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Delayed BCG Vaccination Results in Minimal Alterations in T cell Immunogenicity of Acellular Pertussis and Tetanus Immunizations in HIV-Exposed Infants

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Abstract

Background—Bacille Calmette-Guerin (BCG) is effective in preventing disseminated tuberculosis (TB) in children but may also have non-specific benefits, and is thought to improve immunity to unrelated antigens through trained innate immunity. In HIV-infected infants, there is a risk of BCG-associated adverse events. We aimed to explore whether delaying BCG vaccination by 8 weeks, when in utero HIV is excluded, affected T-cell responses to *B. pertussis* (BP) and tetanus toxoid (TT), in HIV-exposed, uninfected infants.

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CONFLICTS OF INTEREST

No authors have any conflicts of interest to declare.

Methods—Infants were randomized to receive BCG vaccination at birth or 8 weeks of age. At 8 and 14 weeks, T cell proliferation and intracellular cytokine (IL-2, IL-13, IL-17, and IFN-γ) expression was analyzed in response to BP, TT and Staphylococcal enterotoxin B (SEB) antigens.

Results—Delaying BCG vaccination did not alter T-cell proliferation to BP or TT antigens. Infants immunized with BCG at birth had higher CD4+ T cell proliferation to SEB at 14 weeks of age (p=0.018). Birth-vaccinated infants had increased CD8+ IL-2 expression in response to BP, but not TT or SEB, at 8 weeks. Infants vaccinated with BCG at 8 weeks had significantly lower IL-13 expression by BP-specific CD4+ and CD8+ T cells at 14 weeks (p=0.032 and p=0.0035 respectively). There were no observed differences in multifunctional cytokine response to TT, BP or SEB between infants vaccinated with BCG at birth versus 8 weeks of age.

Conclusion—Delaying BCG vaccination until 8 weeks of age results in robust T-cellular responses to BP and TT in HIV-exposed infants.

Clinical Trial Registry—NCT02062580

Keywords

BCG vaccine; T cell; Tetanus; Pertussis; Infants; HIV

INTRODUCTION

Tuberculosis (TB) remains a global health burden, with an estimated 500,000 pediatric cases in 2013[1]. One quarter of new cases occur in Africa, where bacille Calmette-Guerin (BCG) vaccine, a live, attenuated *Mycobacterium bovis* strain, is routinely given at birth to infants regardless of maternal HIV status. When administered at birth, BCG induces robust T-helper type I (Th1) responses in neonates, although this age group is typically thought to have a Th2-biased immune system [2]. Although the efficacy of BCG varies by age and region, BCG has been consistently shown to prevent disseminated TB in HIV-unexposed children [3].

BCG may also have non-specific beneficial effects. In West Africa, BCG vaccination was associated with a 28% reduction in all-cause mortality, however, a systematic review of all the evidence failed to convincingly corroborate these findings [4, 5]. BCG vaccine has been associated with serious adverse events in HIV-infected infants, including BCG immune reconstitution inflammatory syndrome (IRIS) and disseminated BCG disease [6, 7]. Thus, the WHO now considers known HIV infection as a contraindication to BCG vaccination [8]. However, this recommendation is not implemented in settings with high burden of HIV and TB, as infant status is typically unknown at birth [9].

Optimal timing of BCG vaccination would provide enough time to confirm HIV infection status in HIV-exposed infants while retaining the protective effects of the vaccine. The effect of delaying BCG vaccination on HIV-exposed infants' responses to the other routine vaccinations has been largely unexplored. In HIV-unexposed infants, BCG vaccination administered at birth improved humoral responses to pneumococcal, Haemophilus influenza type B (Hib) and tetanus toxoid antigens and increased cytokine responses to tetanus toxoid and polio antigens [10, 11]. Delaying BCG vaccination to 2 months of age increased cellular

and humoral response to the hepatitis B vaccine, increased antibody response to polio, but had limited effect on cytokine response to tetanus toxoid, and no effect on antibody responses to tetanus and diphtheria toxoids [12]. Delaying BCG vaccination to 10 weeks of age has been shown to increase CD4+ T cell polyfunctional cytokine response to BCG, but also to phytohemagglutinin (PHA) and SEB, two T-cell stimulating antigens[13]. Thus, while altering the timing of BCG vaccination in infants may not attenuate immunogenicity to *M. tuberculosis* [14], it may augment the ability of infants to respond to other antigens.

We previously reported that delaying BCG vaccination until 8 weeks of age in HIV-exposed infants did not compromise immunogenicity of the vaccine and resulted in robust BCG-specific T-cell responses [14]. Using this same cohort, we aimed to identify whether delaying BCG vaccination until 8 weeks of age affected T-cell responses to two other routine vaccinations: acellular pertussis and tetanus toxoid. We hypothesized that HIV-exposed infants who received BCG vaccine at birth would have higher Th1 responses to pertussis and tetanus antigens than the infants in the delayed vaccination arm due to early induction of Th1 responses by BCG.

MATERIALS AND METHODS

Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki [15]. The University of Cape Town and Stellenbosch University research ethics committees and the University of Washington Institutional Review Board approved the study. All mothers provided written informed consent.

Participant Recruitment and Vaccination

HIV-exposed infants were recruited from a community health centre in Khayelitsha, Western Cape Province, South Africa, as previously described[12]. Khayelitsha has a TB notification rate of 1,389 cases per 100,000 people, and an antenatal maternal HIV prevalence of 30.1%[16].

