available parameters to provide a perspective on the effect of vaccination on these parameters in the following way. The direction of effect was calculated for each parameter by creating a ratio of the response in those vaccinated compared to the response in the unvaccinated participants. The response could be, the median, geometric mean or fold-rise depending on the statistics reported in the paper. No formal combination of these ratios (such as in a meta-analysis) has been conducted since the ratios are statistically non-comparable. Plots as designed to give a 'feel' for the overall diversity of responses and point to any general trends that may be occurring in the data. The size of each study cohort is represented by the size of the data point 'bubble'. For papers which reported comparisons at multiple time points for the same children the first comparison only is plotted so that each cohort of children is only reported once per study per parameter.

These ratios for all unstimulated assays and PHA stimulation are displayed below (Figures 2 and 3). No general patterns according to pro-inflammatory or anti-inflammatory classifications were observable for either plot. Results for both unstimulated (unstimulated cultures and total cell counts) and PHA stimulated assays show a range of both increases and decreases in response for most parameters.

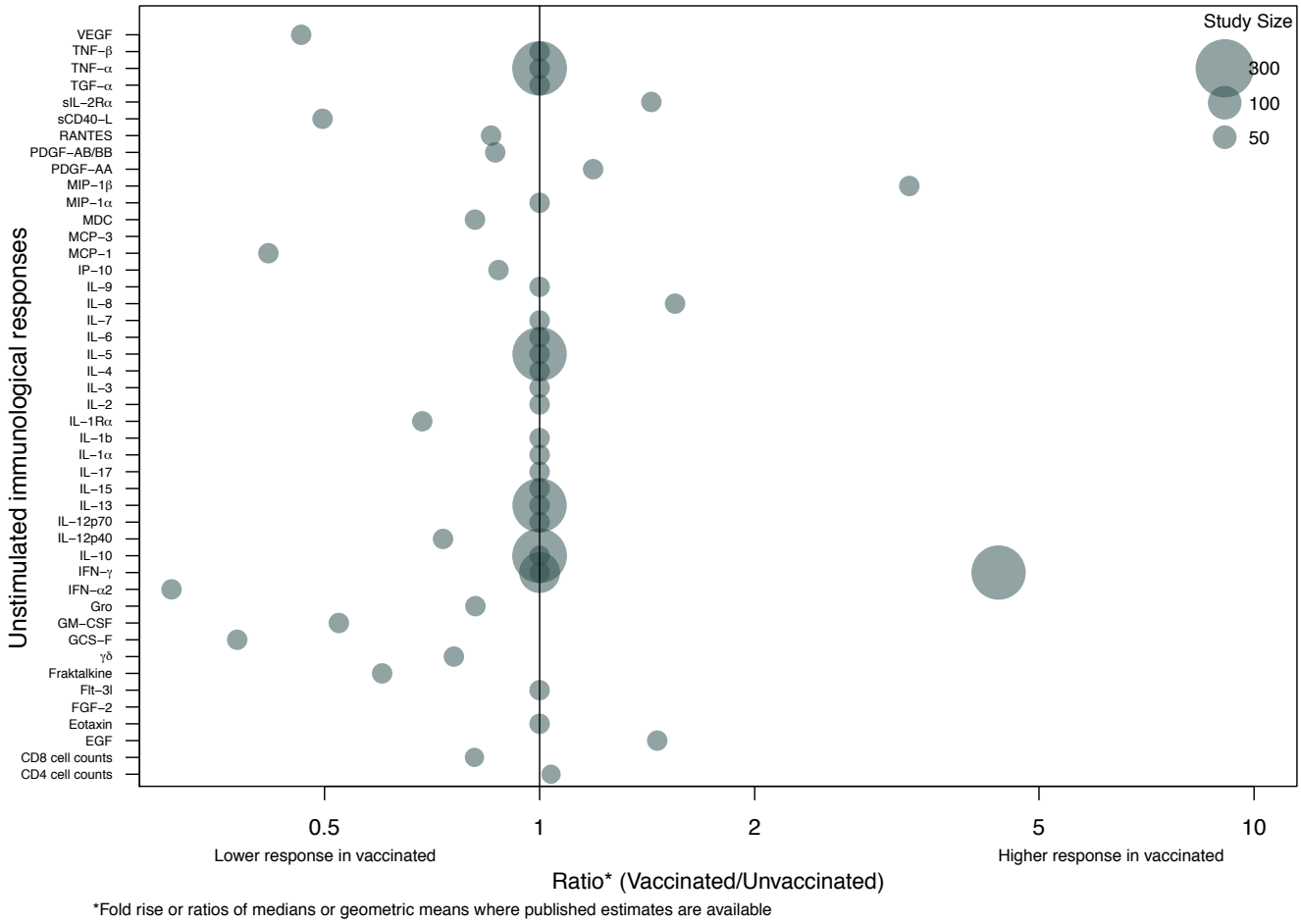


Figure 2. Leukocyte count and unstimulated culture response ratios, comparing vaccinated to unvaccinated, from included BCG studies reporting non-specific immunological effects.

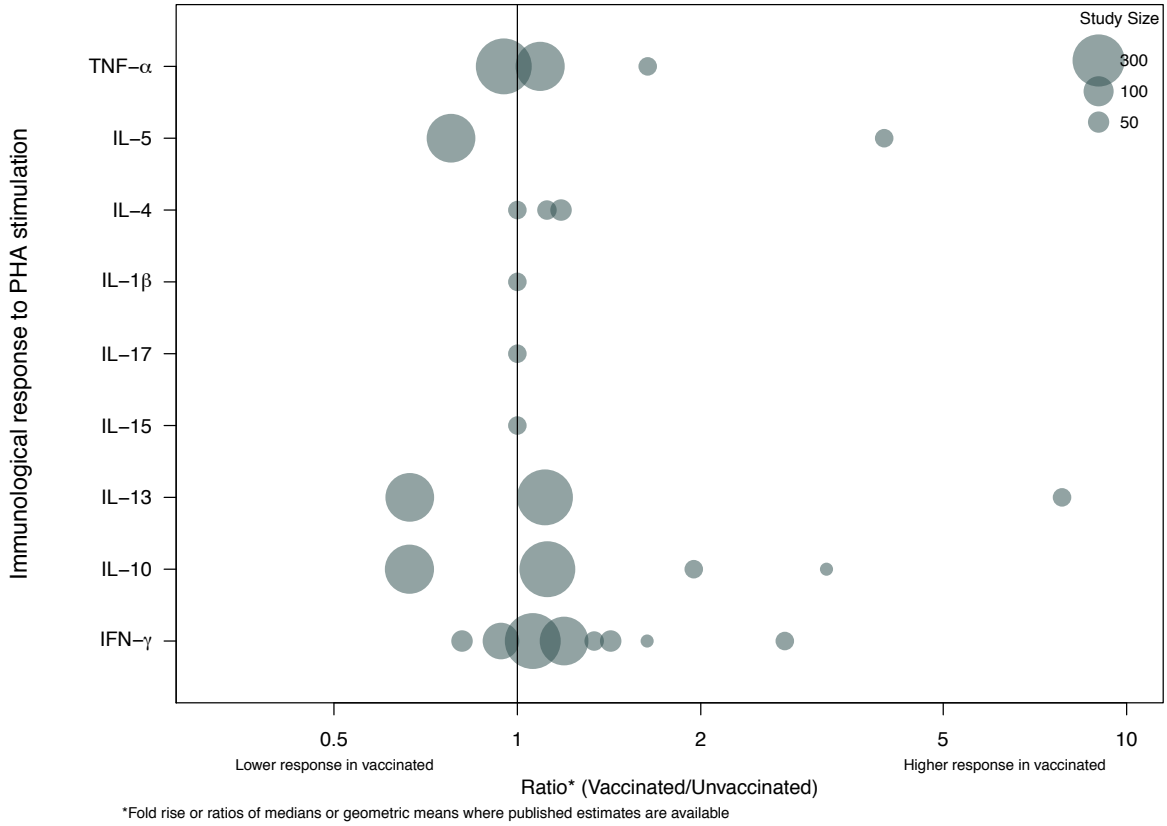


Figure 3. PHA stimulated culture response ratios, comparing vaccinated to unvaccinated, from included BCG studies reporting non-specific immunological effects.

IFN-γ

IFN-γ was the most commonly reported parameter. Data could be extracted from 11 papers and one paper supplied unpublished raw data from unstimulated assays upon request. Stimulants included *C. albicans*, HBsAg, LPS, *M. leprae*, PHA, *S. aureus*, SK/SD, PMA, Tetanus toxoid and unstimulated assays. Results from 6 papers reported results in using PHA stimulation were available (Figure 4). One cohort study (Djuardi *et al* 2010) reported a significant increase 24 months after vaccination in 98 children at birth, but no significant differences at 5 or 12 months after vaccination. The remaining 5 studies reported no significant differences between vaccinated and unvaccinated children.

