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Maturation of Innate Responses to Mycobacteria over the First Nine Months of Life

Muki S. Shey,†,‡,§,∥ Elisa Nemes,*## Wendy Whatney,* Marwou de Kock,* Hadn Africa,* Charlene Barnard,* Michele van Rooyen,* Lynnett Stone,* Catherine Riou,∥ Tobias Kollmann,∥ Thomas R. Hawn,∥∥ Thomas J. Scriba,*## and Willem A. Hanekom*†,∥

Newborns and young infants are particularly susceptible to infections, including Mycobacterium tuberculosis. Further, immunogenicity of vaccines against tuberculosis and other infectious diseases appears suboptimal early in life compared with later in life. We hypothesized that developmental changes in innate immunity would underlie these observations. To determine the evolution of innate responses to mycobacteria early in life, whole blood or PBMC from newborns, as well as 10- and 36-wk-old infants, was incubated with viable Mycobacterium bovis bacillus Calmette–Gue´rin or TLR ligands. Innate cell expression of cytokines and maturation markers was assessed, as well as activation of the proinflammatory NF-kB– and MAPK-signaling pathways. Bacillus Calmette–Gue´rin–induced production of the proinflammatory cytokines TNF-α, IL-6, and IL-12p40 increased from the newborn period to 9 mo of age in monocytes but not in myeloid dendritic cells. No changes in production of anti-inflammatory IL-10 were observed. CD40 expression increased with age in both cell populations. Older infants displayed substantial activation of all three signal transduction molecules: degradation of NF-kB inhibitor IκBα and phosphorylation of MAPK Erk and p38 upon TLR1/2 triggering, compared with predominant activation of only one of any of these molecules in newborns. Maturation of innate proinflammatory responses during the first 9 mo of life may underlie more effective control of mycobacteria and other pathogens observed later in infancy and age-related differential induction of Th1 responses by vaccination. The Journal of Immunology, 2014, 192: 000–000.

Innate cells, such as granulocytes, macrophages/monocytes, and dendritic cells (DCs), form a cornerstone of host defense against infection with bacteria, such as Mycobacterium tuberculosis. These cells recognize conserved pathogen-associated molecular patterns through germ-line encoded pattern recognition receptors (PRRs) (1), such as TLRs (2). PRR triggering leads to the activation of complex signaling networks, including MAPKs and NF-kB pathways (3, 4), and regulates phagocytosis (5), antimicrobial activities, cytokine production (6), and the induction and nature of adaptive immune responses (7).

The neonatal immune system is specifically adapted for transition from a sterile intrauterine compartment to an environment ripe with microbial challenges. At birth, the innate response to pathogens is less vigorous, characterized by lower proinflammatory cytokine production and greater regulatory cytokine production, than that typically observed in adults (reviewed in Refs. 8, 9). Cord blood monocytes and DCs express low levels of costimulatory molecules and are relatively unresponsive to LPS or IFN-γ compared with monocytes and DCs from adults (10). Neonatal monocytes also have reduced capacity to differentiate into DCs in vitro, and cord blood monocyte-derived DCs produce lower levels of IL-12 in response to TLR and CD40 signaling than do adult monocytes (9, 11). Most of these responses were observed upon stimulation with purified TLR agonists; it is not known how innate cells respond to mycobacteria, which express multiple ligands recognized by various PRRs. The focus of this study was to evaluate innate immune responses to the live Mycobacterium, attenuated M. bovis bacillus Calmette–Gue´rin (BCG), which is the currently licensed vaccine against tuberculosis (TB) during the first 9 mo of life.

TB is a major cause of morbidity and mortality in young children in developing countries (12, 13). At least 74,000 HIV-uninfected children died of TB in 2012, and children account for half a million new TB cases every year (14). Newborns and older infants up to 6 mo of age are more likely to develop pulmonary and disseminated forms of TB disease than are older children (12, 13, 15, 16). Before TB treatment was available, mortality of infants < 6 mo

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Abbreviations used in this article: BCG, bacillus Calmette–Gue´rin; BCG-GFP, bacillus Calmette–Gue´rin–expressing GFP; BCG-GFP, bacillus Calmette–Gue´rin–expressing GFP; DC, dendritic cell; iMFI, integrated median fluorescence intensity; mDC, myeloid DC; MFI, median fluorescence intensity; PAM3, Pam3CSK4; PRR, pattern recognition receptor; TB, tuberculosis.