The eligibility criteria for inclusion were: maternal HIV-infection, uncomplicated pregnancy or labor, vaginal delivery, term gestation (>36 weeks), infant birth weight of >2.4 kg and no known close TB contacts. Infants were excluded if they tested positive for HIV DNA by PCR either at birth or at 6 weeks of age.

Infants were randomly assigned via a computer-generated list to receive BCG vaccination (Danish strain 1331; Statens Serum Institut, intradermal), either upon availability of HIV DNA PCR results (at 2–4 days of age, “standard vaccination arm”), or at 8 weeks of age (“delayed vaccination arm”). Infants in both arms of the study received all other routine vaccines according to the South African Expanded Program on Immunization (EPI) schedule [17].

Whole Blood Assay

The whole blood assay was performed as previously described [18]. Whole blood was diluted 1:10 in RPMI and incubated with the following antigens: 0.16 IU tetanus toxoid (TT)

(TETAVAX, Aventis Pharma Ltd.) or 0.16 IU whole cell inactivated pertussis antigen (BP) (Difco Bordetella Pertussis Antigen, BD).

Ki67 Proliferation and Intracellular Cytokine Assays

Cells were thawed, permeabilized and stained for viability (VIVID), proliferation, using Ki67[18], and the following intracellular cytokines with optimized concentrations of fluorescence-conjugated antibodies: anti-CD3-allophycocyanin (APC)-cyanine 7 (Cy7; UCHT1) (Biolegend), anti-CD8-peridinin chlorophyll protein-Cy5.5 (Sk1) (BD), anti-Ki67-fluorescein isothiocyanate (B56) (BD), anti-IFN-γ-Alexa Fluor 700 (B27) (Biolegend), anti-IL-2-APC (5344.111) (BD), anti-IL-13-pycoerythrin (JES10-5A2) (BD), anti-IL-17-phycoerythrin-Cy7 (BL168) (Biolegend), and VIVID-Pacific Blue (Invitrogen). Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA).

Data Analysis

Upon acquisition, compensation was performed using FlowJo v9.4 (Treestar, Ashland, OR). The gating strategy has been previously described and is shown in Supplementary Figure 1 [19]. Cells expressing IFN-γ, IL-2, IL-13, and IL-17 were gated within the Ki67+CD8+ and Ki67+CD8− cell populations. T-cells that were CD8− were considered to be CD4+[20]. Pestle V1.7 software (Vaccine Research Center, National Institutes of Health) was used for background subtraction of the negative control responses, and Spice V5.3 software, for analysis of multiple cytokine expression.

Sample size and power were calculated as previously reported[14], and were based on the comparison of the median number of CD4+Ki67+ cells per microliter in HIV-unexposed infants in response to tetanus toxoid. A sample size of 28 infants per arm allowed 80% power to detect a 5% different in the number of CD4+Ki67+ cells per microliter, using a 2-tailed t test with P=0.05. Samples were excluded if positive control responses were below the cutoff (median value of SEB plus 3 median absolute deviations from negative controls value) or the number of Ki67+ proliferating cells was less than the cutoff of 20.

Proliferation levels were calculated following subtraction of the Ki67+ cell frequency in the negative control. Mann-Whitney nonparametric testing was used to compare proliferation and cytokine expression levels between standard and delayed arms. The overall difference in expression of cytokine polyfunctionality was compared using a 2-sided analysis of variance (ANOVA) using Spice.

RESULTS

Study Participant Characteristics

Between June 2010 and December 2012, 149 HIV-exposed infants were recruited as previously described by Tchakoute *et al* [14]. Two infants inadvertently received BCG vaccine at birth and were subsequently excluded from the study. Two infants died of conditions unrelated to tuberculosis or BCG. A total of 140 infants were eligible for randomization, none with reported TB signs or symptoms, of which 122 completed the

study. There were no differences in baseline characteristics between standard and delayed vaccination arms[14].

Delaying BCG Vaccination Does Not Affect T-Cell Proliferation in Response to BP or TT Stimulation

CD4+ and CD8+ T-cell proliferation in the standard and delayed BCG arms was evaluated at 8 weeks of age (2 weeks after first dose of TT and BP vaccines) and 14 weeks of age (8 weeks after first dose, 4 weeks after second dose of both TT and BP vaccines) using Ki67 as a marker of proliferation. At 8 weeks of age, i.e. 8 weeks after BCG vaccination in the standard arm and prior to BCG vaccination in the delayed arm, infants in the standard and delayed arms had similar frequencies of proliferating CD4+ T-cells in response to BP and TT (Figure 1a, P=0.36 and P=0.74, respectively).

Additionally, CD4+ T-cell responses to SEB, a bacterial superantigen which served as a positive control, were equivalent in the standard and delayed vaccination arms (Figure 1a, P=0.85) but comparatively much higher than the response to BP and TT. CD8+ T-cell responses were also equivalent for BP, TT and SEB at 8 weeks for standard and delayed arms (Figure 1a, P=0.55, P=0.82 and P=0.87).

At 14 weeks of age, i.e. 14 weeks after BCG vaccination in the standard arm and 6 weeks post-vaccination in the delayed vaccination arm, there was no difference in either CD4+ or CD8+ T cell response to BP or TT (Figure 1b). The standard vaccination arm had higher CD4+ T-cell responses to SEB (29.4% [8.25%; 56.7%]) than the delayed arm (16.5% [6.75%; 21.3%]) (P=0.018) although CD8+ T-cell responses were equivalent between groups (Figure 1b).