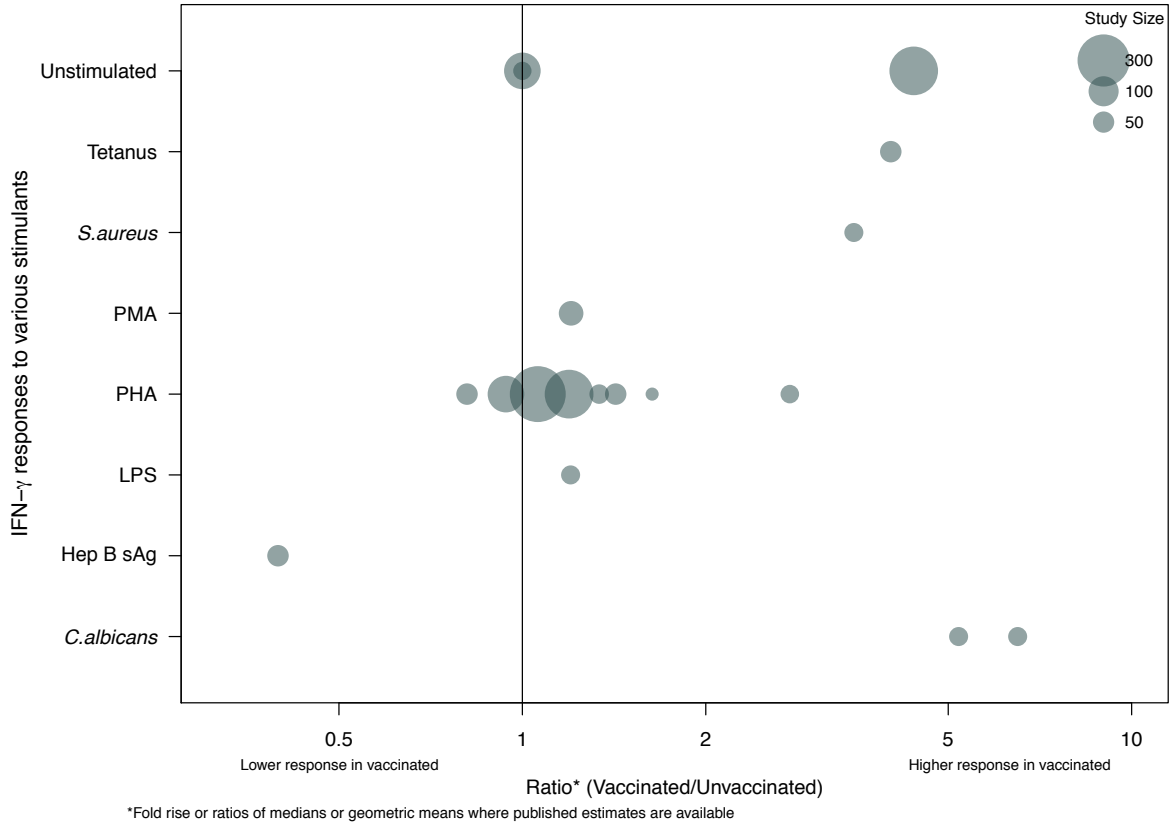


Figure 4. IFN-γ response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

Two studies reported data, which could be extracted for unstimulated IFN-γ responses, and one study provided raw data for this same assay. All studies reported similarly large numbers of results below the level of detection of the assay. One study (Black *et al* 2002) reported low percentages (6% and 2%) of responses above 62 pg/mL in both Malawian and UK vaccinated teens respectively. Djuardi *et al* 2010 reported no differences between vaccination at birth and results at 5, 12, or 24 months and a further study supplied raw data for which the large majority of responses were below the level of detection (<3.2 pg/mL) for vaccinated and unvaccinated children in Malawi and UK. No statistically significant differences were reported.

The remaining studies with extracted data reported IFN-γ responses to LPS (one study: no significant difference), *C. albicans* (one study – significant differences between pre- and post-vaccination in adults), HBsAg (one study: no significant difference) and PMA (one study: no statistical comparison between vaccinated and unvaccinated).

IL-2

IL-2 responses to the mitogen ConA and unstimulated assays, demonstrated a statistically significantly higher response in the vaccinated group for Con A. There was no effect for the unstimulated assay (Figure 5).

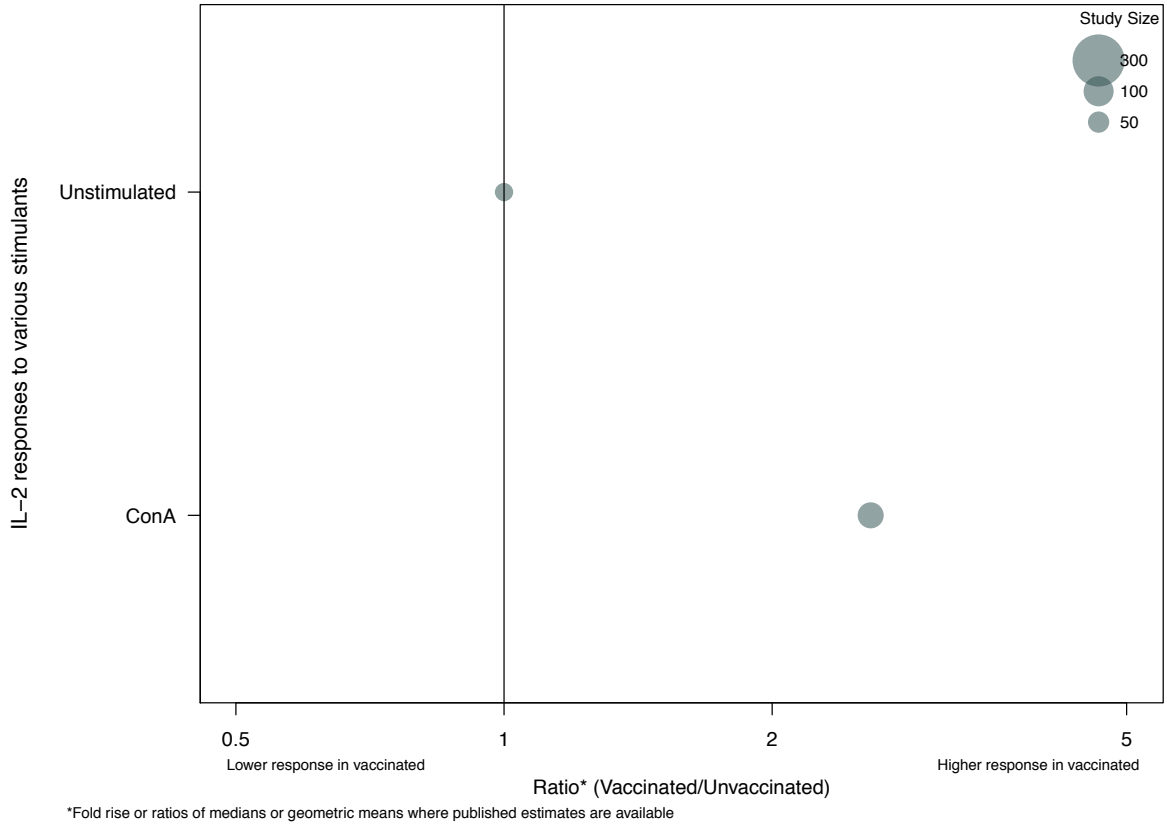


Figure 5. IL-2 response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

IL-4

Hoft *et al* 1999, Marchant *et al* 1999 and Vargas *et al* 2004 reported data for IL-4 responses. IL-4 responses to the mitogens PMA was not statistically compared between vaccinated and control groups in Vargas *et al* 2004. PHA and unstimulated responses did not show any statistically significant difference between vaccinated and unvaccinated responses (Figure 6).

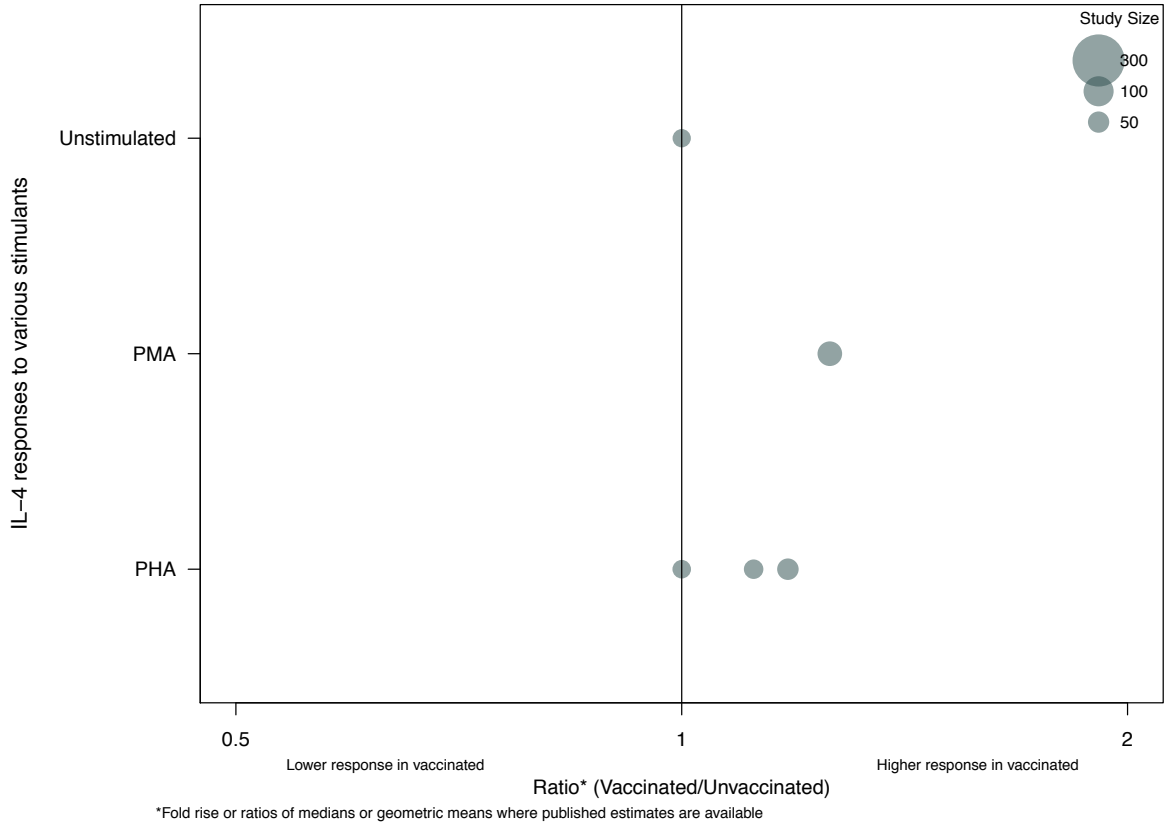


Figure 6. IL-4 response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

IL-10

IL-10 results were extracted from 6 papers. Stimulants used in these assays included HDM, LPS, *M. avium* PPD (CVL), *M. avium* PPD (SSI), *M. intracellulare* PPD (SSI), *M. intracellulare* PPD-B, *M. scrofulaceum* PPD (SSI), PHA and unstimulated (medium alone). Four papers reported results using PHA stimulation (Figure 7). Three of these studies reported no significant differences between vaccinated and unvaccinated children. One study (Akkoc *et al* 2010) reported significant differences but no consistent effect with a significant increase compared to pre-vaccination levels 2 months following vaccination and a significant decrease 8 months after vaccination in 10 infants vaccinated at birth. No significant differences were observed between pre- and post-vaccination in the 9 infants vaccinated at two months of age in this same study and no statistically significant difference was observed between 9 unvaccinated children at 2 months of age compared to 10 children vaccinated at birth and measured at two months. Figure 7 includes this last comparison of vaccinated versus unvaccinated children only.