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The Journal of Immunology
of age was ~55%, compared with ~30% in children aged 1–to 2 y (17). The immature innate immune response in early life may contribute to the higher susceptibility of newborns and older infants to infections, including *M. tuberculosis* (18, 19).

Vaccination-induced immunity is less effective for many vaccines when administered during the first months of life than at older ages (8). The only vaccine available for the prevention of TB, BCG, is given at birth in most countries endemic for TB. BCG protects infants against disseminated forms of TB disease but confers limited protection against pulmonary disease at all ages (20). We previously showed that frequencies of BCG-specific T cell responses measured in 1-y-old infants were higher when BCG vaccination was delayed to 10 wk of age compared with administration at birth (21).

If our hypothesis of a causal relationship between innate immune status and susceptibility to *M. tuberculosis* and other infections were correct, we would expect that as the risk for disease decreases over the first year of life, innate immune responses to mycobacteria should become more adult-like. To test this hypothesis, we investigated production of pro- and anti-inflammatory cytokines, expression of costimulatory molecules, phagocytosis of mycobacteria, and TLR-induced signaling pathways in innate cells over the first 9 mo of life.

### Materials and Methods

#### Participants and blood collection

This study was completed at the South African Tuberculosis Vaccine Initiative site in the Cape Town region of South Africa. The aim was to enroll 25 pregnant mothers for collection of cord blood immediately after giving birth by Cesarean section, a separate group of 35 infants aged 10 wk, and a third group of 15 infants aged 10 mo. Cord blood was collected into sterile, heparinized blood collection tubes or bags, immediately transported to the laboratory, and processed within 30 min of collection. A total of 180 µl whole blood was added to wells containing 20 µl RPMI 1640 (Lonza), with individual TLR agonists or BCG. Polymyxin B (BioChemika; 10 µg/ml) was added to the wells containing PAM3 to minimize possible effects of LPS contamination. RPMI 1640 only was used as medium control (no Ag). Incubation proceeded at 37°C, 5% CO2 in humidified conditions for 6 h for an intracellular cytokine staining assay (unstimulated, BCG, and LPS), as previously optimized (22), and for 18 h to measure maturation marker expression on innate cells and soluble cytokine production (unstimulated, BCG, PAM3, and LPS). For the 6-h intracellular cytokine staining assay, brefeldin A (10 µg/ml; Sigma-Aldrich) was added to each well after 3 h of incubation; no brefeldin A was added for the 18-h assay. Further, after a total incubation of 18 h, 100 µl assay supernatant was removed and stored at ~80°C. Whole blood from 25 infants of each age group was stimulated in duplicate wells, and cells and supernatants from each duplicate were pooled for analyses. After incubation, 2 mM EDTA (final concentration; Sigma-Aldrich) was added for 10 min, followed by lysis of white blood cells with FACS Lysing Solution (BD Biosciences) for 10 min, repeated once after a wash. Fixed white cells were cryopreserved in 10% DMSO in heat-inactivated FCS in liquid nitrogen.

#### Staining of cells and flow cytometric acquisition

Cryopreserved, stimulated cells were thawed in batches and washed twice with PBS (without Ca and Mg) or BD Perm/Wash buffer (BD Biosciences). Samples from the 6-h incubation were stained with fluorescent Ab mixtures in BD Perm/Wash at 4°C for 1 h. Stained cells were washed, and 1 million cells were acquired on a BD LSRII flow cytometer. Results from samples with an acquired total leukocyte number exceeding 500,000 were included in the analysis. None failed to meet this criterion.

The cells yielded by the longer incubation were washed twice in PBS. A two-step staining protocol was used; cells were stained first with non–Qdot-conjugated Abs in PBS, for 1 h followed by a second 1 h staining step with Qdot-conjugated Abs in BD Perm/Wash buffer. We have previously observed that PBS adversely affects fluorescence of Qdot-conjugated Abs (23). Stained cells were washed and acquired on a BD LSRII flow cytometer. Only results from samples with an acquired total leukocyte number > 500,000 were included in the analysis. None failed to meet this criterion.