Delaying BCG vaccination results in increased IL-2 expressing CD8+ T cells in response to BP

IL-2 and IFN-γ expression in proliferating CD4+ and CD8+ T-cells were evaluated in the standard and delayed vaccination arms at 8 and 14 weeks of age as markers of Th1/Tc1 bias[21]. Infants receiving BCG vaccination at birth had a higher proportion of IL-2 expressing CD8+ T cells at 8 weeks of age in response to stimulation with BP antigen (0.465% [0%; 1.89%]) than infants in the delayed vaccination arm (0% [0%; 0.431%]) (Figure 2a, P=0.045) but not to TT antigen (Figure 2b) or polyclonal SEB (Figure 2c). There were no differences in proportion of IL-2 expressing CD4+ T cells in response to any antigen at 8 weeks of age. By 14 weeks of age this difference in the proportion of CD8+ IL-2 expressing T-cells in the standard versus delayed arms in response to BP were no longer evident, nor were there any differences in the proportions of CD4+ or CD8+ IL-2 expressing proliferating T-cells in response to any of the antigens (Figure 2). There were no differences in IFN-γ expression by T cells to any antigen at either time point (Figure 2).

Delaying BCG Vaccination Results in Lower T cellular IL-13 expression in response to BP Stimulation at 14 Weeks of Age

IL-13 expression in proliferating CD4+ and CD8+ T-cells was evaluated in the standard and delayed vaccination arms at 8 and 14 weeks of age as a measure of Th2-type responses[22].

At 8 weeks of age, no differences in the proportion of IL-13 expressing CD4+ and CD8+ T-cells between the standard and delayed vaccination arms to BP, TT or SEB antigens were observed (Figure 3). At 14 weeks of age, there were no differences in the proportion of IL-13 expressing CD4+ or CD8+ T-cells in the standard versus delayed arms in response to TT (Figure 3b) or SEB (Figure 3c) antigens. However, at 14 weeks of age, the standard vaccination arm, who had received BCG vaccination 14 weeks earlier, had higher proportions of IL-13 expressing CD4+ (0% [0%; 0.118%] vs. 0% [0%; 0%] (P=0.032) and CD8+ (1.40% [0.187%; 6.27%] vs. 0% [0%; 0.838%]) (P=0.0035) T-cells in response to BP compared to the delayed vaccination arm, who had received BCG 6 weeks earlier (Figure 3a).

Delaying BCG Vaccination Does Not Alter T cellular IL-17 Responses to BP, TT or SEB Stimulation at 8 or 14 weeks

IL-17 expression in proliferating CD4+ and CD8+ T-cells were evaluated for the standard and delayed vaccination arms in response to BP, TT and SEB. No differences in the standard versus delayed vaccination arms were observed for IL-17 expression in CD4+ or CD8+ proliferating T-cells at 8 or 14 weeks (data not shown).

Delaying BCG Vaccination Has No Effect on T-cell Cytokine Polyfunctionality in Response to BP or TT

The polyfunctionality of proliferating cells in response to TT and BP to express various combinations of the measured cytokines was compared between infants in the standard and delayed vaccination arms at 8 weeks of age (Figure 4). Overall, there were no significant differences in response to BP or TT in the standard versus delayed arm for either CD4+ or CD8+ T cells. In response to BP, the majority of proliferating CD4+ T cells from both the standard and delayed arms were single cytokine-expressing (Figure 4a). The proliferating CD8+ T cells from the standard arm were predominantly dual cytokine-expressing, whereas the delayed arm was predominantly single cytokine-expressing (Figure 4a), however this difference was not significantly different (p=0.85). There were no overall differences in polyfunctionality for CD4+ or CD8+ T cells in response to TT (P=0.77 and 0.31 respectively) (Figure 4b). Specifically, in response to TT, the proliferating CD4+ cells in the delayed arm had a larger proportion of cells producing three cytokines than in the standard arm (Figure 4b), although this difference was not statistically significant (p=0.75). The CD8+ T cells in the standard arm had a larger proportion of cells producing two cytokines than those in the delayed arm, though not statistically significant (p=0.64). The breakdown of CD4+ and CD8+ T-cells producing single cytokines or combinations of cytokines is shown in Figure 4c–d. There were no significant differences in cytokine functionality between infants in the standard and delayed vaccination arms at 14 weeks (data not shown).

Delaying BCG Vaccination Has No Effect on T-cell Cytokine Polyfunctionality in Response to SEB

The functionality of proliferating cells in response to SEB making 0 cytokines, any single cytokine, or any combination of 2, 3 or 4 cytokines was compared between infants in the standard and delayed vaccination arms at 8 (data not shown) and 14 weeks of age (Figure 5). Although we observed differences in the proportion of proliferating CD4+ T-cells in

response to SEB stimulation at 14 weeks of age (Figure 1a), there were no differences in polyfunctional CD4+ or CD8+ (Figure 5a) T cells at 14 weeks of age. There were similarly no differences at 8 weeks of age (data not shown) and of note, the majority of the proliferating T-cells were expressing none of the measured cytokines. The range of specific cytokine production by CD4+ and CD8+ T-cells in response to SEB is shown in Figure 5b.

DISCUSSION

The overall goal of the randomized clinical trial was to seek an alternative BCG vaccination strategy for HIV-exposed infants that would allow for adequate time to confirm HIV infection status while preserving the protective effects of the vaccine for uninfected young infants [14]. Of equal importance is whether varying the timing of BCG vaccination might impact responses to other vaccines in the routine EPI schedule. This is the first study to examine the effects of delaying BCG vaccination on T-cell responses to two other routine infant vaccinations, pertussis and tetanus, in HIV-exposed uninfected infants. Our results indicate that delaying BCG vaccination minimally altered the immunogenic properties of either TT or BP, as measured by CD4+ or CD8+ T-cell proliferation and cytokine functionality.

