Lack of broad functional differences in immunity in fully vaccinated vs. unvaccinated children

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BACKGROUND: Concerns have been raised that with an increase in the number of vaccines administered early in life, immune development could be altered, leading to either increased or decreased immune reactivity.

METHODS: We investigated the impact of vaccination on immune status, contrasting the immune response to general, nonantigen-specific stimuli in a cohort of entirely unvaccinated vs. fully vaccinated children at 3–5 y of age. Innate immunity was assessed by quantifying bulk and cell-type-specific cytokine production in response to stimulation with pathogen associated microbial patterns. Adaptive immune status was characterized by assessing lymphocyte proliferation and cytokine production in response to generic T cell stimuli.

RESULTS: Our investigations failed to reveal a broadly evident alteration of either innate or adaptive immunity in vaccinated children. Equivalently robust innate and adaptive responses to pathogen associated microbial patterns and generic T cell stimulants were observed in both groups.

CONCLUSION: Although our sample size was small, our data suggest that standard childhood vaccinations do not lead to long-lasting gross alterations of the immune system.

Vaccines offer protection against specific infectious diseases, preventing 2–3 million deaths every year (1). The success of this disease-specific, prophylactic approach has led to an increase in the number of pathogens targeted by vaccines, and a corresponding increase in the number of vaccines administered. This is especially so in early life, when infections represent a major cause of morbidity and mortality (2). Parallel to the increase in number of vaccines recommended in childhood, concerns about a potential negative impact of vaccines have increased (3). A frequent concern expressed relates to the increase in total vaccinations and that this increase especially with the more complex combination vaccines might overwhelm the young, developing immune systems (4,5). The common theme among these concerns is the idea of vaccines causing nonantigen-specific immune modulation that is maintained in the vaccine for prolonged periods of time. Such an overwhelmed phenotype could manifest as diminished, slowed, or absent reactivity to a broad range of subsequent stimuli in vaccinated individuals, or altered reactivity resulting in generation of a different type of cytokine and cell response. This hypothesis is testable, since such an effect could be apparent in assays that measure innate and adaptive immune responses to broad, generic stimuli in vaccinated vs. unvaccinated individuals. To our knowledge no investigation to date has examined this in the necessary detail in fully-vaccinated vs. fully-unvaccinated subjects.

The innate immune system recognizes and responds to broad classes of molecules associated with pathogens and host cell damage (pathogen/danger associated molecular patterns or PAMPs and DAMPs, respectively). Cytokines produced by innate cells activate adaptive immune responses including proliferation and cytokine or antibody production by T cells and B cells. Cytokine-type and -amount can be quantified by assays performed on cell supernatants, and the specific types of cells producing each cytokine can be determined by complementary flow cytometric assays. Stimulation with panels of PAMPs and DAMPs for innate cells and antigen-independent T cell stimuli provides a broad and nonantigen specific characterization of the immune status. Our group has developed immunological assays that comprehensively assess an individual's innate and adaptive immunological status in this manner. We had employed these assays previously to characterize the ontogeny of childhood immunological development (6,7), and to compare immune responses between geographically distinct cohorts (8).

To address concerns about the perceived risk for vaccine-mediated effects on immune development, we applied these robust and validated assays to empirically assess effects of the modern Canadian vaccine schedule on immune status in early life; this schedule involves exposure to 35 individual vaccines over four visits. A highly unique cohort of fully vaccinated vs. fully unvaccinated subjects was recruited between the ages of 3 and 5 y old, i.e., 2–3 y after completion of primary immunization series according to the schedule of British Columbia, Canada (see **Supplementary Table S1** online). We compared the capacity for innate and adaptive immune responses of these participants using our validated assays.

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METHODS

Participant Recruitment and Enrollment

Participants were recruited through pediatrics offices within the Vancouver, British Columbia, Canada area. The subjects were all recruited from the Vancouver area, and there were no differences in ethnicity. Eligible children were between 3 and 5 y old, clinically healthy, and had received either all, or none of the vaccines recommended for children in British Columbia. Vaccination status was confirmed by checking each participant's vaccine records.

Blood Collection

Peripheral blood (3–5 ml) was drawn by a trained phlebotomist via sterile venipuncture into vacutainers containing sodium-heparin (BD Biosciences, San Jose, CA), and processed within 4 h as described previously (8–10).

Whole Blood Stimulation

Master mixes of all reagents sufficient for all subjects were sterilely prepared, aliquoted, and frozen at −80°C until use, according to our well-established, validated, and quality-controlled innate immune phenotyping protocol (8,9). Toll-like receptor (TLR) ligands were prepared in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, CA), at concentrations shown in **Supplementary Table S2** online. Whole blood was diluted 1 : 1 with RPMI, added to the TLR ligand plates, and incubated for 24 h at 37°C in 5% CO_2. After centrifugation, 100 µl of supernatant was removed for bulk cytokine analysis. For intracellular cytokine staining, blood was cultured for an additional 6 h in the presence of Brefeldin A (BFA, 10 µg/ml, Sigma-Aldrich, St. Louis, MO), treated with 2 mmol/l EDTA for 15 min at 37°C, centrifuged, and frozen in fluorescence activated cell sorting (FACS) Lysing Solution (BD Biosciences).