#### Flow cytometric measurement of signaling pathways in PBMCs

PBMCs were isolated by gradient centrifugation of infant blood (cord blood, n = 10; 10-wk old, n = 7; 36-wk old, n = 7) and cryopreserved in FCS containing 10% DMSO within 4 h of phlebotomy. Cryopreserved PBMCs were thawed, washed, and rested in RPMI 1640 containing 1% L-Glutamine (Lonza), 10% human AB serum (Sigma), and DNase (30 µg/ml; Sigma) for 6 h at 37°C, 5% CO2 in humidified conditions. PBMCs were washed and rested in the absence of serum for an additional 30–60 min in RPMI 1640 containing 1% L-Glutamine. PBMC viability was assessed by trypan blue exclusion. Cells were left unstimulated or incubated with PAM3 for exactly 30 min at 37°C and then fixed in PBS containing 2% parafomaldehyde. Fixed PBMCs were washed with ice-cold PBS and permeabilized using Perm Buffer IV 0.5% (BD Phosphor) for 20 min. Cells were washed twice in PBS containing 1% FCS and stained for 1 h at room temperature with Abs. Stained cells were washed and acquired on a BD LSRII flow cytometer.

#### Secreted cytokine measurement

Supernatants from whole blood stimulated for 18 h were thawed. Concentrations of IL-12p40, IL-12p70, IL-10, IL-6, and TNF-α were measured for all samples by Milliplex MAP Multiplex Immunoassay (based on Luminex MAP technology; Millipore) on a Bio-Rad Luminex 100 Bio-Plex Suspension Array System (Bio-Plex 200, Bio-Rad). Measurements were performed according to the manufacturer’s instruction. The following concentrations of the standards in 200 µl assay buffer were used: 10,000, 2,000, 400, 80, 16, and 3.2 pg/ml. A sample volume of 25 µl, diluted 2- or 25-fold, was used.

#### Data analysis

Flow cytometry data were analyzed using FlowJo v9.2 software. Results from single-stained and unstained mouse beads were used to calculate compensation for each run. Cell doublets were run. Cells were gated using forward scatter-area versus forward scatter-height parameters. Cytokine coexpression by monocytes and DCs and signaling molecule coactivation by monocytes were assessed by Boolean gating. Subtraction of background cytokine expression (unstimulated samples) was done using Pestle v1.6.2, whereas data sorting and analysis were done with Spice v5.1 (http://exon.niaid.nih.gov/spice, accessed February 25th, 2011). The median fluorescence intensity for each cytokine was determined by FlowJo software, and samples were excluded if there were <25 cells in a given cytokine gate.
For analysis, the Milliplex MAP Multiplex Immunoassay data, standard curve values were considered as outliers and excluded if the observed/expected \times 100 was outside the range of 100 ± 30%, according to the manufacturer’s instruction. Cytokine values below the lowest level of detection were assigned half of the lower value of the lowest standard.

For intracellular signaling assays, only samples showing >50% viable cells after resting were processed further and only those for which >1000 monocytes were acquired were included in the analysis.

GraphPad Prism v5 was used for data presentation and statistical analysis. Comparisons within the same age group were done using a Wilcoxon matched-pairs signed-rank test. Comparisons among the different age groups were assessed with a Kruskal–Wallis test, followed by a Mann–Whitney U test. The \( p \) values < 0.05 were considered statistically significant.

Results

Participants

We enrolled three groups of participants: newborns, infants aged 10 wk, and infants aged 36 wk (Table I). Gender distributions between the groups were similar. Newborns were from black African, South African mixed ancestry, and white ethnic groups, whereas all 10- and 36-wk-old infants were from the mixed race group.

Proinflammatory cytokine production in response to mycobacteria over the first 9 mo of life

To investigate changes in innate immunity during the first 9 mo of life, we evaluated intracellular expression of the proinflammatory cytokines TNF-\( \alpha \), IL-6, and the p40 subunit of IL-12/IL-23 (referred to as IL-12) by monocytes following incubation of whole blood with BCG or LPS (Fig. 1A, Supplemental Fig. 1).

Frequencies of monocytes expressing any combination of TNF-\( \alpha \), IL-6, and IL-12 or any single cytokine in response to BCG increased over the study period (Fig. 1B, 1C). At all ages, frequencies of monocytes expressing IL-6 and/or TNF-\( \alpha \) were higher than those expressing IL-12. Coexpression of IL-6 and TNF-\( \alpha \) and single expression of IL-6 dominated among possible cytokine combinations (Fig. 1B), regardless of age.

