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Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria

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Abstract

Innate cells are essential for host defense against invading pathogens, and the induction and direction of adaptive immune responses to infection. We developed and optimized a flow cytometric assay that allows measurement of intracellular cytokine expression by monocytes, dendritic cells (DC) and granulocytes, as well as cellular uptake of green-fluorescent protein (GFP)-expressing mycobacteria, in very small volumes of peripheral blood.

We show that innate cell stimulation resulted in increased granularity of monocytes and mDCs and decreased granulocyte granularity that precluded flow cytometric discernment of granulocytes from monocytes and myeloid DC by forward and side scatter gating. Anti-CD66a/c/e antibody staining allowed reliable identification and exclusion of granulocytes for subsequent delineation of monocytes and myeloid DC. Intracellular cytokine expression by granulocytes, monocytes and mDC was remarkably sensitive to the dose of mycobacterial inoculum. Moreover, activation of monocytes and mDCs with live BCG reduced expression levels of CD14 and CD11c, respectively, necessitating optimization of staining conditions to reliably measure these lineage markers. Finally, we characterized expression of IL-12/23p40, TNF- α , IL-6, and IL-10, by GFP⁺ and GFP⁻ monocytes and mDC from 25 healthy adults.

This assay may be applied to the study of innate cell responses to any GFP-expressing pathogen, and can be performed on blood volumes as low as 200 μ L per condition, making the assay particularly suitable for pediatric studies.

Keywords

Mycobacteria; flow cytometry; monocytes; dendritic cells; granulocytes; innate cytokines

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1. INTRODUCTION

Innate immunity is critical for host defense against invading pathogens, and the induction and direction of adaptive immune responses to infection (Kollmann et al., 2009). Innate phagocytic cells, such as macrophages and neutrophils, constitute the first line of defense. Upon recognition of pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), these cells become activated and may phagocytose, kill and eliminate the infecting microorganism (Dorhoi et al., 2011; Eum et al., 2010). Activated cells also secrete cytokines and chemokines, which mediate recruitment of additional cells and orchestrate an inflammatory response (Dorhoi et al., 2011).

In addition, antigen-presenting cells that have internalized pathogen, such as dendritic cells (DC), traffic to draining lymph nodes where they present processed antigens to naïve T cells (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). The quality and magnitude of the ensuing T cell response may depend on a number of factors, including the number of invading organisms, the type and combination of PRR(s) triggered, and the subsequent efficiency of phagocytosis, innate cell activation, pathogen killing and processing, and the release of inflammatory mediators (Dorhoi et al., 2010; Kapsenberg, 2003; Mazzoni and Segal, 2004). Intracellular pathogens, such as mycobacteria, possess a number of escape pathways that arrest phagosome acidification and maturation to allow survival and replication within the phagocyte (Russell, 2011; Flynn and Chan, 2003). Optimal cytokine and chemokine-mediated cross-talk between innate and adaptive cells is required for optimal activation of macrophages to overcome this subversion and mediate pathogen killing (Flynn and Chan, 2003).

Several studies have described flow cytometric methods for *ex vivo* characterization of peripheral blood monocytes and DCs (Autissier et al 2010; Fung et al., 2010; Ida et al., 2006; Wang et al., 2006; Wang et al., 2009). In these studies granulocytes are typically excluded based on their unique size and granularity, before identifying monocytes and mDC using lineage markers, such as CD14 and CD11c.

We developed and optimized a flow cytometric assay that measures intracellular expression of key pro- and anti-inflammatory cytokines by peripheral blood innate cells in response to live mycobacteria. We show that upon activation with viable mycobacteria or LPS, changes to several properties of innate cells have to be accounted for to accurately delineate peripheral blood innate cell subsets and measure intracellular cytokine expression. We describe multiple important factors for assay success and apply this intracellular cytokine staining assay to characterize the innate cell response to the live mycobacterium, *M. bovis* Bacille Calmette-Guerin (BCG), using 200µL of whole blood per condition.

2. MATERIALS AND METHODS

2.1. Participant recruitment and enrollment

Healthy adults, aged 18–50 years, were enrolled at the South African Tuberculosis Vaccine Initiative Field Site in the Cape Town region of South Africa. Exclusion criteria included pregnancy, HIV-1 infection, *M.tb* infection and any other acute or chronic infection. HIV-1 infection was diagnosed by rapid HIV antibody test (HIV Determine 1&2), while *M.tb* infection was defined as a positive interferon gamma (IFN-γ) response to ESAT6/CFP-10 protein, measured by ELISA, as described previously (Kagina et al., 2009). The study protocol was approved by the Research Ethics Committee of the University of Cape Town, and all participants provided written informed consent.