Two of the papers reporting responses to PHA also reported responses to LPS. No statistically significant differences were reported.

Other stimulants reported include HDM, *M. avium* PPD (CVL), *M. avium* PPD (SSI), *M. intracellulare* PPD (SSI), and *M. intracellulare* PPD-B which were all reported in one paper and were all $p > 0.05$. IL-10 responses to *M. scrofulaceum* PPD (SSI) were reported once with $p=0.015$ in Malawian infants and $p=0.838$ in UK infants. Response to HDM was significantly lower in those vaccinated ($p<0.0001$).

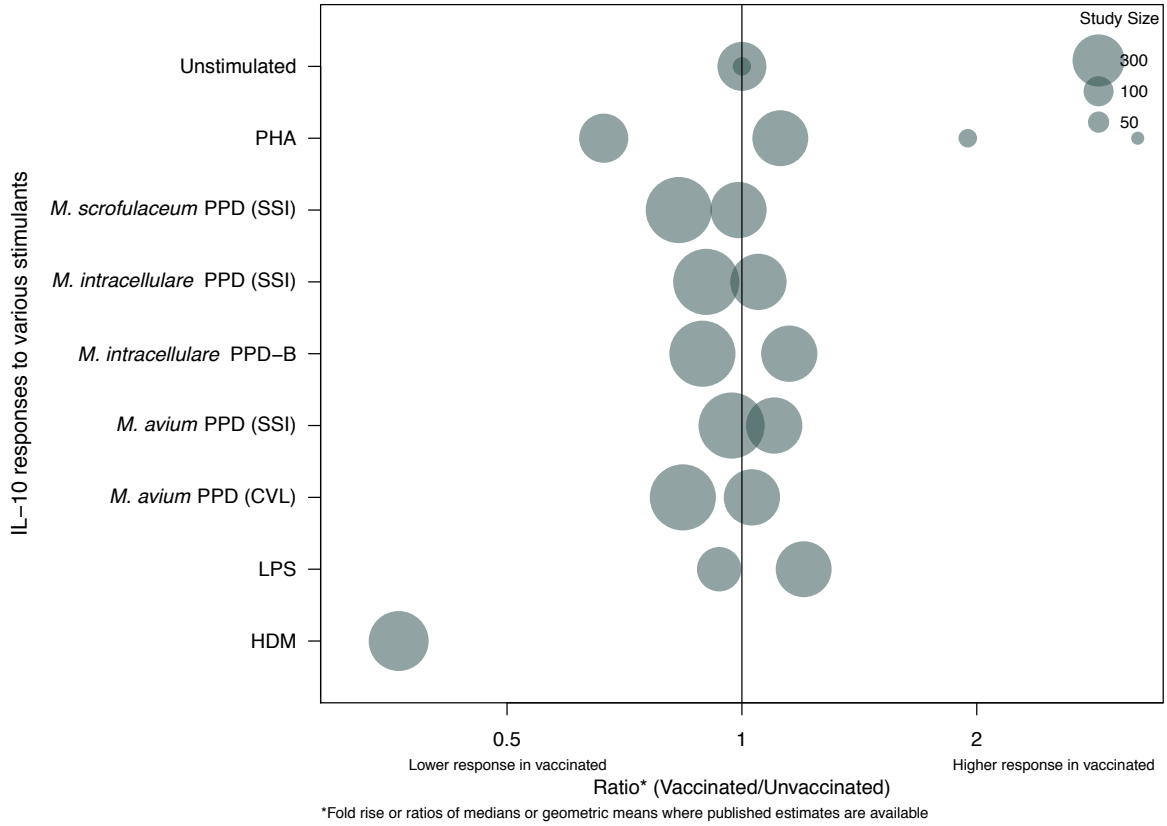


Figure 7. IL-10 response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

TNF-α

TNF-α results were extracted from five papers and one paper supplied raw data. Stimulants used in these assays included *C. albicans*, *E. coli* LPS, *M. avium* PPD (CVL), *M. avium* PPD (SSI), *M. intracellulare* PPD (SSI), *M. intracellulare* PPD-B, *M. scrofulaceum* PPD (SSI), PHA, *S. aureus* and unstimulated.

Three studies reported results using PHA stimulation (Figure 8). Two of these studies also reported results for LPS stimulation. There were no significant differences reported in these three studies.

One study reported unstimulated TNF-α responses and one supplied raw data for this same assay. The large majority of responses were below the limit of detection and no significant differences were reported.

Other reported responses were to the following stimulants (All of which were non-significant): *C. albicans*, *E. coli*, *M. avium* PPD (CVL), *M. avium* PPD (SSI), *M. intracellulare* PPD (SSI), *M. intracellulare* PPD-B, *M. scrofulaceum* PPD (SSI) and *S. aureus.*

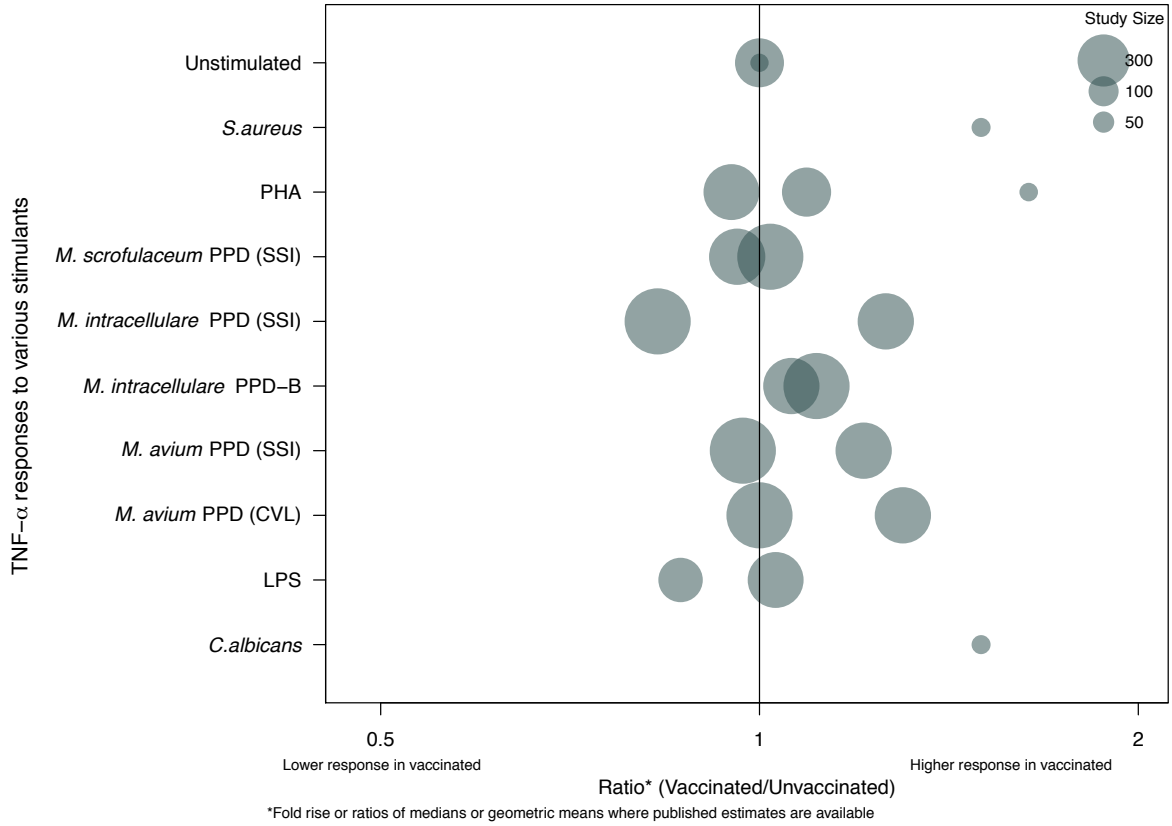


Figure 8. TNF-α response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

IL-13

IL-13 results were extracted from four papers and one paper supplied raw data. Stimulants used in these assays included PHA and unstimulated (medium alone). Results from 3 papers, reported data using PHA stimulation (Figure 9). One of these papers (Djuardi *et al* 2010) reported a significant decrease in IL-13 at 5 months (but not at 12 or 24 months) in infants vaccinated at birth whilst the remaining two studies reported no differences.

Unstimulated responses were reported in one paper and one study supplied the raw data. The large majority of these responses were below the limit of detection and no significant differences were reported.