The median fluorescence intensity (MFI) of TNF-\( \alpha \) and of IL-12 expression among monocytes positive for these cytokines also increased from the newborn period to 36 wk of age (Supplemental Fig. 2, middle panel; data not shown); IL-6 MFI did not change (data not shown). To enable measurement of age-related changes in both frequencies and relative cytokine expression levels of innate cells, we calculated the product of cytokine frequency and their MFIs, the so-called “integrated MFI” (iMFI, Supplemental Fig. 2), described previously (23). iMFI levels of TNF-\( \alpha \), IL-6, and IL-12 expression in BCG-stimulated monocytes were lowest in newborns and increased with age (Fig. 1D).

Similar age-associated increases in monocyte expression of proinflammatory cytokines upon LPS stimulation were observed. These increases were not necessarily stepwise from birth to 10 wk and then 36 wk of age (Supplemental Table I).

Similar to monocytes, the myeloid DCs (mDCs) response to BCG was characterized by a predominance of cells expressing IL-6 alone or coexpressing IL-6 and TNF-\( \alpha \) (data not shown). IL-12 expression also was relatively low but detectable in mDCs in only a subset of participants (Fig. 2B).

In contrast to monocytes, frequencies and iMFIs of these cytokines in mDCs following incubation with BCG were not different in newborns and 10- and 36-wk-old infants (Fig. 2C, data not shown).

In contrast, in response to LPS, higher expression of IL-12 and TNF-\( \alpha \) by both monocytes and mDCs was observed in older infants, as early as at 10 wk of age, compared with newborns (Supplemental Table I). Also, LPS-induced frequencies of mDCs coexpressing two or three cytokines were higher in older infants compared with newborns, whereas the frequencies of single cytokine–expressing mDCs were lower in older infants compared with newborns (Supplemental Table I).

Next, we measured soluble levels of secreted proinflammatory cytokines after stimulation of whole blood for 18 h with live BCG or with PAM3. Consistent with our results with regard to monocyte responses, levels of TNF-\( \alpha \) and IL-6 were lowest in newborns and increased with age in response to both BCG and PAM3 (Fig. 3). Levels of IL-12 did not increase significantly with age upon BCG stimulation (Fig. 3A), but they were higher in response to PAM3 in 36-wk-old infants compared with younger infants (Fig. 3B). Soluble levels of TNF-\( \alpha \), IL-6, and IL-12 after LPS stimulation were again lower in newborns and increased with age (data not shown). Levels of these proinflammatory cytokines in response to BCG and LPS were not different in 10- and 36-wk-old infants; however, in response to PAM3, these proinflammatory cytokines were higher at 36 wk of age (\( p = 0.02 \) for IL-6; \( p = 0.04 \) for IL-12; and \( p = 0.003 \) for TNF-\( \alpha \); data not shown).

Taken together, our results suggest that the antituberculosis proinflammatory cytokine response by peripheral blood mononuclear innate cells is lowest at birth and increases during the first 9 mo of life. The differences in responses observed between BCG and purified TLR ligands could be attributed to a complex array of ligands expressed by BCG that could be both activating and inhibitory to the immune system.

No change in anti-inflammatory IL-10 response to mycobacteria over the first 9 mo of life

IL-10 is a key anti-inflammatory cytokine with a well-described role as a suppressive regulator of proinflammatory immune responses during infection (24). To determine whether this anti-inflammatory response to mycobacteria changes during the first 9 mo of life, we measured monocyte and mDC expression of IL-10 upon

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incubation of whole blood with BCG. Low levels of IL-10–expressing monocytes and mDCs were detected after BCG stimulation (Fig. 4A, 4B). In contrast to the proinflammatory monocyte response to BCG, frequencies and iMFI levels of IL-10–expressing monocytes and mDCs were not different in newborns and older infants (Fig. 4C, data not shown). Similarly, levels of secreted IL-10 after incubation with BCG for 18 h also were not different in newborns and older infants (Fig. 4D). However, lower levels of secreted IL-10 were observed in newborns compared with older infants after stimulation with PAM3 or LPS (Fig. 4D).

**FIGURE 1.** Monocyte expression of proinflammatory cytokines upon BCG stimulation of whole blood from newborns and infants. **(A)** Monocyte expression of TNF-α, IL-6, and/or IL-12 from a representative infant at 36 wk of age. Frequencies (%) of cells falling into each gate are shown for each plot. **(B)** Frequencies of monocyte subsets coexpressing proinflammatory cytokines upon BCG stimulation of whole blood from newborns and 10- and 36-wk-old infants (n = 25 for each group). **(C)** Frequencies of BCG-stimulated monocytes expressing all three proinflammatory cytokines (polyfunctional) (left panel), any two of the three cytokines (bifunctional) (middle panel), or any one of the three cytokines (monofunctional) (right panel). **(D)** iMFI of BCG-induced TNF-α, IL-6, and IL-12 expression by monocytes, representing the product of the frequency and the MFI of cytokine-positive monocytes, from newborns and 10- and 36-wk-old infants. For box and whisker plots, horizontal lines represent the median, boxes represent the interquartile range, and whiskers represent the range. Group comparisons were done using the Kruskal–Wallis test (overall effect), followed by the Mann–Whitney test.
LPS-induced intracellular IL-10 expression by monocytes or mDCs in newborns and 10- and 36-wk-old infants also was not different (Supplemental Table I).