Lymphocyte Stimulation

Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and resuspended in R10 media (RPMI with 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, MA), beta-mercaptoethanol (50 µmol/l, Sigma-Aldrich), and 1X penicillin/streptomycin (Life Technologies)). Cells were washed, DNased (60 µg/ml, Sigma-Aldrich), and incubated overnight at 37°C in 5% CO_2. EDTA (2 mmol/l, Sigma-Aldrich) was added, and cells were washed then passed through a 70 µm filter. Cells were then incubated in Oregon green staining buffer (OG, 5 µmol/l, Life Technologies) for 5 min, washed, and inoculated into 96-well plates at 5×10^5 cells/well. Cells were stimulated with costimulatory reagents anti-CD49d and anti-CD28 (1 µg/ml each, CD49/28, eBioscience, San Diego, CA) phytohaemagglutinin (PHA, 5 µg/ml, Sigma-Aldrich), or Staphylococcal enterotoxin B (SEB, 0.25 µg/ml, Sigma-Aldrich). After 7 d, cells were centrifuged and 50 µl supernatant was removed for bulk cytokine analysis. Then, cells were restimulated for an additional 6 h with phorbol-12-myristate-13-acetate (PMA, 10 ng/ml, Sigma-Aldrich) and ionomycin (1 µmol/l, Sigma-Aldrich) in the presence of BFA and stained as described below.

Staining and Flow Cytometry

Plates were thawed and stained as previously described (8,10,11). Briefly, for innate flow cytometric analysis, cells were incubated in FACS Permeabilizing Solution (BD Biosciences) at room temperature (RT) for 10 min, washed in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide (PBSAN), stained with surface and intracellular antibodies for 30–60 min at RT, then washed and resuspended in PBSAN. For the adaptive flow cytometry assay, cells were stained with surface antibodies for 40 min at RT, centrifuged, incubated in 1X Fixation/ Permeabilization Solution (eBioscience) for 20 min at 4°C, then in 1X Permeabilization Buffer for 10 min at RT. Cells were centrifuged, stained with intracellular antibody mix in 1X Permeabilization Buffer for 40 min at RT, washed and resuspended in PBSAN. Antibody panels are detailed in **Supplementary Tables S3 and S4** online. Compensation was performed using Anti-Mouse Ig Comp Beads and anti-FBS negative control beads (BD Biosciences). Data were collected on an LSRII Flow Cytometer (BD Biosciences) configured with ultraviolet (355 m, 20 mW), violet (405 nm, 25 mW), blue (488 nm, 20 mW), and red (637 nm, 25 mW) lasers, and analyzed using FlowJo 9.7.5 (Treestar, Ashland, OR).

Cells were gated as previously published (11) for the innate assay, or as indicated in **Supplementary Figure S1** online for the adaptive assay. Cells were gated as: monocytes $CD14^+HLADR^+$, conventional dendritic cells (cDCs) $CD14^-HLADR^+CD11c^+$, and plasmacytoid dendritic cells (pDCs) $CD14^-HLADR^+CD123^+$. T cells were identified as $CD3^+$, then gated as either $CD8^+$ or $CD4^+$ T cells, with $CD4^+$ T cells gated as $CD3^+$ $CD8^-$ lymphocytes, due to down-regulation of CD4 expression following PMA/ionomycin stimulation. T cells were then divided into OG^{LOW} (proliferators) or OG^{HIGH} (nonproliferators). Cytokine gates were set using the CD49/28 negative control sample. Proliferation indices were determined using the FlowJo Proliferation Platform, using the CD49/28 negative control samples to set the nonproliferator gate.

Bulk Cytokine Measurement

Supernatants were assayed by multiplex assay (Luminex for innate; Procarta for adaptive) using the high-biotin protocol with overnight 4°C incubation. Levels of the following cytokines were measured for the innate assay: IFN-α2, IFN-γ, CXCL10, IL-12p70, IL-12p40, IL-6, TNF-α, IL-1β, CXCL8, CCL3, CCL4, and IL-10. All analytes except CCL22, CXCL10, and CCL4 were produced at levels above background and within the linear range of the assay. For the adaptive assay the following cytokines were measured: Granzyme B, IL-5, IL-13, TNF-α, and IFN-γ. Samples were diluted to fall within the standard curve, and data were within the linear range for all analytes except for Granzyme B. Beadlytes, biotin, and streptavidin-phycoerythrin were used at half the manufacturer's recommended concentrations. Assays were read using Luminex 200 Total System (Luminex, Austin, TX) running MasterPlex (MiraiBio, San Bruno, CA) software, and downstream analysis was performed in Excel (Microsoft, Redmond, WA) and an in-house database.

Analysis

Graphs were prepared using Prism 5 (GraphPad, La Jolla, CA). Limitations in sample size precluded statistical analysis, due to the high chance of Type I and Type II errors in nonparametric analysis of populations of this size. Previous data from our immune profiling assays predicted that a sample size of 30 would allow detection of a twofold difference in cell subsets or cytokine responses at a power of 76%. We thus aimed for a sample size of 30 subjects in each arm.