Sampling the standard and delayed vaccination arms at 8 and 14 weeks of age provided an opportunity to examine different immunological scenarios. At 8 weeks of age, infants in the standard vaccination arm had received the BCG vaccine 8 weeks earlier but those in the delayed arm had not yet received BCG. In contrast, at 14 weeks of age, infants in the standard arm had received BCG 14 weeks earlier, and those in the delayed arm had received BCG 6 weeks earlier. Although the fetal and neonatal immune system is heavily Th2/ Tc2 biased [23–25], it does not appear that receiving BCG vaccination, a Th1-driven response, alters this profile. At 8 weeks of age there was a higher proportion of IL-2 expressing, proliferating CD8+ T cells in the standard vaccination arm than the delayed arm in response to BP, but not SEB or TT. The immunogenicity of acellular pertussis vaccination likely relies on a heterogeneous input from both Th1 and Th2 cells [26, 27]. Although increased IFN-γ ELISPOT responses have been noted in response to unrelated vaccinations in infants receiving BCG at birth, the cellular origin of these responses have not been identified [10]. T cells of BCG vaccinated adults produce increased IFN-γ and IL-17 in response to *in vitro* stimulation to heterologous antigens[28]. However, in the present study, BCG vaccination did not appear to induce significant Th1 or Th17 enhancement to unrelated antigens *in vivo*.

At 14 weeks of age, there was no difference in Th1 response to SEB, BP or TT in the standard versus delayed vaccination arms. However, there was an increased Th2 response to BP, but not TT or SEB, in the standard BCG vaccination arm. We are limited in making strong conclusions about differential T cell responses to TT at 14 weeks of age due to a low number of infants in the standard vaccination arm at this time point. It is important to consider that for this study, we are limited in making definitive claims that there are no differences between groups due to our low sample size for some of the antigens. Nonetheless, infants vaccinated at birth may have enhanced B-cell activation and maturation in response to BP due to BCG priming. Although it is widely believed that antibody is the correlate of protection against tetanus, for acellular pertussis, induction of cellular responses

is important [29, 30]. We did not measure humoral responses to vaccination in this study, however previous studies of the effect of BCG on humoral responses to tetanus show mixed results [11, 12, 31]. The few studies that have examined the effect of BCG timing on humoral responses to pertussis showed no effect [32].

Recent developments in the mechanisms underlying BCG vaccination-enhanced immunity to heterologous antigens indicate that these changes may be caused by innate cellular epigenetic reprogramming [33]. In adults, BCG vaccination was found to induce increased H3K4 methylation, through the NOD2 receptor, of IFN-γ, IL-1β and TNF-α. Epigenetic changes were observed 3 months and up to one year after BCG vaccination [28]. Enhanced pro-inflammatory cytokine production was reflected four weeks after immunization in low birth weight, BCG-vaccinated infants in Guinea-Bissau upon heterologous challenge [34]. However, other studies have not observed these differences when infants were BCG vaccinated at birth [13]. These studies were performed in HIV-unexposed populations, and there is evidence that HIV-exposed, uninfected infants may have different Th1 and Th2 cytokine profiles to BCG vaccination [35, 36]. We previously observed that HIV exposure results in reduced breadth and magnitude of cytokine production to BCG and acellular Pertussis vaccination in infants [19]. HIV-exposed infants also display increased monocyte and cDC production of pro-inflammatory cytokines in response to PAMPs [37], thus perhaps would have different epigenetics than unexposed infants. The effects of delayed BCG vaccination on immunity in HIV-exposed infants warrants further study.

We show that delaying BCG vaccination until 8 weeks of age results in minor alterations in immune responses to TT, BP, and SEB (non-specific T cellular activation). Prior studies of HIV-unexposed infants in high TB burden settings have observed low rates of *M. tuberculosis* infection prior to 8 weeks of age, indicating that it may be safe to postpone vaccination [13]. In most developing countries, BCG vaccine is routinely administered at birth, despite WHO recommendations that HIV infection is a contraindication for this vaccine. The optimal BCG vaccination schedule would leave adequate time to test for HIV infection and maintain immunogenicity of BCG as well as any potential non-specific enhancement that BCG may have on immune response to other routine childhood vaccines. Although follow up was short in our study, there were minimal effects on T cell immunogenicity to BP and TT by delaying BCG vaccination. These experiments warrant long-term, comprehensive studies of the risks and benefits of delayed administration of the BCG vaccine to HIV-exposed infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Delaying BCG until 8 weeks did not alter T-cell proliferation to BP or TT
- Infants immunized with BCG at birth had higher CD4+ T cell proliferation to SEB
- Infants BCG-immunized at birth had higher CD8+ IL-2 expression to BP
- Infants BCG-immunized at birth had higher CD4+ and CD8+ IL-13 expressions to BP
- Delaying BCG had no effect on overall T cell cytokine polyfunctionality