**Changes in expression of costimulatory molecules over the first 9 mo of life**

Upregulation of costimulatory molecules in innate cells upon PRR triggering is critical for optimal interaction between the innate and adaptive immune response. To determine whether expression and/or stimulation-induced upregulation of costimulatory molecules changed during the first 9 mo of life, we evaluated expression of CD40, CD83, and CD86 on monocytes and mDCs after incubation of whole blood with BCG, LPS, or media (Fig. 5).

Levels of basal CD40 expression increased consistently with age on both unstimulated monocytes and mDCs ($p < 0.0001$ and $p = 0.003$, respectively; Fig. 5B, 5C). Little or no upregulation of CD40 was observed after BCG stimulation on monocytes or mDCs in newborns ($p = 0.04$ and $p = 0.727$, respectively; Fig. 5B, 5C). However, monocytes and mDCs from 10- and 36-wk-old infants upregulated CD40 expression in response to BCG ($p < 0.001$ for all; Fig. 5B, 5C). This age dependency appeared to be unique to mycobacterial stimulation, because LPS-induced CD40 upregulation was already observed in cord blood mDCs ($p = 0.0006$; Fig. 5C).

CD83 expression on unstimulated mDCs did not change with age. Interestingly, mDCs from newborns significantly upregulated CD83 expression upon BCG stimulation, whereas expression of this molecule was not upregulated upon stimulation in the older age groups (Fig. 5D). Although CD86 expression on unstimulated mDCs increased with age, significant upregulation of CD86 expression upon BCG stimulation was not observed at any age on mDCs (Fig. 5E) or monocytes (data not shown). However, CD86 upregulation was observed upon LPS stimulation in cord blood mDCs (Fig. 5E).

**Mechanisms for differential innate responses over the first 9 mo of life**

To determine whether differential BCG uptake and binding by monocytes was associated with observed age-dependent differential cytokine and CD40 expression, we quantified the proportions of cell subsets that phagocytosed or bound GFP-expressing BCG upon whole-blood incubation for 6 h (Fig. 6A). Higher proportions of GFP+ monocytes and mDCs were observed in newborns compared with older infants (Fig. 6B, 6C), suggesting that the greater proinflammatory monocyte response in older infants is not driven by greater binding or uptake of BCG by their innate cells.

We also compared cytokine expression in cells that had bound/ internalized BCG (BCG-GFP+ with BCG-GFP− cells. As previously reported (22), the BCG-GFP+ and BCG-GFP− monocyte and mDC subsets expressed IL-6, TNF-α, and IL-12, as well as IL-10. These data suggest that bystander activation of innate cells in the absence of direct pathogen recognition may lead to cytokine expression. However, significantly higher frequencies of BCG-GFP+ monocytes and mDCs expressed these cytokines, relative to BCG-GFP− cells, at all ages (data not shown) (22).

We next investigated whether differential activation of TLR signaling through NF-κB and MAPK pathways may underlie the greater proinflammatory responses observed in older infants. Only cryopreserved PBMCs were available for these signaling experiments, and we evaluated BCG and PAM3 stimulation in pilot studies. Stimulation of thawed PBMCs with BCG yielded variable responses, even in cells from the same donor, whereas PAM3-induced signaling was highly reproducible. Because a limited amount of PBMCs was available from infants, we prioritized PAM3 stimulation. We measured activation of the NF-κB pathway by detecting degradation of IκBα, an NF-κB inhibitor, in TLR1/2 ligand PAM3-stimulated PBMCs in comparison with unstimulated controls (Fig. 7A, Supplemental Fig. 3A, 3B). Activation of the MAPK pathway was measured by detecting phosphorylation of p38 and Erk.