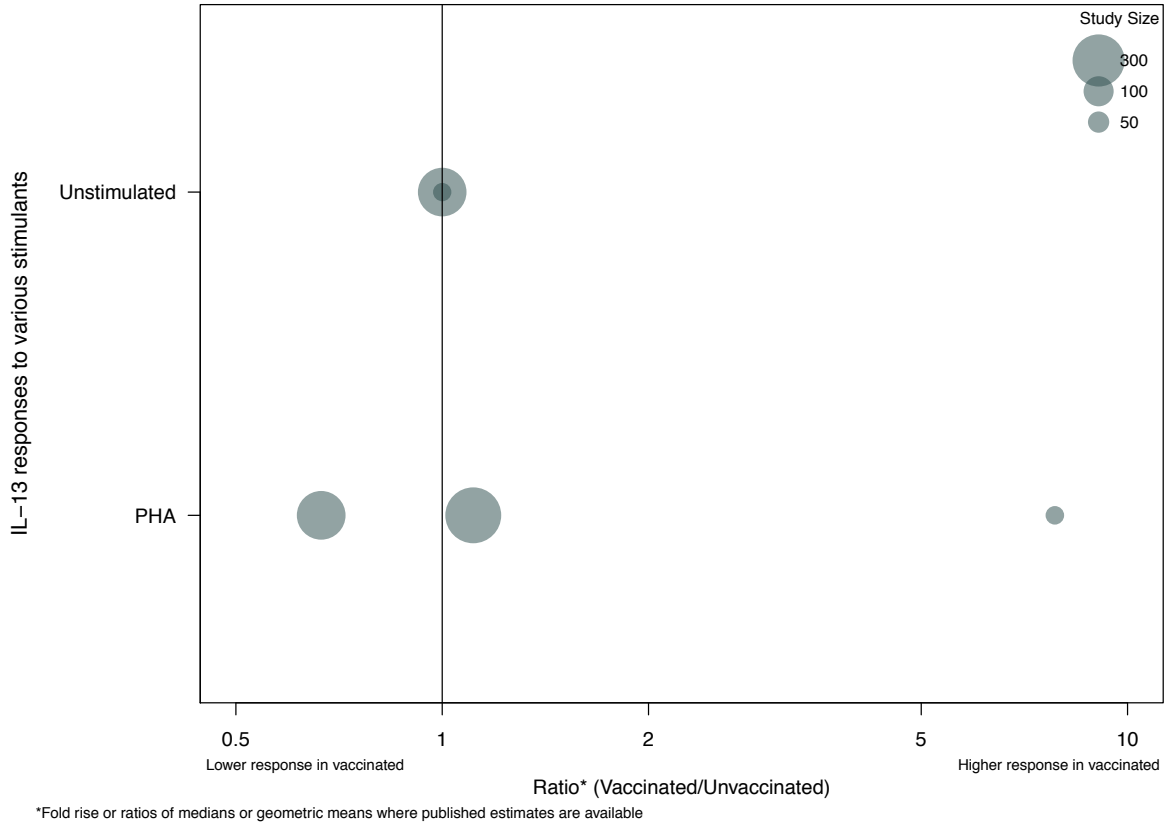


Figure 9. IL-13 response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

IL-5

Data from two studies containing IL-5 responses to PHA stimulation were extracted and graphically represented (Figure 10). There were no significant differences reported in these studies. Responses to HDM, reported by Marks *et al* 2003 also showed no difference between vaccinated and unvaccinated.

Unstimulated responses from one published paper, and from data supplied in raw format demonstrated the majority of readings to be below the limit of detection with no reported differences seen.

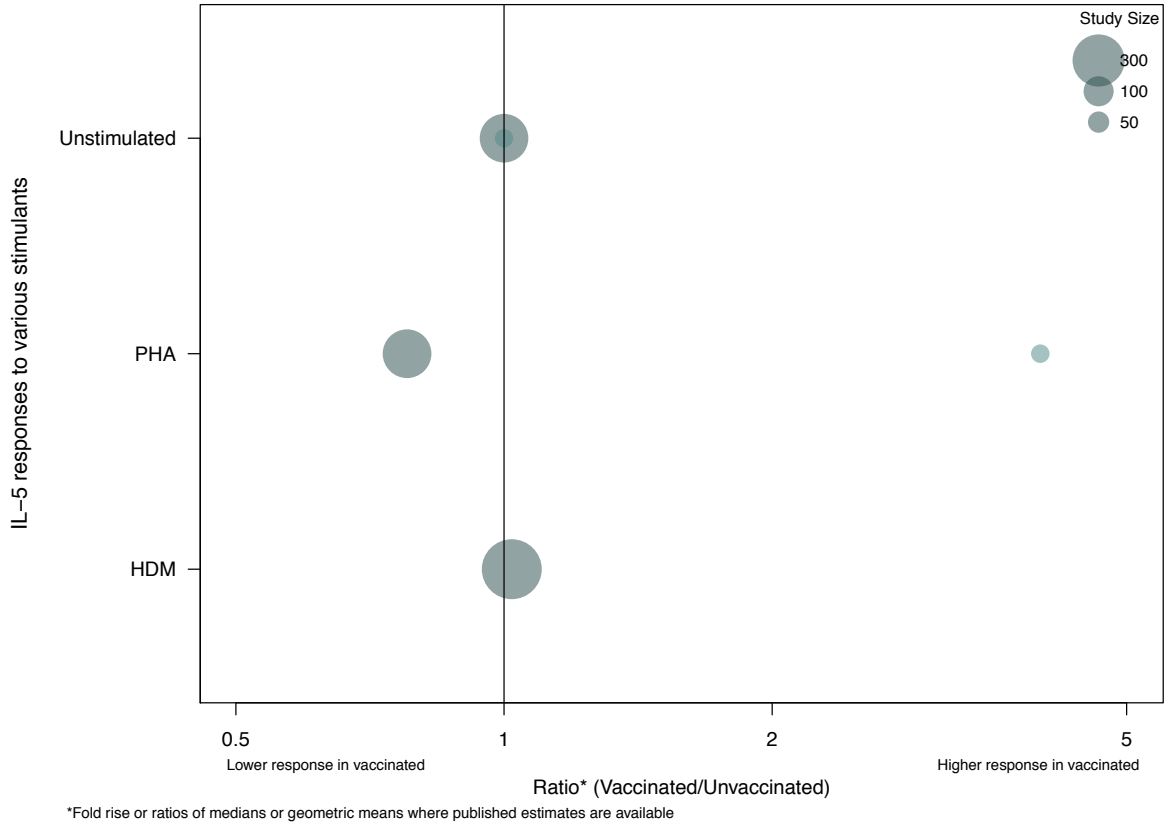


Figure 10. IL-5 response ratios, comparing vaccinated to unvaccinated, following non-specific antigen stimulation of cultures from included BCG studies.

Leukocytes

Two studies reported total leukocyte and eosinophil counts (Figure 11). No significant differences were reported in Steenhuis *et al* 2007 whilst no statistical comparison between vaccinated and unvaccinated children was reported in Vargas *et al* 2004.

Tarstan *et al* 2005 presents results for total lymphocyte responses that were significantly increased post-vaccination at both 2 and 4 months of age in children vaccinated at 48 hours and 2 months respectively. Results for aβ+ T lymphocytes were significantly reduced post-vaccination in both groups whereas gδ+ T lymphocytes were significantly decreased in the group vaccinated at 48 hours but were no different in the group vaccinated at 2 months.

In contrast gδ+ T lymphocytes were significantly increased in Hoft *et al* 1998 in response to IPP plus IL-2 stimulation but not in response to tetanus stimulation nor for unstimulated media responses.

CD4 and CD8 T cell responses were all measured in relatively small sample sizes (Hoft *et al* 1998). Both CD4 and CD8 T cell responses to stimulation with IPP together with IL-2 were significantly higher in the vaccinated group. CD4 and CD8 T cell responses to Tetanus and in unstimulated cultures were similar in the vaccinated and unvaccinated group and no statistical differences were reported.

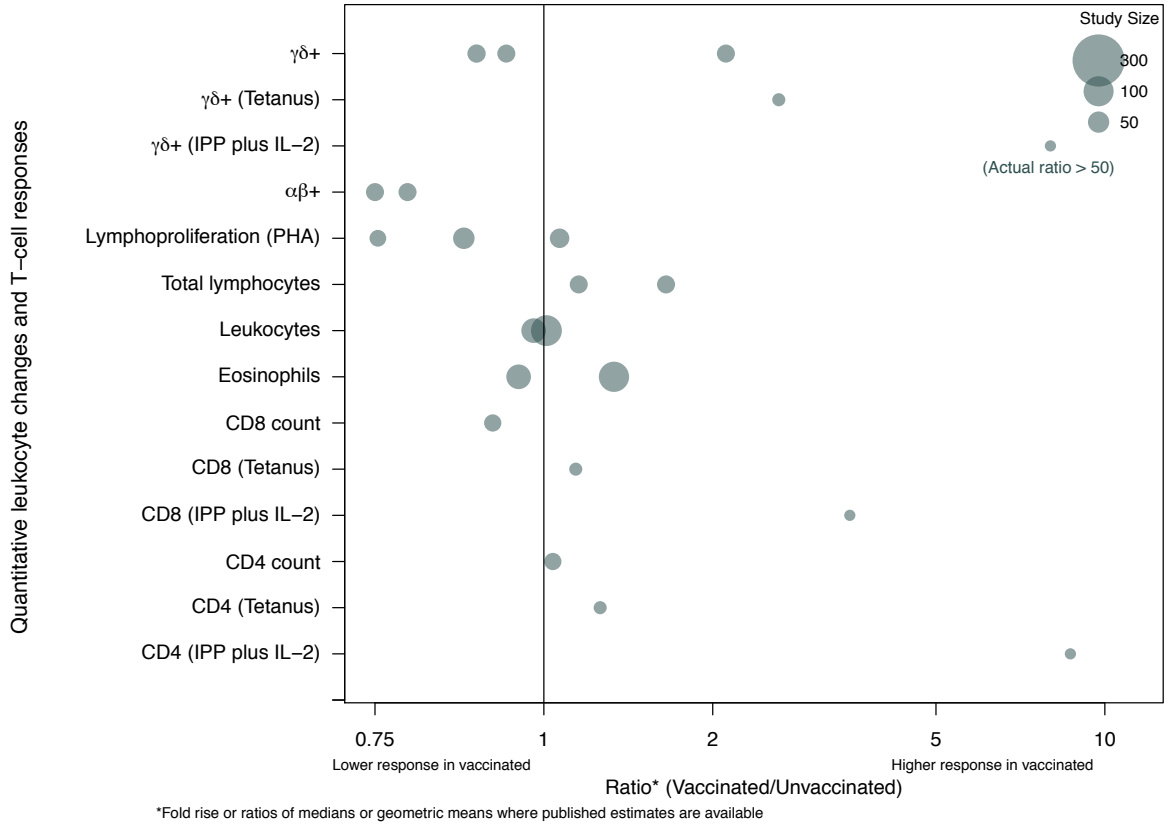


Figure 11. Responses rations of lymphoproliferation following non-specific antigen stimulation and Leukocyte counts, in vaccinated compared to unvaccinated groups, from included BCG studies.