Ethics Statement

This study was conducted according to the principles expressed in the Good Clinical Practice Guidelines, and the Declaration of Helsinki. This study was approved by the University of British Columbia Ethics Board. Informed written consent was obtained from a parent or guardian of each study participant.

RESULTS

Cohort Description

Eighteen children between the ages of 3 and 5 y old were recruited who had either received all recommended vaccines (n = 9), or received no vaccines at all (n = 9). The characteristics of the study population at enrolment are presented in **Table 1**.

Innate Immune Phenotype

We first chose to investigate potential differences between unvaccinated and vaccinated children at the level of the innate immune system, utilizing PAMPs to stimulate pattern recognition receptors. We performed bulk cytokine quantification for a panel of 13 cytokines encompassing the following broad functional categories: proinflammatory (**Figure 1a,b**, TNF-α and IL-1β), T_H1-supporting (**Figure 1c–e**, IFN-γ, IFN-α, and IL-12p70), regulatory (**Figure 1f**, IL-10), T_H17-promoting (**Figure 1g,h**, IL-6 and IL-12p40), and chemokines (**Figure 1i,j** and data not shown, CCL3, CXCL8, CXCL10, CCL4, and

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Table 1. Demographics of the children in each cohort

	Unvaccinated	Vaccinated
N	9	9
Mean age (mo), mean ± SD	49.06 ± 8.84	56.44 ± 2.76
Mean weight (kg), mean ± SD	17.38 ± 2.66	18.92 ± 3.30
Mean height (cm), mean ± SD	106.71 ± 7.13	108.46 ± 6.59
Mean arm circumference (cm), mean ± SD	17.11 ± 1.08	17.58 ± 1.25
Sex (male : female)	7:2	4:5

CCL22). These are generalized categories that do not fully capture the multiple roles played by some cytokines in distinct contexts (e.g., IL-10 can be T_H2-promoting as well as regulatory, IL-12p40 can promote T_H1 as well as T_H17 responses). The majority of cytokines quantified were most highly produced in response to stimulation with the TLR7/8 agonist resiquimod (R848). Lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (polyI:C), and peptidoglycan (PGN) (TLR4, TLR3, and NOD1/2 agonists, respectively) generated a range of responses depending on the specific cytokine, while stimulation with the TLR2/1 agonist PAM and NOD2 agonist muramyl dipeptide induced production of low levels of most cytokines (**Figure 1a–j**).

Vaccinated and unvaccinated individuals showed equally robust production of the T_H1-supporting cytokines IFN-α, IFN-γ, and IL-12p70 in response to R848 and polyI:C (**Figure 1c–e**). Individuals within the two groups also synthesized similar levels of the proinflammatory cytokines TNF-α and IL-1β in response to LPS, R848, and PGN (**Figure 1a,b**). LPS and R848 stimulated equivalent production of the T_H17-promoting cytokines IL-6 and IL-12p40, while IL-6 was made in response to PGN at similar levels, in most individuals of both groups (**Figure 1g,h**). The chemokine CXCL8 was made most robustly in response to PGN and to a lesser extent to LPS by individuals within each group, while CCL4 was produced similarly in both groups in response to LPS and R848 (**Figure 1i,j**). Finally, production of the regulatory cytokine IL-10 in response to LPS, R848, and PGN was observed at similar levels in both vaccinated and unvaccinated individuals (**Figure 1f**). In summary, no obvious differences were detected in the intensity or type of innate immune reactivity between vaccinated vs. unvaccinated individuals, based on bulk production of the selected cytokines.

To assess potential differences in cytokine production by specific cell types, we performed intracellular cytokine staining followed by flow cytometry. Overall, similar to the analysis of secreted cytokines, the percentage of each cell subset producing cytokine in response to PAMPs was similar between vaccinated and unvaccinated individuals (**Figure 2**). Monocytes (**Figure 2a**) and cDCs (**Figure 2b**) produced IFN-γ, IL-12p40, IL-6, and TNF-α, with the strongest responses to R848 and LPS in most individuals. The percentage of monocytes producing TNF-α and IL-6 in response to PGN was particularly variable between individuals. We noted a trend toward increased production of these two cytokines by monocytes from vaccinated vs. unvaccinated individuals, though substantial overlap between the two groups was observed. PAM stimulation also generated robust TNF-α and IL-6 production by cDCs. While the percentage of cDCs producing IL-12p40, IL-6, and TNF-α in response to R848 was highly varied between vaccinated individuals, the unvaccinated group demonstrated universally high responses. pDCs from individuals in both groups responded only to R848, and generally produced only IFN-α and TNF-α (**Figure 2c**). These results revealed no apparent differences in the strength or type of cell-specific innate immune reactivity to broad, generic stimuli in vaccinated vs. unvaccinated individuals.