After resting, median PBMC viability was 90% (interquartile range, 76.7–94%). Lower basal activation of TLR-signaling pathways in unstimulated monocytes was observed in newborns compared with older infants. Newborns had higher frequencies of IκBα− monocytes (Fig. 7B) and generally displayed lower proportions of activated monocytes, as reflected by p38 and Erk phosphorylation, compared with older infants (Fig. 7C).

When cells were stimulated with PAM3, IκBα degradation and p38 and Erk phosphorylation were observed in monocytes at all ages (Supplemental Fig. 3C). However, IκBα degradation was significantly lower in monocytes from newborns compared with older infants (Fig. 7D). By contrast, higher Erk phosphorylation was observed in monocytes from newborns compared with those from older infants (Fig. 7E). Phosphorylation of p38 did not differ according to age (Fig. 7F). No activation or activation of only one signal-transduction molecule was observed in PAM3-stimulated monocytes from newborns (Fig. 7G). A markedly higher proportion of monocytes with simultaneous activation of two or three signal-transduction molecules was observed in 10- and 36-wk-old infants (Fig. 7G, 7H). No significant differences in ac-

![FIGURE 2. mDC expression of proinflammatory cytokines upon BCG stimulation of whole blood from newborns and infants.](http://www.jimmunol.org/)

(A) mDC expression of TNF-α, IL-6, and/or IL-12 from a representative infant at 36 wk of age. Frequencies of cells falling into each gate are shown for each plot. (B) Frequencies of mDCs expressing IL-12 in unstimulated (Nil) or BCG- or LPS-stimulated whole blood from 36-wk-old infants ($n = 25$ for each group). (C) Frequencies of BCG-stimulated mDCs expressing any combination of TNF-α, IL-6, and/or IL-12. In scatter plots, horizontal lines represent the median, and error bars represent the interquartile range. Intragroup comparisons were done using the Wilcoxon test, whereas intergroup comparisons were done using the Kruskal–Wallis test (overall effect).
tivation of signal-transduction molecules were observed between 10- and 36-wk-old infants. Our data indicate that, upon TLR1/2 triggering, monocytes from newborns activate proinflammatory signaling pathways to a lower degree than do monocytes from older infants.

Discussion

Newborns are exquisitely susceptible to infection, including \textit{M. tuberculosis}, and respond poorly to vaccination. To explore possible immunological mechanisms underlying these observations, we investigated changes in innate immune responses to BCG and mycobacterial ligands during the first 9 mo of life. Three key observations suggest an increasing capacity for proinflammatory monocyte responses to mycobacteria or mycobacterial ligands as infants mature. 1) BCG-induced proportions of monocytes expressing TNF-\(\alpha\), IL-6, and/or IL-12, as well as the relative expression levels of these proinflammatory cytokines, were lower in newborns than in older infants. 2) Upregulation of the innate cell maturation marker CD40 upon BCG stimulation was lower in monocytes and mDCs from newborns compared with those from older infants. 3) These functional and phenotypic differences were possibly due to differential activation of key proinflammatory signal-transduction pathways downstream of mycobacterial recognition receptor triggering; specifically, reduced TLR1/2-mediated coactivation of monocyte NF-\(\kappa\)B and MAPK pathways was observed in newborns compared with older infants.

The magnitude and nature of innate immune responses are critical components of host resistance to infection and the generation of adaptive immunity. Several characteristics of the monocyte proinflammatory responses to BCG were lowest in newborns and increased during the first 9 mo of life. These included the proportions of TNF-\(\alpha\), IL-6, and/or IL-12-expressing monocytes; the capacity of these cytokine-positive monocytes to simultaneously express multiple proinflammatory cytokines; the relative amounts of TNF-\(\alpha\), IL-6, and/or IL-12 on a per-cell level; and the levels of TNF-\(\alpha\) and IL-6 released upon BCG stimulation. Taken together, our study is the first, to our knowledge, to show that innate responses to live \textit{Mycobacterium} develop a more proinflammatory capacity in early life. These findings are consistent with the previously described development of innate immune responses in response to other purified TLR agonists (25, 26).

We also observed an increase in CD40 expression by monocytes and mDCs during the first 9 mo of life. CD40, which belongs to the TNFR superfamily, binds to its ligand CD154, expressed on T cells, providing costimulatory signals to CD40-expressing cells (27, 28). This signaling is required for long-term DC activation, cytokine production, and T cell polarization (27, 29, 30), implying that these processes may be relatively immature in newborns.