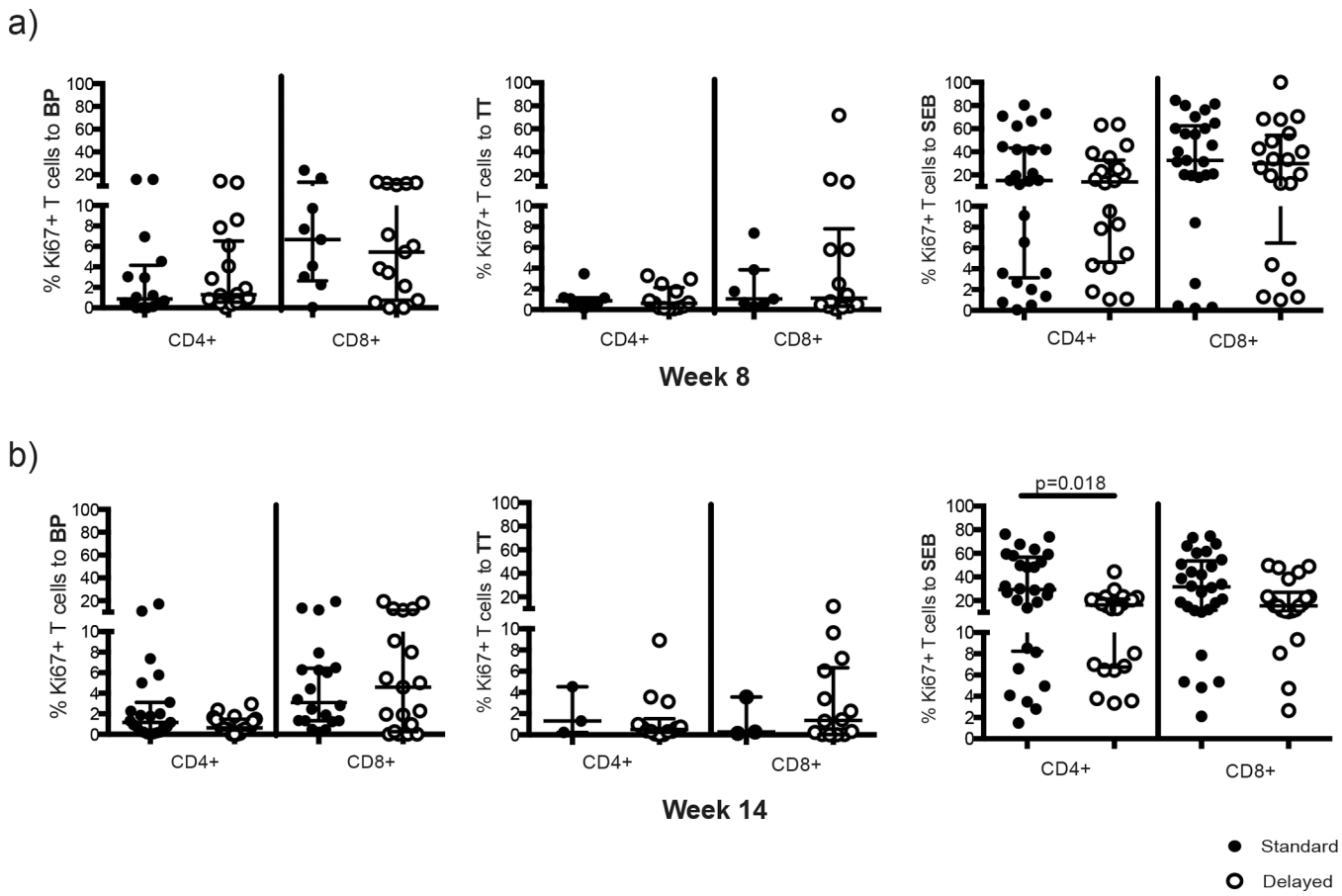

Figure 1. T cell proliferation at 8 (a) and 14 (b) weeks of age in response to BP, TT, and SEB

Frequency of proliferating (Ki67+) T cells in whole blood of infants in standard (black circles) and delayed (white circles) BCG vaccination arms. Frequencies reported as percentage of total CD4+ or CD8+ T cells. Bars indicate median ± IQR. Sample sizes for the early and delayed arms respectively at 8 weeks were: BP (n=16 and n=18), TT (n=8 and n=15), SEB (n=25 and n=20) and at 14 weeks were: BP (n=23 and n=20), TT (n=3 and n=14) and SEB (n=28 and n=22). Statistical analysis was performed using Mann-Whitney U test with alpha = 0.05.