Adaptive Immune Phenotype

To characterize potential differences between unvaccinated and vaccinated children at the level of the adaptive immune response, we stimulated PBMCs with generic T cell stimuli to generate a nonantigen-specific response as a measure of general lymphocyte responsiveness. Bulk cytokine production was quantified for the following cytokines, selected to represent the spectrum of adaptive immune response: T_H1 response (IFN-γ and TNF-α), T_H2 response (IL-5 and IL-13), regulatory (IL-10), and cytotoxic (Granzyme B). Both T cell stimuli generated robust production of IL-5 by individuals within both groups (**Figure 3**). Production of IL-10, IL-13, and TNF-α was comparable in vaccinated and unvaccinated individuals, and strongest in response to SEB with intermediate responses to PHA. IFN-γ production in response to SEB exceeded the range of detection for the dilution tested, but was minimal following PHA stimulation (data not shown). Slightly higher cytokine production of certain cytokines (IL-10, TNF-α) was observed in some vaccinated individuals; however, the range of response between groups overlapped substantially. Our bulk cytokine production results indicate that non-antigen-specific T cell reactivity appeared similar in both intensity and type in unvaccinated and vaccinated individuals.

To quantify proliferation and cytokine production within specific cell types, we utilized OG dye dilution and intracellular cytokine staining followed by flow cytometry. The following cytokines were assessed in proliferating cells vs. nonproliferating cells: proliferative (IL-2), T_H1 response (IFN-γ, TNF-α), T_H2 response (IL-4, IL-13), and regulatory (IL-10). Multiple successive OG dilution peaks were clearly visualized following stimulation with PHA and SEB for both $CD4^+$ and $CD8^+$ T cells, indicating robust and synchronized lymphocyte proliferation in response to these stimuli (**Figure 4a**). The number of divided cells, as indicated by the percentage of OG^{LOW} cells after stimulation, was highly similar between unvaccinated and vaccinated individuals for all stimuli in both $CD4^+$ and $CD8^+$ cells. A more detailed analysis using FlowJo's Proliferation analysis platform revealed no differences in either the average number of divisions among proliferating cells, or the percentage of cells in the original plated population that divided at least once (referred to as proliferation index and precursor frequency by FlowJo), between vaccinated and unvaccinated individuals (**Figure 4b**). Additionally, evaluation of resting cell

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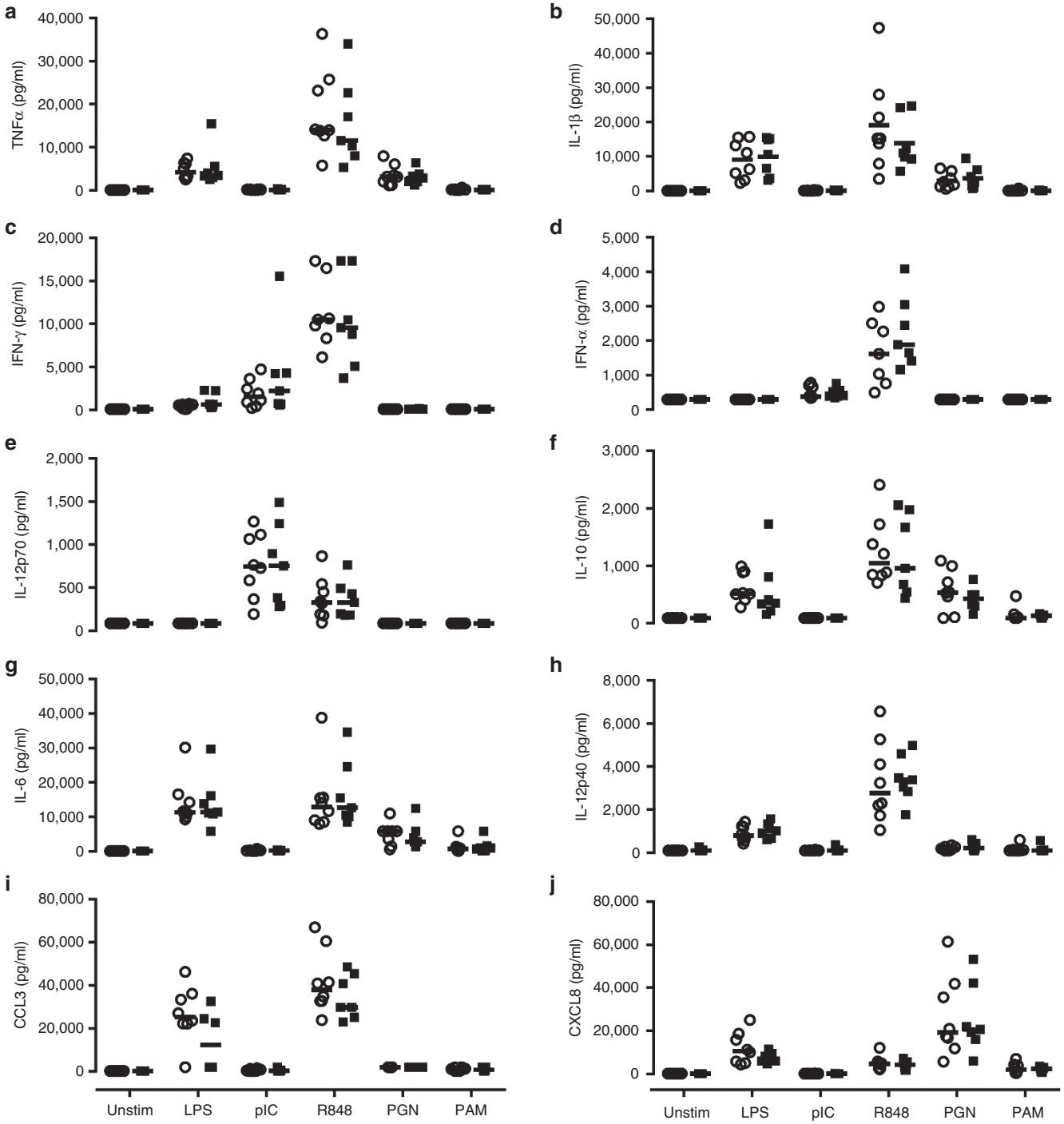