Tetanus

There were 10 papers found reporting responses to non-specific stimuli with data either reported in table format or that could be extracted from graphs. These papers reported 21 different immunological parameters the main ones being proliferation (^{3}H-thymidine incorporation), CD25 and CD69, antigen reactive cells, Area of Skin Reactivity, B population, blastic transformation , IFN-γ , IL-10, IL-13, IL-17, IL-2, IL-4, IL-5, Stimulation Index, T1 population, T2 population, TNF-α, total proliferation and 25-hydroxyvitamin D, T and B lymphocytes and T-derived lymphocytes.

There were 14 types of stimulants used in the above assays (Candida-E, Candida-I, Cell electrophoresis, ConA, ERFC, HSV, Mumps-E, Mumps-I, PBS, PHA, PPD, PWM, unstimulated) resulting in 36 unique combinations of the above.

Only one study, Borut *et al* 1980, involved children under the age of 5 years, who only made up a fraction of the total study cohort.

No two papers reported the same parameters. No meta-analysis was possible nor plots created.

The following results were reported:

- Armitage *et al* 1993 report mitogen-induced blastogenesis in lymphocytes cultured with the mitogens PHA and ConA from 17 elderly and 17 young subjects. Differences were observed between the age groups with younger participants having higher responses. No comparison to baseline was made.
- Chollet *et al* 1979 report results for cell electrophoresis (B population, T1 population and T2 population) and non-specific blastic transformation with mitogens PHA, ConA and PWM in 6 individuals over 8 days after TT booster. B, T1 and T2 populations all increased from baseline to Day 2 and Day 3. T2 population was also significantly increased at Day 8. No differences were reported in non-specific blastic transformation.

- Cooper *et al* 1998 report peripheral blood mononuclear cell proliferation and cytokine production (IFN-γ, IL-4, IL-5, IL-10) to purified protein derivative of tuberculin before and 6 months after vaccination in 19 *O. volvulus*–infected subjects and 20 comparable non-infected controls. IL-4, IL-5 and IL-10 responses were negligible. No significant differences were reported.
- Gentile *et al* 2006 report PHA stimulated IFN-γ and IL-13 responses in 15 subjects with allergic rhinitis (AR) and 15 similar healthy subjects. Significant increases from baseline were reported in those without AR but not in those with AR.
- Donnenberg *et al* 1984 report a 'modest but significant' rise in HSV-specific antigen reactive cells at 28 days post immunization in 15 individuals. No p values or confidence intervals for the fold rise from baseline are reported and it is unclear whether the fold rise at 7 days is significant.
- Fevrier *et al* 1977 reported ^{3}H-thymidine incorporation by either total blood lymphocytes, purified B cells, purified T cells or a mixture of purified B and T cells stimulated with mitomycin-treated allogeneic lymphocytes. No statistical tests were performed
- Heine *et al* 2007 report staphylococcus enterotoxin B (SEB) stimulated cytokine responses (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10) and 25-hydroxyvitamin D in 32 individuals randomised to receive 2000 IU of vitamin D or placebo and followed for 10 weeks are TT booster immunization. The paper reports that Vitamin D supplementation did not affect SEB stimulated responses however 25-hydroxyvitamin D levels differed as would be expected. After booster TT immunization 25-hydroxyvitamin D levels decreased ($p \leq 0.0007$) within the placebo (no Vitamin D supplementation) group alone when compared to pre-booster levels.
- Livingston *et al* 2013 reported PBS antigen-specific cytokine secretion (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, IL-13, IL17) by PBMC cultures before and after booster immunization with tetanus toxoid in previously vaccinated individuals who had not been boosted in at least 5 years as well as intracellular cytokine expression by CD4+ T-cells. Results are compared to responses to TT stimulation (which were higher). Changes in PBS results are plotted but not specifically tested for changes in those results alone.
- Mahalingam *et al* 2010 reported a significant rise in ConA stimulated IFN-γ in 108 healthy female adults, 56 days after vaccination when compared to baseline levels, but not at 28 days after vaccination. This rise in ConA stimulated IFN-γ was more substantial in those receiving 400mg of vitamin E supplementation (tocotrienol-rich fraction (TRF).

Data could not be extracted from the following papers;

- Di Genova *et al* 2006 reported proliferative responses to unrelated antigens PPD and *C. albicans* in 12 individuals at variable time points (between 1 and 12 weeks) after vaccination. Individuals' results are plotted but group summary statistics are not presented.
- Fernandez *et al* 1994 reported lymphoproliferation activity for 3 individuals to different levels of PPD stimulation. No comparisons are reported.
- Fryauff *et al* 1999 conducted lymphoproliferation assays stimulated with PHA however the data were not shown.
- Chui *et al* 2004 described the conduction of lymphoproliferation assays to unstimulated and HBsAg stimulation, but did not report the results. IFN-ϒ ELIspots were also performed using PHA, HBsAg and unstimulated controls, however these results were also not reported.

Measles

There were 8 papers reporting non-specific effects with data in a format that could be extracted. These papers report 23 different immunological parameters the main ones being B cells, β2-microglobin, CD4, CD4:CD8 ratio, CD8, IFN-γ, IL-10, IL-2, sIL-2Ra, IL-4, IL-6, lymphocytes, lymphoproliferation, malaria parasites, MIP-1β, Neopterin, sCD4, sCD8, T-cell proliferation, TNF-α, and WBC (Figures 12, 13 and 14).

There were 6 types of stimulants used in the above assays (Candida, PHA, tetanus toxoid, and unstimulated) resulting in 31 unique combinations of the above.

All of the papers contained participants who were under five years of age however there were no consistent findings for the main parameters (IFN-γ, IL-10, sIL-2Ra, IL-2, CD4, CD8) reported in papers where data could be extracted.

In addition to the studies described below the following studies conducted assays for non-specific immunological outcomes to measles vaccine, but did not report the results:

- Gans *et al* 2004 conducted T cell proliferation assays to PHA following vaccination with monovalent measles vaccine, however the results were not reported.
- Jaye *et al* 2014 conducted assays examining cytotoxic T cell responses following vaccination, however the non-specific outcome data were not reported.
- Okada *et al* 2001 report that on day 7 after measles vaccination, average numbers of total lymphocytes were relatively decreased to the lower limit of normal ranges before rising again by day 30. No p-values are reported and it is unclear what statistical testing was conducted.

In addition to the studies described above the following studies described non-specific immunological outcomes to measles vaccine with non-significant changes:

- Bertley *et al* 2004 reported unstimulated (vero) lympoproliferative responses in children following measles vaccination with either one or two doses, with no significant changes between groups noted.
- Gans *et al* 1999 conducted assays measuring IFN-γ in unstimulated culture supernatants, but did not report the results. T cell proliferative responses to PHA were also conducted with no significant differences reported between or within the study groups comparing before to after vaccination. Measles vaccine was given initially with MMR subsequently given at 12 months.
- Pabst *et al* 1999 measured non-specific proliferative responses to unstimulated (vero), tetanus toxoid, Candida and reported that there were no decreases in any of these responses after vaccination with AIK-C or CLL in 6 month olds. Additionally, there were no differences between groups or vaccines.

In addition to the studies described above the following studies described non-specific immunological outcomes to measles vaccines with significant changes noted for at least one parameter:

- Hennino *et al* 2007 reported eczema severity, utilising a scoring system in infants vaccinated with measles against placebo, with no significant change noted. Serum levels of CCL18 were significantly decreased in 2 measles vaccinated individuals compared to baseline. There were no significant differences in any individuals for serum E-selectin levels.
- Hussey *et al* 1996 reported PBMC proliferation to PHA at time-points following measles vaccination with either E-Z or Schwarz strains. There was a significant decrease from baseline to 3 months, within the group that received Schwarz at 6 months. There was also a significant decrease from baseline to 2 weeks and 3 months within the group that received Schwarz at 9 months. No significant changes were noted within the E-Z group. In the group that received Schwarz at 6 months there was a significant decrease in lymphoproliferation to PHA at 3 months compared to baseline. In the groups that received Schwarz at 9 months there was a significant decrease in lymphoproliferation at 2 weeks and 3 months compared to baseline. There was increased soluble CD8 and β2 microglobulin at 2 weeks and 3 months compared to baseline and also increased Neopoterin at 2 weeks compared to baseline in this study group. All other parameters in the three study groups were not significant.
- Samb *et al* 1995 report a significantly lower response to Rabies antibodies in 32 girls who received high-titre E-Z compared to 31 girls receiving Schwarz measles vaccine after all children had received 2 doses of rabies vaccine 4 weeks apart at age 36-44 months. No difference was seen in boys or overall. No differences were reported for Yellow fever antibodies.
- Ovsyannikova *et al* 2003 report on PHA stimulated TNF-α, IL-2, IL-4 and IL-6 (all non-significant when comparisons between younger children and older children). Unstimulated TNF-α responses

following vaccination were significantly higher in infants compared to older children but IL-4 and IL-6 were not.