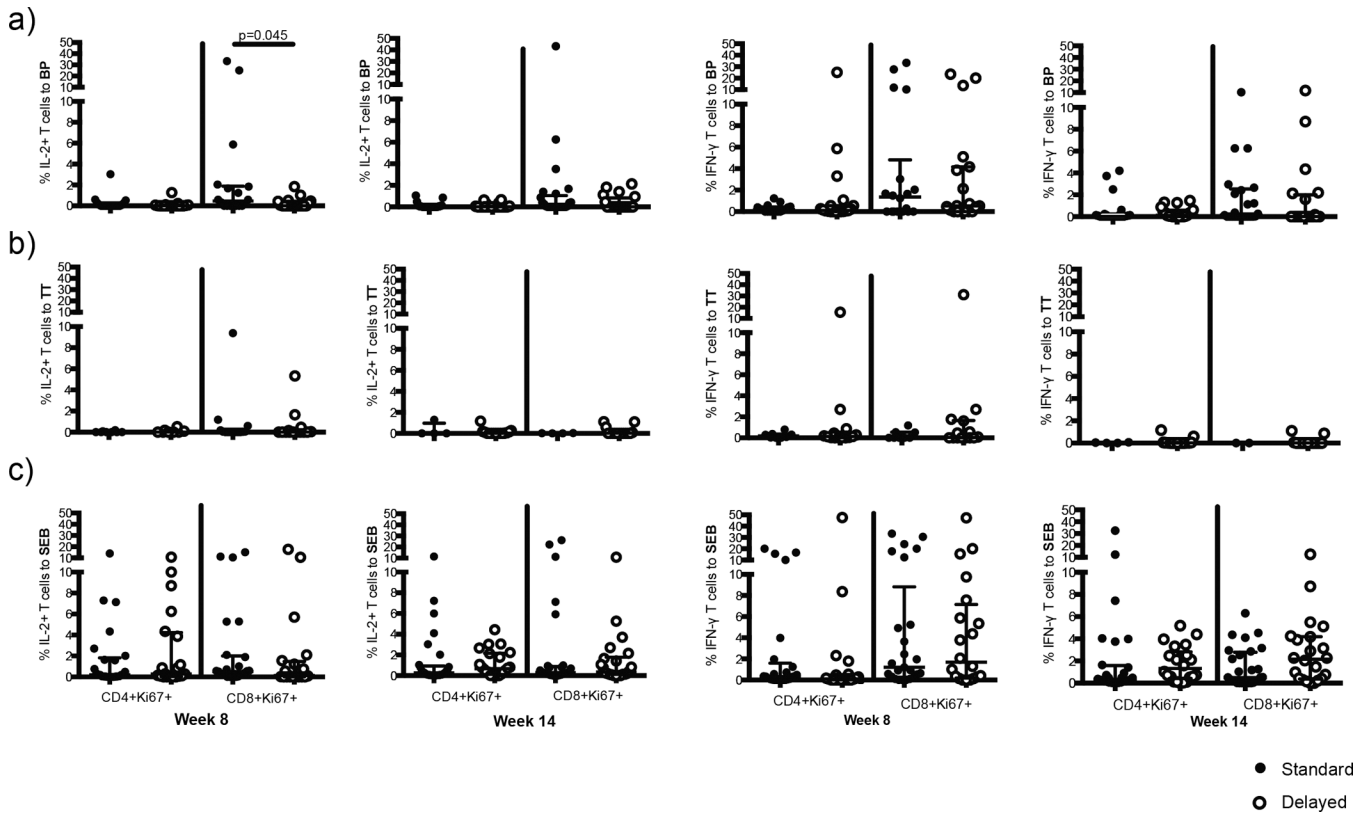

Figure 2. Frequency of IL-2 and IFN-γ producing proliferating CD4+ and CD8+ T cells in response to BP (a) TT (b) SEB (c) at 8 and 14 weeks of age

Proliferating (Ki67+) T cells in whole blood of infants in standard (black circles) and delayed (white circles) BCG vaccination arms. Frequencies of IL-2 and IFN-γ producing T cells reported as percentage of total CD4+ or CD8+ T cells. Bars indicate median ± IQR. Sample sizes for the early and delayed arms respectively at 8 weeks were: BP (n=18 and n=19), TT (n=10 and n=14), SEB (n=25 and n=20) and at 14 weeks were: BP (n=25 and n=20), TT (n=4 and n=15), SEB (n=29 and n=22). Statistical analysis was performed using Mann-Whitney U test with alpha = 0.05.