Figure 1. Bulk cytokine production of vaccinated vs. unvaccinated children in response to innate stimuli. Whole blood was cultured in the presence of toll-like receptor (TLR) and NOD stimuli for 24 h, then Luminex multiplex cytokine analysis was utilized to quantify cytokines in culture supernatants from the following broad categories: proinflammatory (**a**, **b**), T_H1-supporting (**c**-**e**), regulatory (**f**), T_H17-promoting (**g**, **h**), and chemokines (**i**, **j**). Open circles are unvaccinated individuals ($n = 8$), filled squares are vaccinated individuals ($n = 7$), and lines represent median values.

phenotypes revealed no differences in the relative number of CD4+ and CD8+ T cells in vaccinated vs. unvaccinated individuals (data not shown).

We evaluated cytokine production by total, proliferated (OG^{LOW}), nonproliferated (OG^{HIGH}) $CD4^+$, and $CD8^+$ T cells. $CD4^+$ T cells produced IL-2 in response to both SEB and PHA, while IFN-γ and TNF-α were primarily produced in response to SEB (**Figure 4c**). IFN-γ, TNF-α, and IL-2 were produced primarily by $CD4^+$ T cells that had proliferated (**Supplementary Figure S2** online). Notably, the T_H2 cytokine IL-4 was expressed in $CD4^+$ T cells in the CD49/28 negative control; a small induction of IL-4 expression in PHA and SEB stimulated cells was primarily observed in nonproliferating $CD4^+$ T cells. In $CD8^+$ T cells, IFN-γ and TNF-α production

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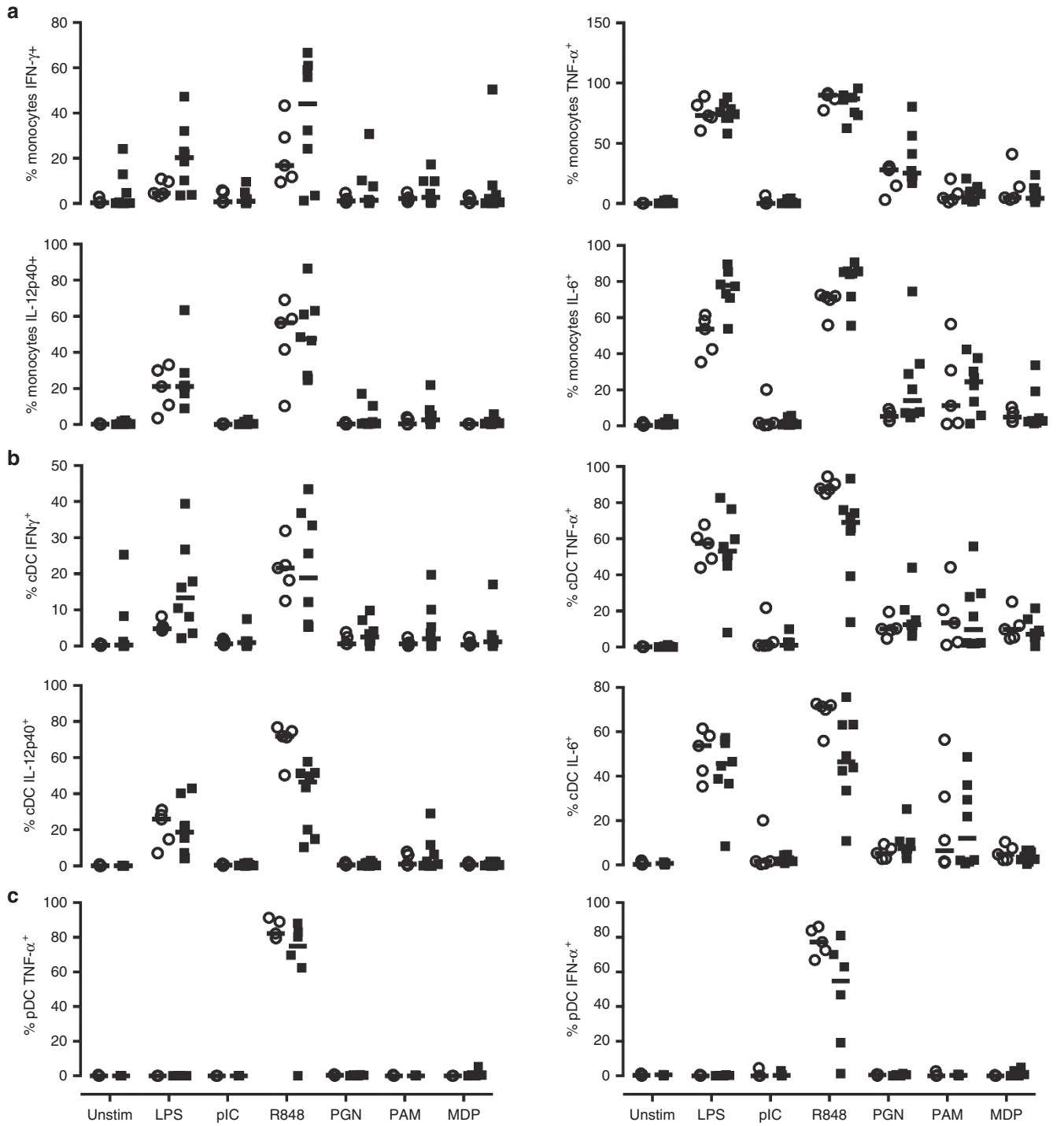