The increasing proinflammatory capacity and CD40 expression during the first 9 mo of life suggest that BCG vaccination at birth may elicit poorer inflammatory responses than does vaccination.
weeks or months after birth, when the infant immune system has matured to be more proinflammatory. This may underlie our previous observation that BCG vaccination at birth induced lower frequencies of specific Th1 responses, measured at 1 y of age, compared with BCG vaccination at 10 wk of age (21). These results also may explain why infants who are exposed to M. tuberculosis in the first few months of life are highly likely to progress to TB disease.

Our findings of reduced proinflammatory cytokine production in response to whole mycobacteria in newborns compared with older infants build on the previous observations showing lower levels of purified TLR agonist–induced IL-12 and TNF-α in newborns compared with older infants and/or adults (25, 31–33, 36, 38). In contrast to our results, Reikie et al. (36) recently characterized innate responses during the first year of life and reported decreasing IL-6 and no changes in TNF-α expression in PAM3-stimulated whole blood. The reasons underlying these differences are not clear, but they likely include the well-recognized immune subversion strategies used by mycobacteria, including BCG (37), and concomitant activation of multiple pathogen-associated molecular patterns and signaling pathways triggered by BCG. Our results of increasing PAM3 and LPS-induced soluble IL-10 with age are at odds with several studies (25, 32, 33, 36, 38) reporting higher IL-10 expression in response to TLR agonists in newborns compared with older infants or adults. Belderbos et al. (39) observed greater IL-10 production in newborns compared with older infants for some TLR agonists, whereas the opposite pattern was found for other TLR agonists. Genetic variations in populations, epidemiological factors (e.g., exposure to cigarette smoking and helminths) (40–45), and differences in experimental protocols also may underlie...
FIGURE 7. Differential proinflammatory cell signaling in monocytes from newborns and older infants. Degradation of \( \text{I}\kappa\text{B}\alpha \) and phosphorylation of Erk (p-Erk) and p38 (p-p38) were measured by flow cytometry (gating strategy is shown in Supplemental Fig. 3A and 3B) in monocytes from newborns (\( n = 10 \)), 10-wk-old infants (\( n = 7 \)), and 36-wk-old infants (\( n = 7 \)). (A) Representative \( \text{I}\kappa\text{B}\alpha \), p-Erk, and p-p38 staining in unstimulated (UNS) and PAM3-stimulated monocytes from a single 36-wk-old infant. Dashed lines represent the threshold between positive and negative staining. (B) Basal expression of \( \text{I}\kappa\text{B}\alpha \) in unstimulated monocytes. (C) Simultaneous activation of proinflammatory signaling pathways in unstimulated monocytes from infants at different ages. Pie charts represent the total monocyte population. Slices of each pie show the median proportion of monocytes exhibiting simultaneous activation of three (red), any two (blue), one (green), or no (yellow) signaling pathways (i.e., \( \text{I}\kappa\text{B}\alpha \) degradation, phosphorylation of Erk or p38). Proportions of monocytes expressing \( \text{I}\kappa\text{B}\alpha \) (D), p-Erk (E), and p-p38 (F) after PAM3 stimulation, expressed as fold induction (FI) over marker-positive monocyte proportions in unstimulated samples. The dotted line represents no change in expression. (G) Simultaneous activation of (Figure legend continues)
We did not assess the role of these CD71+ erythroid cells, but they triggered by colonization of neonate with commensal microbes. A fundamental adaptation to protect against excessive inflammation active immunosuppression during the perinatal period may represent are enriched in the neonatal period. This study suggested that active immunosuppression during the perinatal period may represent a fundamental adaptation to protect against excessive inflammation triggered by colonization of the neonate with commensal microbes. We did not assess the role of these CD71+ erythroid cells, but they should be investigated in future studies of mycobacterial immunity in newborns and infants.

Given the discordance between higher acquisition of BCG-GFP and lower cytokine expression by monocytes from newborns compared with older infants, as well as previous studies showing that levels of TLR expression may not change markedly during the first 9 mo of life (48, 49), we investigated proinflammatory signaling pathways downstream of TLR1/2 triggering. We observed lower basal activation of proinflammatory signaling pathways in unstimulated monocytes from newborns compared with older infants. Activation of signaling pathways after birth may result from sensitization to foreign Ags and/or inflammation due to infections, vaccinations, and ingested substances or simply to lower levels of inhibitory molecules (e.g., adenosine). Interestingly, changes in epigenetic regulation of genes involved in the NF-κB and MAPK pathways have been observed during the perinatal period (50). Moreover, BCG-induced epigenetic changes in monocytes that may augment proinflammatory responses to other pathogens were recently described (51). Whether such BCG-induced epigenetic reprogramming is driving activation of proinflammatory responses in monocytes from infants is the focus of ongoing studies.