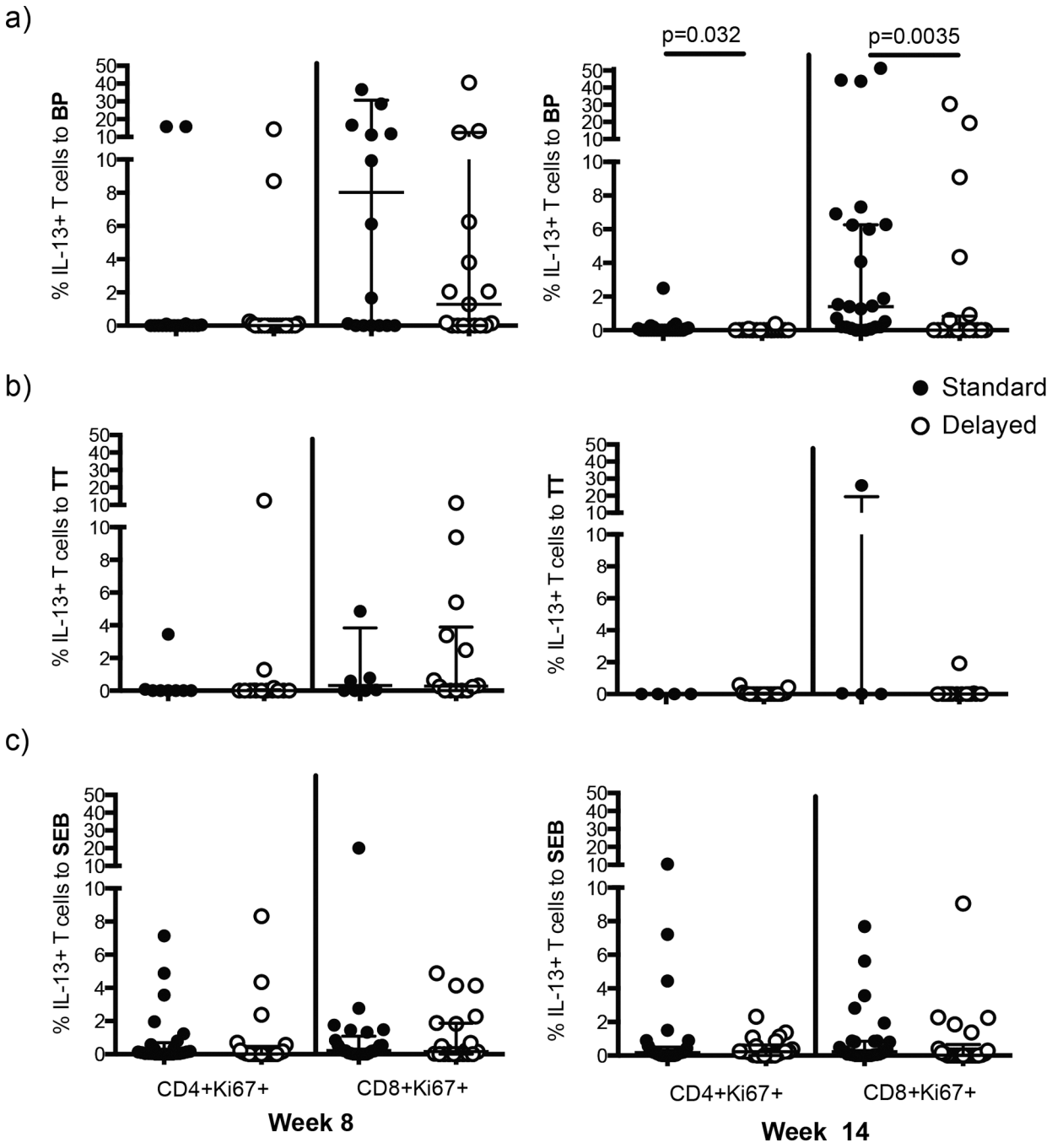

Figure 3. Frequency of IL-13 producing proliferating CD4+ and CD8+ T cells in response to BP (a), TT (b) and SEB (c) at 8 and 14 weeks of age

Proliferating (Ki67+) T cells in whole blood of infants in standard (black circles) and delayed (white circles) BCG vaccination arms. Frequencies of IL-13 producing T cells reported as percentage of total CD4+ or CD8+ T cells. Bars indicate median ± IQR. Sample sizes for the early and delayed arms respectively at 8 weeks were: BP (n=18 and n=19), TT (n=10 and n=14), SEB (n=25 and n=20) and at 14 weeks were: BP (n=25 and n=20), TT (n=4 and n=15), SEB (n=29 and n=22). Statistical analysis was performed using Mann-Whitney U test with alpha = 0.05.