Figure 2. Cell-type-specific cytokine production of vaccinated vs. unvaccinated children in response to innate stimuli. Whole blood was cultured in the presence of toll-like receptor (TLR) and NOD stimuli plus Brefeldin A (BFA) for 6 h prior to staining for intracellular cytokines and surface markers and quantification by flow cytometry. Cytokine production was quantified for (**a**) monocytes, (**b**) conventional dendritic cell (cDC), and (**c**) plasmacytoid dendritic cell (pDC). Open circles are unvaccinated individuals ($n = 5$), filled squares are vaccinated individuals ($n = 8$ for monocytes, cDC; $n = 6$ for pDC), and lines represent median values.

in response to SEB, and IL-2 production in response to both SEB and PHA (Figure 4d), were driven primarily by proliferating cells (**Supplementary Figure S2** online). As previously reported, the nuclear protein Ki67 was a highly specific marker of proliferation, expressed in ~60–90% of proliferated (OG^{LOW}) cells but in <10% of nonproliferated cells (data not shown) (12). The T_H2 cytokine IL-13 was expressed in only a small percentage of cells overall (<5% of $CD4^+$ or $CD8^+$ T cells) and was not significantly induced by stimulation (data not shown). Similar to our results for innate response, there

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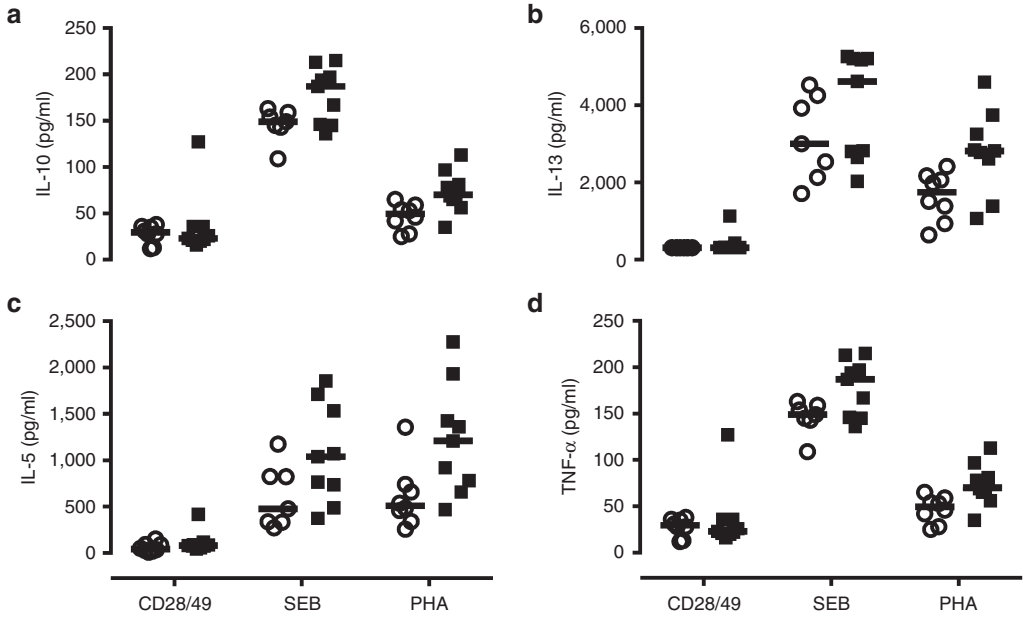

Figure 3. Bulk cytokine production of vaccinated vs. unvaccinated children in response to T cell stimuli. Peripheral blood mononuclear cells (PBMCs) were cultured in the presence of phytohaemagglutinin (PHA), Staphylococcal enterotoxin B (SEB), or costimulatory molecules (CD28/49) for 7 d, then Luminex multiplex cytokine analysis was performed on culture supernatants to quantify (**a**) interleukin (IL)-10, (**b**) IL-13, (**c**) IL-5, (**d**) tumor necrosis factor (TNF)-α. Open circles are unvaccinated individuals ($n = 8$), filled squares are vaccinated individuals ($n = 9$), and lines represent median values.

were no striking differences in the intensity or type of adaptive response, as measured by proliferative capacity or cytokine production, between the adaptive responses of vaccinated vs. unvaccinated subjects.