- Njie-Jobe *et al* 2012 report a drop in unstimulated MIP-1β levels in response to a booster dose of E-Z vaccine at 36 months of age in both those who received a single priming dose of E-Z or 2 priming doses.

IFN-γ

IFN-γ data were extracted from five papers. PHA was the only stimulant reported and unstimulated assays were also conducted. Results from three papers, which reported results using PHA stimulation were plotted. One study (Ovsyannikova *et al* 2003) reported a significant difference between younger children (12-15 months) after one dose of Attenuvax and older children (4-12 years) who had received a second dose. Pabst *et al* 1999 and Schnorr *et al* 2001 did not report any significant differences between vaccinated and unvaccinated children. All three studies reported on different measles vaccines to each other (E-Z, Schwarz, AIK-C, CLL and Attenuvax).

Three studies reported data relating to unstimulated IFN- γ production. Ovsyannikova *et al* 2003 reported a significant difference when comparing production of IFN- γ following two doses of Attenuvax in older children compared to one dose in younger children. Njie-Jobe *et al* 2013 reported no difference in response to E-Z vaccine and Liguori *et al* 1998 also reported no difference (vaccine strain not reported).

IL-10

IL-10 data were extracted from three papers. PHA was the only stimulant reported and unstimulated assays were also conducted. Results from 2 studies that reported results using PHA stimulation were plotted. One study (Schnorr *et al* 2001) reported a significantly higher difference between measles vaccinated children (E-Z or Schwarz strains) at 6 or 9 months of age compared to unvaccinated children who were on average 10 months of age. Pabst *et al* 1999 reported no differences in response to AIK-C and CLL strains 8 weeks after vaccination at 6 months of age. The vaccinated children had received either E-Z or Schwarz vaccine according to local standard procedures.

Njie-Jobe *et al* 2012 report a drop in unstimulated IL-10 in response to a booster dose of E-Z vaccine at 36 months of age in both those who received a single priming dose of E-Z or 2 priming doses.

IL-2

IL-2 data were extracted from two study reports using PHA stimulation. Results from these two studies, which reported results in pg/mL and calculated geometric means or medians with IQR were plotted. One study (Schnorr *et al* 2001) reported a significant higher difference between vaccinated children at 6 or 9 months of age compared to unvaccinated children who were on average 10 months of age. The vaccinated children had received either E-Z or Schwarz vaccine according to local standard procedures. Ovsyannikova *et al* 2003 reported no differences. Both studies reported different measles vaccine strains to each other including E-Z, Schwarz, Attenuvax.

Soluble interleukin-2 receptor alpha subunit (sIL-2Ra)

sIL-2Ra data were extracted from three papers reporting unstimulated or PHA stimulated responses. No meta-analysis of these data was possible. Results from two papers, which reported unstimulated results in pg/mL and calculated medians with IQR were charted. One study (Ovsyannikova 2003) reported a significantly higher difference between younger children (12-15 months) after one dose of Attenuvax and older children (4-12 years) who had received a second dose. Njie-Jobe *et al* 2012 report no significant differences. Both studies reported different measles vaccine strains to each other (E-Z, Attenuvax).

Hussey *et al* 1996 report PHA stimulated sIL-2Ra responses to HT E-Z and Schwarz vaccines (at 6 and 9 months of age). sIL-2Ra values after vaccination were no different to baseline. Comparisons between the vaccine groups (HT E-Z, Schwarz at 6 months and Schwarz at 9 months) were statistically significant at 2 weeks and 3 months after immunization however they were also significantly different at baseline (pre-vaccination) thus these results don't appear to represent the effect of the vaccine.

CD4 and CD8 T lymphocytes

Three manuscripts reported total CD4 and CD8 counts with medians and IQR or means and 95% CIs. Lisse *et al* 1994 reports a follow-up of two separate studies. No significant differences were noted within any of the studies.

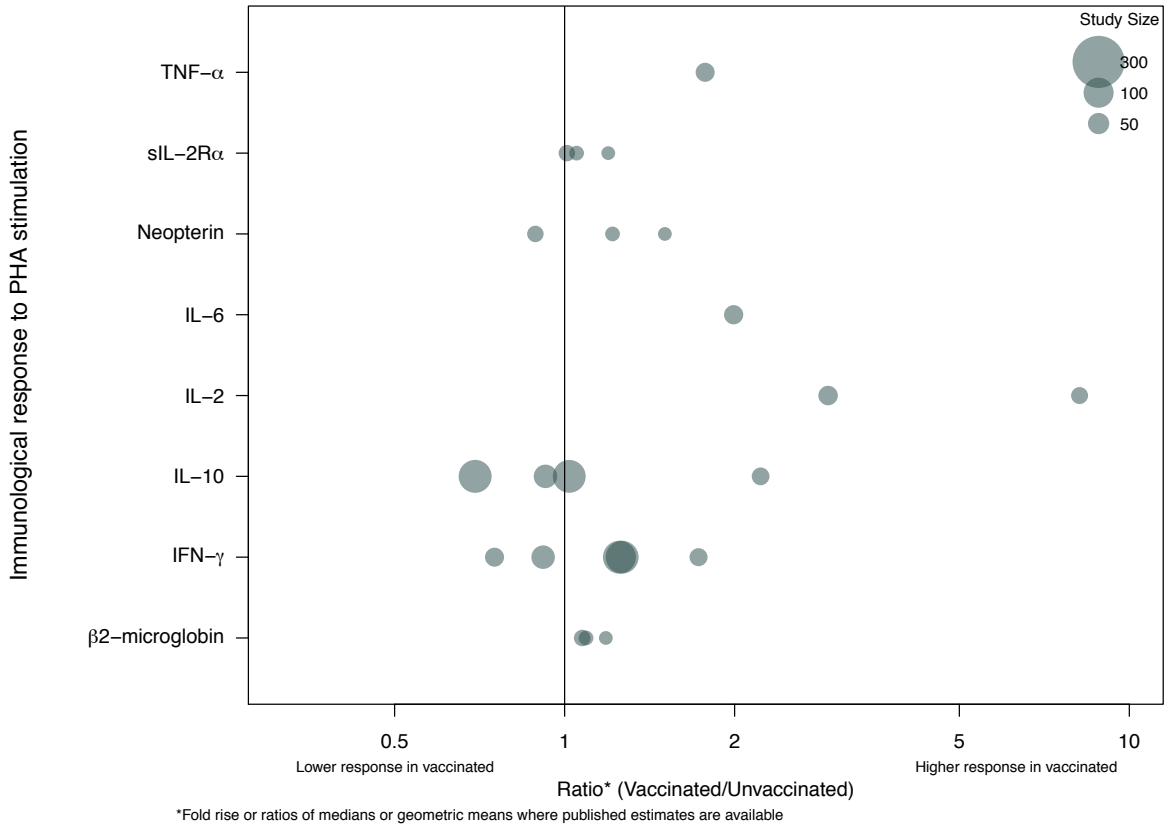


Figure 12. Immunological response ratios, comparing vaccinated to unvaccinated, in PHA stimulated cultures, from included measles vaccine studies.

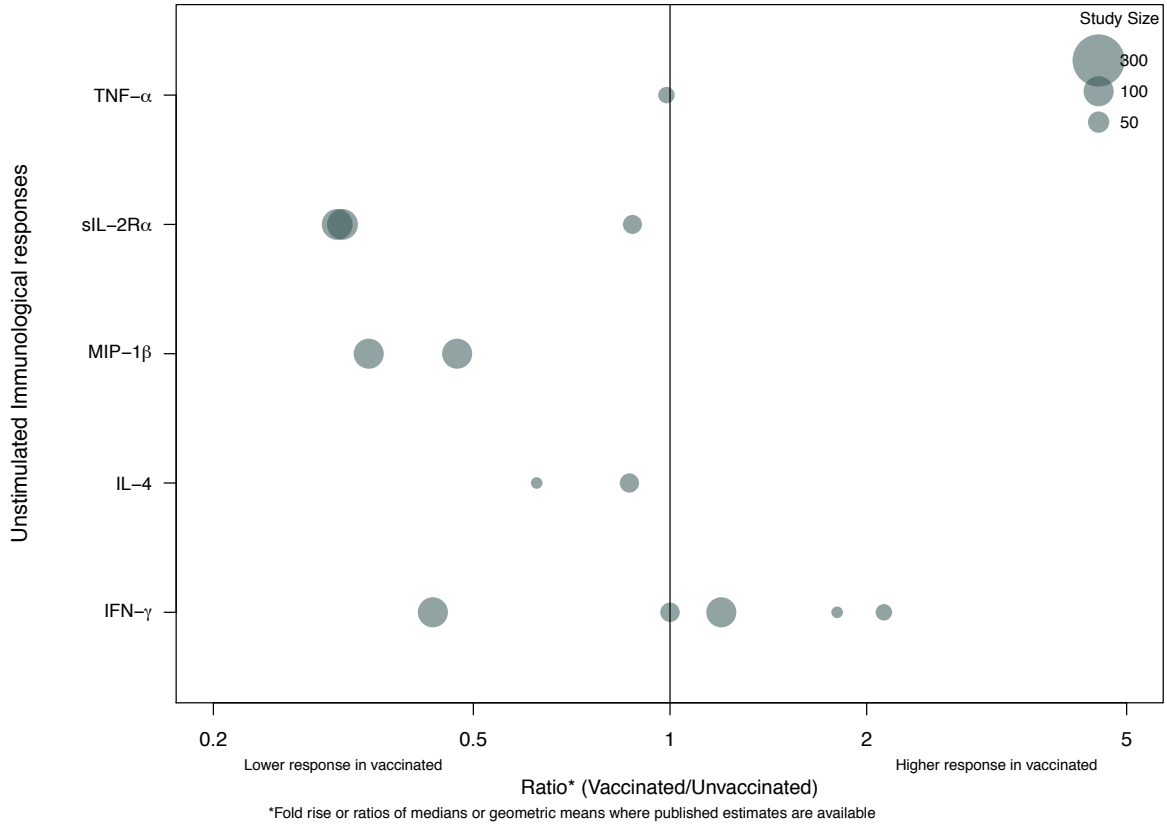


Figure 13. Immunological response ratios, comparing vaccinated to unvaccinated, in unstimulated cultures, from included measles vaccine studies.