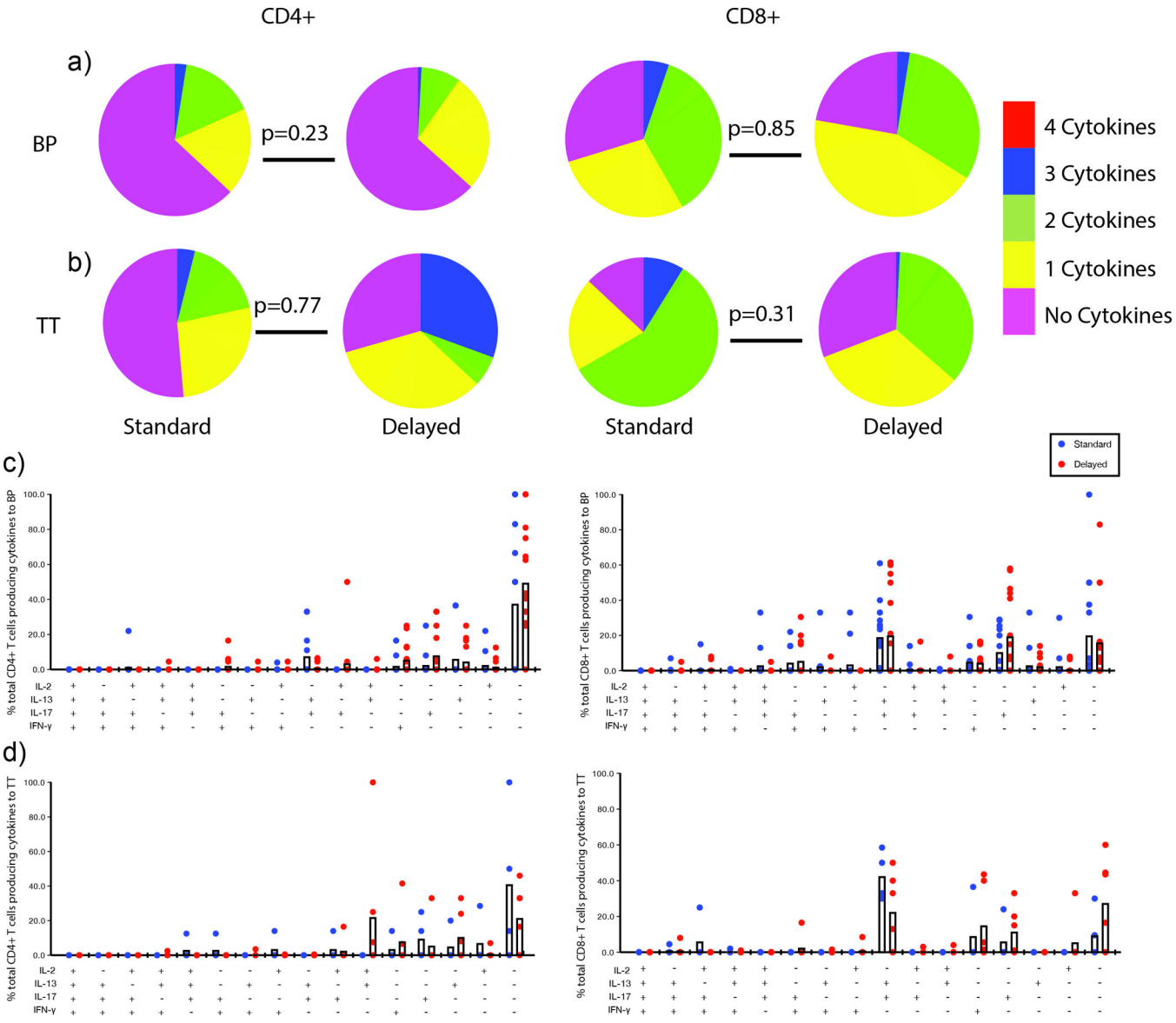

Figure 4. Proportions of proliferating (Ki67+) CD4+ and CD8+ T cells producing no, one, or a combination of cytokine(s) in response to pertussis (a,c) or tetanus (b,d) at 8 weeks of life in standard versus delayed BCG-vaccinated infants

a–b) Comparison of the proportion of proliferating T cells expressing no cytokine (pink), one cytokine (yellow), any combination of two cytokines (green), any combination of three cytokines (blue) or all 4 cytokines (red) based on expression of IL-2, IL-13, IL-17, and/or IFN-γ. Cd) Frequency of proliferating T cell in whole blood of infants in standard (blue) versus delayed (red) BCG vaccination arm. Frequency calculated as percentage of total CD4+ or CD8+ T cells. Sample sizes for the early and delayed arms respectively at 8 weeks were: BP (n=32 and n=55), TT (n=34 and n=28) and at 14 weeks: BP (n=64 and n=60), TT (n=38 and n=27). Box correspond to medians; statistical analysis performed using Wilcoxon signed-rank test and multiple comparison adjustment using the Holm stepdown procedure with alpha=0.05.

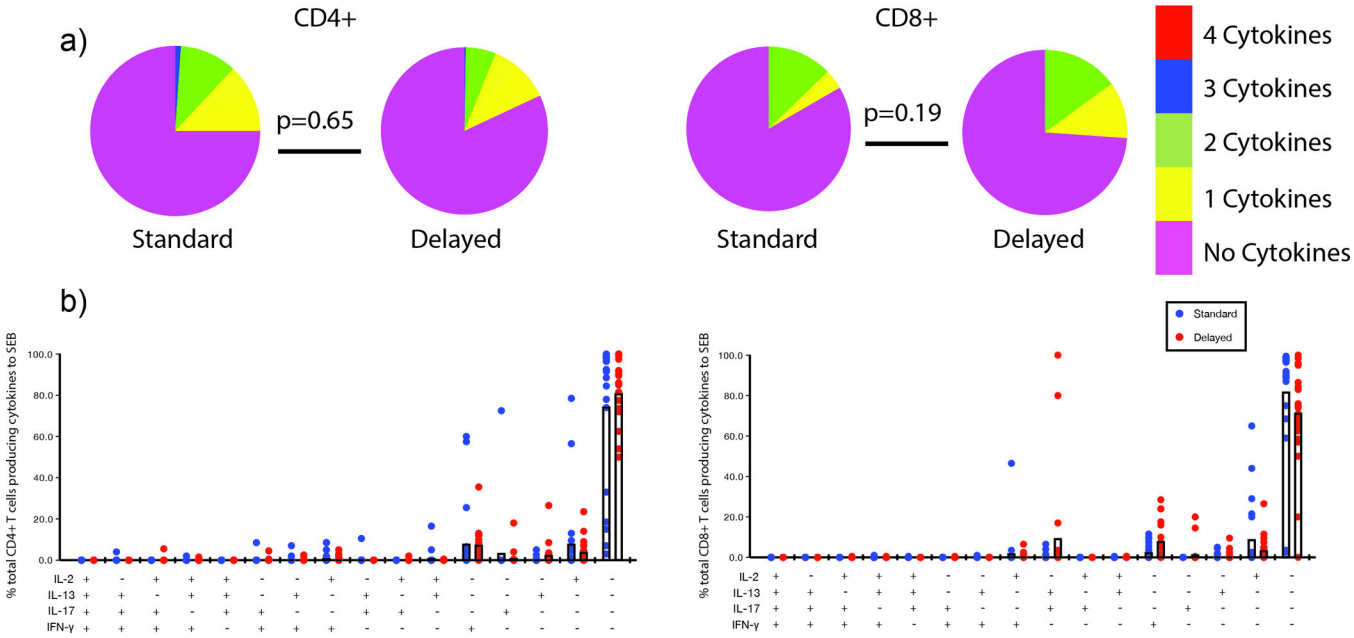

Figure 5. Proportions of proliferating (Ki67+) CD4+ and CD8+ T cells (a) producing no, one, or a combination of cytokine(s) in response to SEB in standard versus delayed BCG-vaccinated infants at 14 weeks of life

a) Comparison of the proportion of proliferating T cells expressing no cytokine (pink), one cytokine (yellow), any combination of two cytokines (green), any combination of three cytokines (blue) or all 4 cytokines (red) based on expression of IL-2, IL-13, IL-17, and/or IFN-γ. b) Frequency of proliferating T cell in whole blood of infants in standard (blue) versus delayed (red) BCG vaccination arm. Frequency calculated as percentage of total CD4+ or CD8+ T cells. Sample size for the early and delayed arms respectively at 8 weeks for SEB was n=63 and n=58, respectively, and at 14 weeks was n=61 and n=53, respectively. Box correspond to medians; statistical analysis performed using Wilcoxon signed-rank test and multiple comparison adjustment using the Holm step-down procedure with alpha=0.05.