DISCUSSION

Parental concerns about vaccination include the view that an increasing number of recommended vaccines might overwhelm developing immune systems of their children (4,13). Such effects would have to involve modulation of the immune system in a nonantigen-specific and long-lasting manner, and likely would manifest as reduced, delayed, or altered reactivity in either innate or adaptive immune responses to a broad range of subsequent stimuli. In this comparative study, we completed the first step guiding future analysis of the immunological status in early life contrasting highly unique cohorts of fully vaccinated vs. entirely unvaccinated subjects. Our investigations failed to reveal a gross impact on immune status 2–3 y following receipt of all recommended infant vaccinations, as measured by standard immune phenotyping assays. This was true for both innate as well as adaptive immune responses, and across a wide range of functional cytokine and proliferative responses. Our assay designs allowed us to directly determine that immune reactivity was not grossly diminished or altered in vaccinated vs. unvaccinated individuals. Due to the single time point analyzed, these assays were unable to directly address whether responses might be delayed. However, the similar immune reactivity observed at the selected time point suggests that responses are not delayed in vaccinated individuals.

This conclusion has to be tempered because of the small sample size in our cohorts. Previous performance of our immune profiling platform predicted that a sample size of ~30 in each group would have allowed us to detect a twofold-or-greater difference in cell subsets or cytokine responses at a power of 76%. However, enrolling entirely unvaccinated children in studies such as ours was a highly complex and resource-consuming undertaking; as a result we did not reach the planned sample size of 30. Given the highly unique cohorts recruited, we nevertheless opted to analyze the samples collected, instead of abandoning this precious opportunity. But our results have to be viewed as hypothesis generating data helping to guide future investigations, not as firm conclusions. More specifically, given the small sample size we cannot conclude that there are no significant differences in the response of vaccinated vs. unvaccinated children. Yet, our current results did predict that a significant difference was unlikely to be reached even with a sample size of 30. Thus, our data suggests that the immune system of vaccinated children was unlikely to be entirely "overwhelmed" as neither a broad inhibition nor increased reactivity of the functional immune status of fully vaccinated children was apparent when contrasted with the immune status of entirely unvaccinated children.

Selected functional differences may exist between the two groups. For example, the percentage of monocytes producing IL-6 and TNF-α following PGN stimulation (**Figure 2a**) possibly could be higher in vaccinated individuals, although the response of the two study populations overlapped substantially. Given that results from our bulk cytokine assay indicated no differences in overall production capacity of these two cytokines between unvaccinated and vaccinated individuals, such cell-specific difference would unlikely amount to a broad functional alteration with an obvious clinical impact. Similarly, production of certain cytokines (**Figure 3**, IL-10 and TNF-α)

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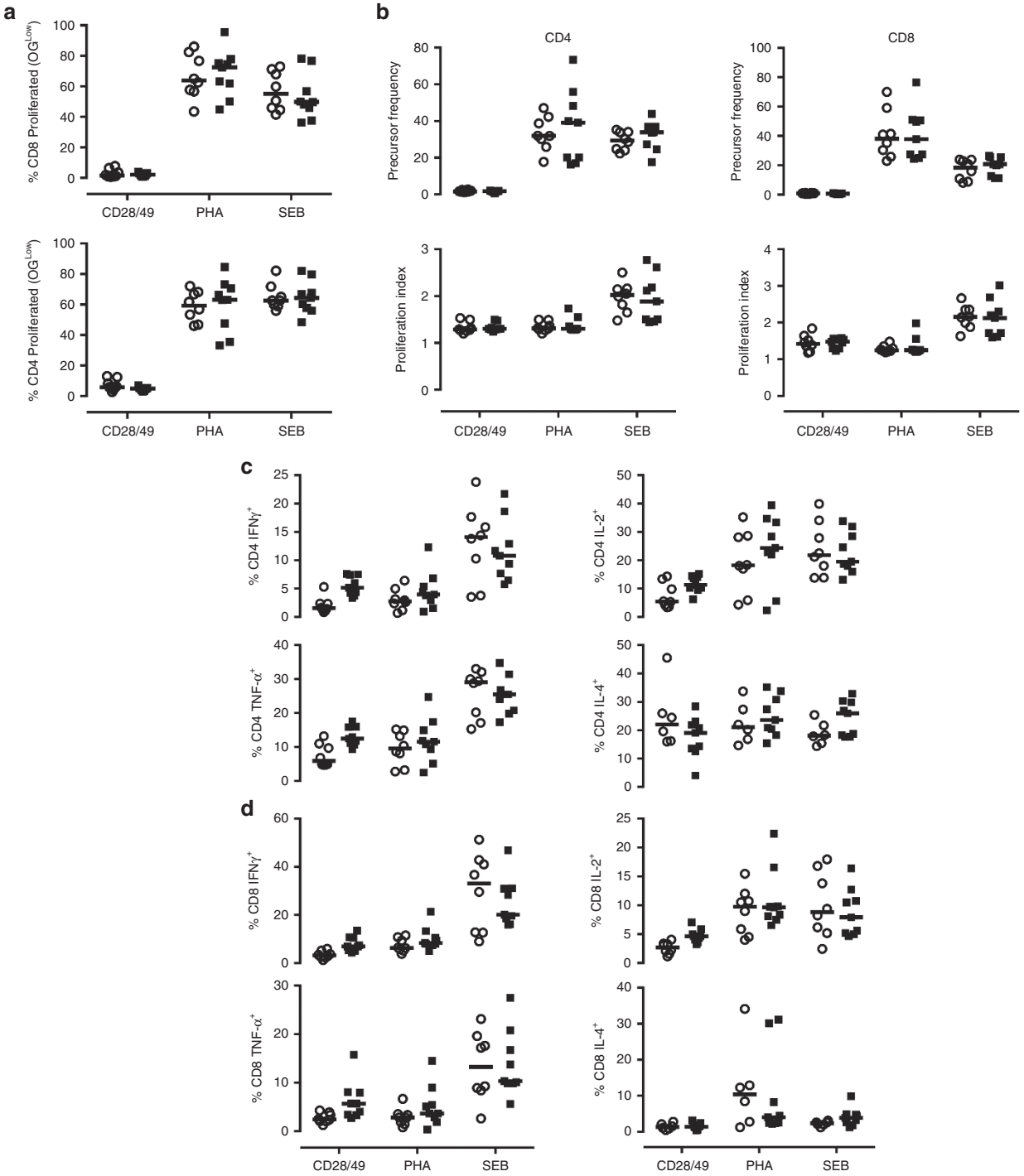