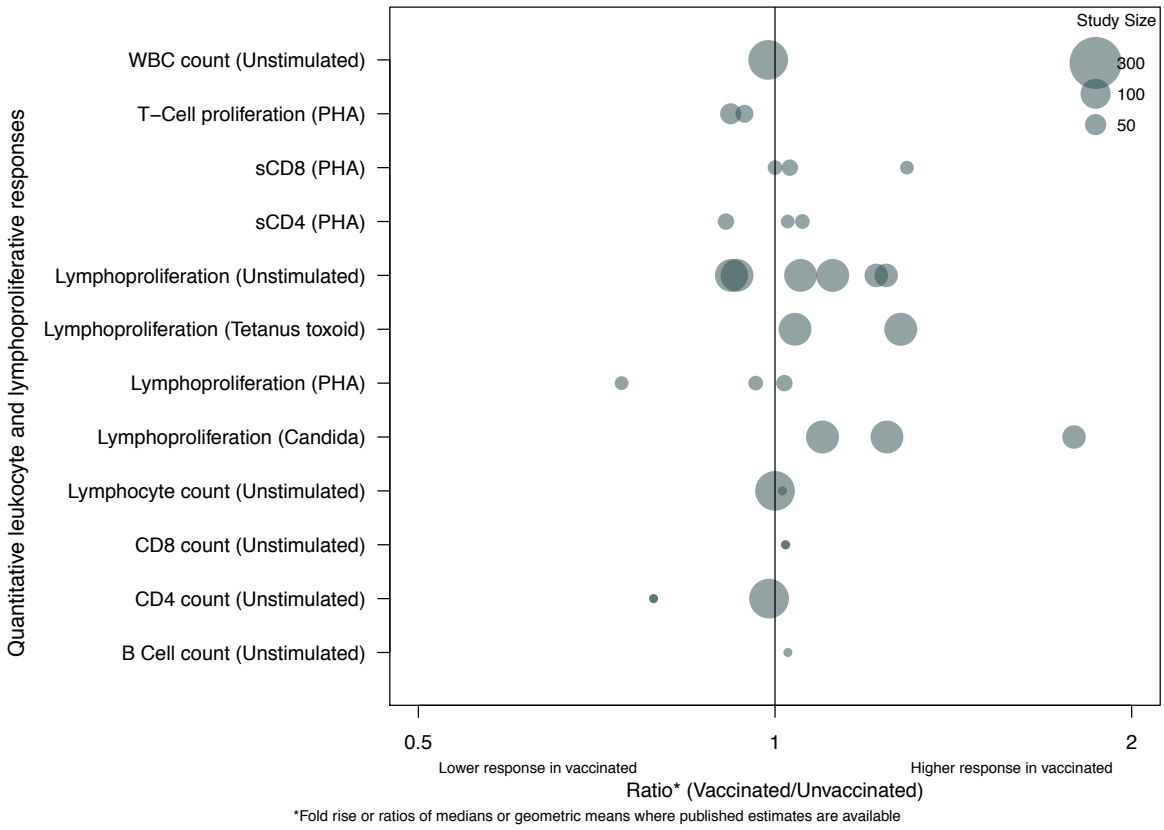


Figure 14. Non-specific antigen stimulated lymphoproliferation and leukocyte count response ratios, comparing vaccinated to unvaccinated, from included measles vaccine studies.

MMR

There were three papers reporting responses to non-specific stimuli with data reported in a format which could be used or extracted. Two papers conducted studies in children less than 5 years, whilst the third followed up vaccinated infants at mean age of 6.14 years. These papers reported 10 different immunological parameters the main ones being, CD4, CD4:CD8, CD56, CD8, IFN-γ, lymphoproliferation, NK, T-Cell proliferation and WBCs.

There were five types of stimulants used in the above assays (Candida, PHA, TT, and unstimulated) resulting in 13 unique combinations of the above.

Described below are the main parameters reported in papers from where data were extracted. Results that were not plotted (due to only being reported in one paper) include;

- Pabst *et al* 1997 report no difference in blast transformation in PBMC in response to tetanus toxoid or no antigens in the month post vaccination. One significant decrease in blast transformation to candida antigen was reported at 22 days after vaccination but was not significant at 14, 30 or 38 days.
- Gans *et al* 1999 report T cell proliferative responses to PHA of infants before and 12 weeks after measles/MMR immunization were no different.
- Rager-Zisman *et al* 2003 report there was a significant increase in mean percent CD56+ cells before and after secondary MMR immunization and proliferative responses to PHA were unchanged (P = 0.158) and to TT (P = 0.006) were improved after immunization in n=28, 6 year olds.
- Assays for IFN-α responses from unstimulated lymphocytes, from children vaccinated with MMR were conducted by Nakayama *et al* 1990, however the results were not reported.

CD4 T Lymphocytes

CD4 counts (%) were extracted from two studies (Figure 15). Both studies reported statistically significant decreases in CD4: Rager-Zisman *et al* 2003 report a significant difference between pre and one-month post booster vaccination in 28 children approximately 6 years of age and Pabst *et al* 1997 report a significant decline in CD4 T lymphocytes from pre to 38 days post-priming vaccination in 33 infants. No significant difference was seen in a separate cohort tested at 30 days post vaccine dose.

CD8 T lymphocytes

CD8 counts (%) were extracted from two papers. Both studies reported statistically significant results but in different directions: Rager-Zisman *et al* 2003 report a significant decline between pre and 1 month post booster vaccination in 28 children approximately 6 years old whereas Pabst *et al* 1997 report a significant increase in CD8 T lymphocytes from pre to 38 days post-priming vaccination in 33 infants. No significant difference was seen in a separate cohort tested at 30 days post dose.

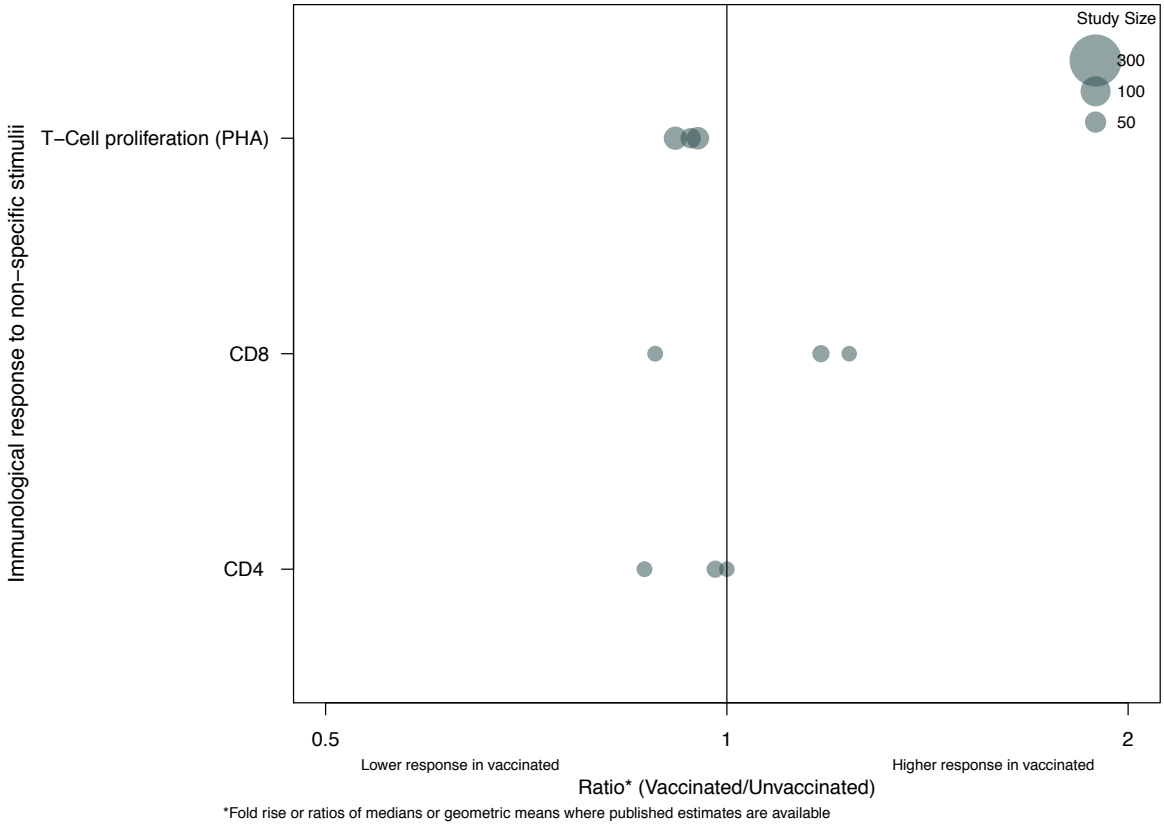


Figure 15. Immunological response ratios, comparing vaccinated to unvaccinated, for T cell proliferation to PHA stimulation and total counts of CD4 and CD8 T cells in included MMR vaccine studies.