To our knowledge, we are the first to demonstrate lower TLR1/2-mediated coactivation of the NF-κB and MAPK pathways in monocytes from newborns compared with older infants. Our findings suggest that coactivation of different pathways may underlie higher innate proinflammatory cytokine responses to mycobacteria observed in older infants compared with newborns. Indeed, other investigators (52–54) showed that coactivation of MAPKs and NF-κB pathways is important for production of proinflammatory cytokines in response to mycobacteria, because simultaneous inhibition of multiple molecules abrogated cytokine production to a larger extent than did blockade of individual pathways. Furthermore, the combination of activated signaling pathways may determine which cytokines are produced: activation of both p38 and Erk pathways is essential for TNF-α production, whereas activation of p38, but not Erk, induces IL-10 in response to M. tuberculosis (55). We could not determine whether cytokine production and monocyte signaling were directly associated. These outcomes were measured in samples from different individuals because only limited volumes of blood could be safely collected from infants. Future studies will focus on cytokine production in whole blood stimulated with BCG and a panel of TLR ligands in the presence or absence of specific cell-signaling inhibitors.

Our study was subject to a number of limitations. A longitudinal study design in which individual newborns are followed up would have been ideal to evaluate changes in maturation of innate responses over the first 9 mo of life. This was not possible within the context of available resources; a cross-sectional design was more practical and feasible. Despite the lower statistical power of our cross-sectional design, we observed striking age-associated differences. Another limitation was that ethnicity distributions of the newborn and older infant groups were not the same. However, innate responses to BCG did not differ between white and mixed-ethnicity infants (data not shown), suggesting that ethnicity is unlikely to be an important confounder in our study.

Another potential confounder in our study resulted from the fact that infants were vaccinated with BCG after birth. Therefore, all analyses at 10 and 36 wk of age were potentially influenced by BCG-induced immune responses, whereas analyses of cord blood, taken before the newborn was vaccinated, were not subjected to the same external influences. In our functional assay, whole blood was stimulated for a total of 6 h, which, in our experience (56), is too short for optimal processing and presentation of BCG-derived Ags. Therefore, activation of BCG-specific memory T cells and downstream effects on innate cells would have been minimal. Further, stimulation with BCG and purified TLR ligands showed similar results. Because it is known that PAM3 and LPS do not directly activate Ag-specific T cells, this further supports that Ag-specific responses did not markedly influence our outcomes. However, we cannot rule out that other effects of BCG vaccination, not detected with these approaches, may have confounded age-associated innate responses. In fact, natural microbiome colonization, as well as other vaccines administered during infancy, are also likely to influence innate immune responses (57). Regardless, given the risk for infant morbidity and mortality due to infectious diseases in developing countries, these routine health interventions should not be withheld. We propose that effects of routine vaccinations be considered part of the normal development of the infant immune system.

Finally, we acknowledge that, in the context of TB, it would have been more appropriate to measure immune responses to M. tuberculosis rather than BCG. We did not have access to a BSL-3 laboratory at our rural clinical site to inoculate blood specimens with virulent M. tuberculosis, so we opted for M. bovis BCG instead.

Taken together, our study is the first, to our knowledge, to show that innate immune responses to BCG, a live Mycobacterium routinely used as vaccine, mature to a more proinflammatory capacity from birth to 9 mo of age. Limited innate proinflammatory capacity at birth may underlie suboptimal responses by newborns to vaccines and their particular susceptibility to infections, including M. tuberculosis. Different vaccine formulations or adjuvants may be required to achieve optimal activation of innate immunity in the perinatal period compared with later in life.

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**Figure 1.** Relative increase (stimulated minus unstimulated) in proportions of monocytes with simultaneous activation of the three signaling pathways in response to PAM3 at different ages. In scatter plots, horizontal lines represent the median, and error bars represent the interquartile range. Group comparisons were done using the Kruskal–Wallis test (overall effect), followed by the Mann–Whitney U test for pairwise comparisons. Pies and slices were compared using permutation test (p values are shown) or t test, respectively. *p < 0.05, versus newborns.
MATURATION OF ANTIMYCOBACTERIAL INNATE IMMUNITY IN INFANTS

Disclosures

The authors have no financial conflicts of interest.

References