Figure 4. Cell-type-specific cytokine production of vaccinated vs. unvaccinated children in response to generic T cell stimuli. Peripheral blood mononuclear cells (PBMCs) were cultured in the presence of anti-CD28/49, phytohaemagglutinin (PHA) or Staphylococcal enterotoxin B (SEB) for 7 d, then restimulated with phorbol-12-myristate-13-acetate (PMA) and ionomycin in the presence of Brefeldin A (BFA) for 6 h prior to staining for surface markers and intracellular cytokines. (**a**) Proliferation of CD4+ and CD8+ T cells by Oregon Green intensity; (**b**) Precursor frequency and proliferation index of CD4+ and CD8+ T cells as determined by FlowJo Proliferation platform; (**c**) Cytokine production by total CD4+ T cells; (**d**) Cytokine production by total CD8+ T cells. Open circles are unvaccinated individuals (n = 8), filled squares are vaccinated individuals (n = 9), and line indicates median.

in response to PHA appeared higher in some vaccinated individuals in the bulk adaptive cytokine assay; however, there was considerable overlap between groups. This potential difference was also not reflected by differences in cell-specific adaptive phenotypes (Figure 4). Verification of whether these small possible differences are reproducible and clinically relevant would require a far larger study.

A major strength of this study beyond the highly unique cohorts recruited is the panel of stringently standardized, robust immunological assays applied to interrogate of a wide range of

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innate and adaptive immune functionalities. These assays have been proven to be able to detect functionally important differences in studies with small sample size (8,14,15). Our study also has several limitations. For example, our study did not assess cell types other than monocytes, dendritic cells and T cells; we thus cannot comment on altered functional status of other cell types such as B cells or NK cells or functions beyond the pathways examined here. Specifically, our study does not rule out an impact of childhood vaccinations on other aspects of innate immunity, as cell subpopulations and innate response pathways involved in e.g., trained immunity are beyond the level detail of assays employed in our study (16). We chose to use nonantigen-dependent stimuli to assess adaptive immune responses, due to the absence of an antigen likely to be universally recognized among unvaccinated individuals. It remains possible, though unlikely, that antigen-specific adaptive immune responses could differ between vaccinated and unvaccinated individuals. The age range of participants in this study (3–5 y old) was selected to optimally analyze any long-lasting vaccine-mediated effects. Our data does not assess whether any transient differences may occur more immediately after vaccination. This study was also limited to participants from a single geographic area (Vancouver, BC) and was not able to capture broad diversity in ethnic backgrounds. As mentioned above, a further limitation of this study is the small sample size available for comparison; this precluded statistical analyses, due to the high risk for Type I and Type II errors and the inability to accurately assess normal distribution of the data points in populations of this size. The primary purpose of this study was to generate initial data in a robust set of standardized assays, sufficient to calculate necessary sample size for future studies to be conducted should distinct, qualitative differences in immunophenotype appear to exist between unvaccinated and fully-vaccinated children. Future studies capable of providing a more robust answer would require a larger number of study participants, including ethnically diverse individuals from geographically distinct locations. However, such qualitative differences were not evident in this study.

A multitude of well-designed studies have demonstrated convincingly that the efficacy of the standard childhood vaccines for preventing infection or disease from the targeted pathogens is high (17). However, very little published literature exists on the potential nonantigen specific effect of vaccination on immunological status of individuals. As parental concerns persist about such nonspecific effects, and often lead to vaccine refusal, the re-emergence of vaccine-preventable infections such as measles and pertussis is an unfortunate but inevitable consequence (18,19). Assessment of the nonantigen specific effect of vaccination on immune development offers to provide the tool necessary to systematically investigate any such nonantigen specific effect. To our knowledge, this is the first study to address this complex question using a broad functional experimental platform. Our findings indicate that vaccination does not grossly alter immune status 2–3 y after infant immunization.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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