DTP and DT

There were ten studies identified that contained assays of non-specific immunological responses following immunization with DTP or DT. There were four studies, which co-administered a polio vaccine, whilst Zorzeto *et al* 2009 did not describe co-administration with polio, however it is likely given the study demographic. No two studies had data that were extractable and could be compared descriptively in a graphical form.

Four of the studies were in children aged below five years of age. Only one study showed a significant increase in IL-5 and IL-13 after DTP in infants, however this was not replicated in any other study.

Dirix *et al* 2009 reported IFN-γ, IL-12p70 and IL-10 responses to PHA stimulation of PBMCs in infants who had received Tetravac. No significant changes over time following vaccination were noted.

Fernandes *et al* 2010 measured B lymphocyte subsets in adults following DT vaccination, with no significant changes noted. Pre-plasma cell IgA and IgG responses to Polio and HSV were also measured with significant increases in HSV IgA, IgG, and Polio IgA at day 7 compared to day -7 and day 28.

Fryauff *et al* 1999 measured lymphocyte proliferation to PHA following DT immunisation in adults, however the results were not reported.

Halasa *et al* 2008 reported polio neutralisation assays, and pneumococcal antibody levels following immunisation of infants with either 4 or 5 doses of DTP. In the 4 dose compared to 5 dose group there was a significantly higher Pneumococcal serotype 14 GMC at 7 months. In the 5 dose compared to 4 dose group there was a significantly higher Polio 1 and 3 GMC at 18 months.

He *et al* 1998 conducted cytokine mRNA expression studies following PHA and medium alone stimulation of PBMCs following DTP vaccination, however the results were not reported. Proliferative responses were also measured with no significant differences noted following immunisation in the medium alone cultures. The data for the PHA stimulated proliferative responses were not reported.

Heine *et al* 2011 conducted T cell activation studies using Staphylococcus enterotoxin and without any stimulation following DT vaccination of adults, with no statistical differences reported. Supplementary data reported a range of immunological parameters, with only the monocyte count being significantly reduced in the placebo group following vaccination.

Lin *et al* 1997 conducted studies examining lymphocyte proliferation to ConA, PHA and PWM following DTP vaccination of adults with no significant changes noted. Cytokine responses from these cultures were also examined with no significant differences noted either.

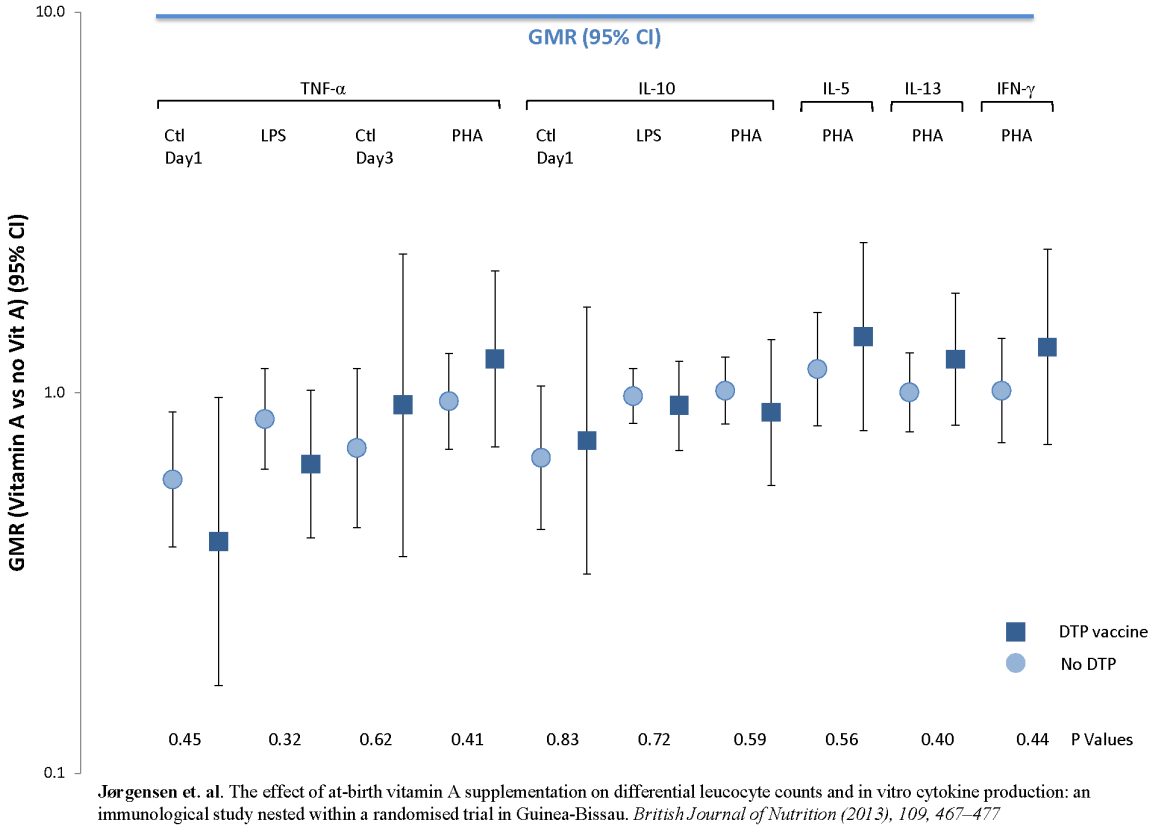
Rowe *et al* 2000 examined cytokine responses by PBMCs to PHA stimulation following DTP vaccination of infants and demonstrated significant increases in IL-5 and IL-13 at 12 months.

Yousfi *et al* 2005 examined serum biochemical markers and leukocyte following vaccination with a DT-Polio-Typhim vaccine in elderly and young adults. There were significant increases in CRP, AGP, Fibrinogen, Haptoglobin and a decrease in Transthyretin following vaccination. There were also significant changes in monocyte (increase), lymphocyte (decrease) and neutrophil (increase) counts following vaccination.

Zorzeto eta la 2009 reported lymphocyte sub-population proliferation and cytokine production to PHA by infants immunised with either a conventional DTP vaccine or a low LPS content DTP vaccine. There were no significant changes noted.

DTP and Vitamin A

One study explored the effect of Vitamin A on cytokine (IFN-γ, TNF-α, IL-10, IL-5, and IL-13) responses in relation to receipt of DTP vaccination (Figure 16). The data were reported as geometric mean ratios of Vitamin A supplementation to no Vitamin A supplementation within each group of DTP vaccinated or unvaccinated subjects. No significant differences were noted.



Jørgensen et. al. The effect of at-birth vitamin A supplementation on differential leucocyte counts and in vitro cytokine production: an immunological study nested within a randomised trial in Guinea-Bissau. *British Journal of Nutrition (2013), 109, 467–477*

Figure 16. Effect of Vitamin A supplementation on cytokine responses to non-specific antigen stimulation of whole blood from DTP vaccinated and DTP unvaccinated infants.

Pertussis

Di Tommaso *et al* 1997 conducted lymphoproliferative assays to TT stimulation and media alone, after vaccinating four healthy adults who failed to previously seroconvert, with a monovalent pertussis toxin vaccine. The TT results were reported but not statistically analysed. The unstimulated controls were not reported. There were no included studies, which reported non-specific immunological effects in children less than 5 years of age.

Interpretation

The improvement in technology for testing immunological parameters (e.g. multiplex assays) allows multiple tests to be assessed at one time using one blood sample and greatly increases the chances of false positive results occurring due to chance alone. The standard arbitrary cut-point used for significance testing in these situations is $p<0.05$ means that there is a 5% chance of a false positive result with every p value computed. If a study reports the results of a multiplex assay testing 42 separate parameters and each one is tested at the $p<0.05$ level then the chances that one of those parameters will show a significant difference where none exists is high.

Our review has shown that there are a multitude of parameters, which have been used to assess potential non-specific immunological effects of vaccines during the past 6 decades. Many of these are only reported once and it is in these situations that single significant p values need to be interpreted with caution. Stronger evidence for any effect can be observed where more than one study has assessed the same parameter and where confirmatory results can be found from different studies.

Overall there is a very heterogeneous spread of study designs that could not be meta- analysed, with a low level of evidence provided by these studies. Thus we could not conclude from the current available data that there are any consistent findings to confirm non-specific immunological effects following vaccination with BCG, diphtheria, pertussis, tetanus or measles containing vaccines. In addition, the data from the included papers were not presented in a form such that the effect of sex on non-specific immunological effects could be analysed. More meaningful conclusions might be drawn if raw data analyses could be conducted using unpublished and published data. If the same summary statistics could be computed for each study then meta-analysis would be possible.

These findings do not exclude the possibility of important non-specific immunological effects of vaccines, which are well described in animal studies and accepted as occurring in humans by most immunologists. However, the human data do not provide the necessary evidence to provide any confidence in the nature, quality, quantity, kinetics or impact of non-specific immunological effects in young children after vaccination. At this stage it is not possible to provide any guidance of an expected effect or when/how to measure it.

To further investigate the subject, future detailed studies might be undertaken using a systems biology approach to capture the functional genomic, genetic, epigenetic and immunological effects of vaccines in a kinetic study, which provides data on the timing, duration, quality and magnitude of such effects. This would entail a rigorous statistical approach within a test cohort with confirmation by replication in an additional cohort of subjects from the same study. It would be of particular importance to gain an understanding of whether any such measurable effects are able to influence future inflammatory or innate/acquired immunological responses to exposure with vaccines or infectious agents. If reproducible signals are identified, these could then be used in large scale studies with relevant epidemiological endpoints to characterize the clinical significance of such vaccine effects.

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