2.2. TLR ligands and bacteria, and antibodies

Ultrapur lipopolysaccharide (LPS, TLR4 ligand, 100ng/mL final concentration), isolated from *Salmonella minnesota*, was obtained from InvivoGen. This LPS concentration was previously found to induce optimal cytokine expression by innate cells in whole blood (Jansen et al., 2008). Viable BCG expressing green fluorescent protein (BCG-GFP, Pasteur strain; 3.5×10^5 CFU/mL final concentration, unless otherwise stated, donated by Muazzam Jacobs, University of Cape Town) was cultured in our laboratory. Bacteria were harvested at log phase 3 weeks after start of culture (optical density of 0.8) and CFU counts determined by plating bacteria on an agar. LPS and bacteria were prepared at 10 times the final concentration in RPMI 1640, and 20 μ L was added into 96 round bottom well plates.

The following antibodies were used, CD14-QDot605 (clone Tuk4, Invitrogen), CD14-Pacific Blue (clone M5E2, BD Pharmingen), TNF- α -PECy7 (clone Mab11, BD Pharmingen), CD11c-PerCPCy5.5 (clone Bu15), HLA DR-AlexaFluor700 (clone L243), IL-12/23p40-Pacific Blue (clone C11.5), IL-10-PE (clone JES3-19F1) and IL-6-APC (clone MQ2-13A5), all from Biolegend. CD66a/c/e (ASL-32, Biolegend) was conjugated in-house to QDot565 (Invitrogen) using the manufacturer's protocol.

2.3. Blood collection and processing

Heparinized blood was collected from each participant and processed immediately (maximum delay between blood collection and incubation with ligands was 30 minutes). We previously investigated the effects of delayed processing of blood and showed that delay by 60 minutes or more affected cytokine expression by mDC (Mendelson et al., 2006). An undiluted blood volume of 180 μ L was added to wells of a 96-well plate containing LPS or bacteria. The cultures were incubated at 37°C, 5% CO₂ in humidified conditions. RPMI 1640 was used as medium control. After 3 hours of incubation, brefeldin A (BFA, 10 μ g/mL, Sigma-Aldrich) was added to each well and the plate was incubated for 3 additional hours as previously optimized (Jansen et al., 2008). After a total incubation of 6 hours, EDTA was added (2mM final concentration, Sigma-Aldrich) and blood incubated for 10 minutes at room temperature in order to detach adherent cells. To lyse red cells and fix white cells, FACS Lysing Solution (BD Biosciences) was added and cells were incubated at room temperature for 10 minutes. This lysing step was repeated to ensure complete red cell lysis. Fixed white cells were either stained immediately or cryopreserved in 10% DMSO in heat inactivated fetal calf serum (10% DMSO/FCS) or in FACS Lysing Solution.

2.4. Intracellular cytokine staining and flow cytometric acquisition

Cryopreserved, stimulated cells were thawed in batch, and cells were washed twice with either phosphate buffered saline (PBS, without calcium and magnesium) or BD Perm/Wash buffer (BD Biosciences). Cells were stained with fluorescent antibodies in a total volume of 30 μ L in either PBS or BD Perm/Wash, at 4°C for 1hr. Stained cells were washed and 1 million cells or the total sample volume were acquired on a BD LSR II flow cytometer.

2.5. Data analysis

Flow cytometry data were analysed using FlowJo v9.2. Results from single-stained and unstained mouse κ beads were used to calculate compensations, for each run. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Cytokine co-expression by innate cell subsets was assessed by boolean gating. Subtraction of background cytokine expression (unstimulated samples) was done using Pestle V1.6.2, while data sorting and analysis were done with Spice V5.1 (Roederer et al., 2011; <http://exon.niaid.nih.gov/spice>, accessed February 25th, 2011). GraphPad Prism v5 was used

for data presentation and statistical analysis. The Mann-Whitney or Wilcoxon signed rank tests were used to compare data sets. P values <0.05 were considered significant.

3. RESULTS

3.1. CD66a/c/e allows identification and exclusion of granulocytes

The different size and granularity of granulocytes, compared with monocytes and mDC, allows identification of these cell subsets by *ex vivo* flow cytometric analysis (Autissier et al 2010; Fung et al., 2010). Upon stimulation with live mycobacterium BCG-GFP, or LPS, we observed a decrease in side scatter fluorescence of granulocytes, while the side scatter fluorescence for mDC and monocytes increased (Fig. 1A). This precluded separation of mDC and monocytes from granulocytes using forward and side scatter parameters.

The CD66 isoforms a, c, d and e are members of the carcinoembryonic antigen (CEA) family of the Ig superfamily, and are exclusively expressed on granulocytes and epithelial cells (Gray-Owen and Blumberg, 2006). Staining with anti-CD66a/c/e antibody allowed identification of peripheral blood granulocytes (CD66a/c/e⁺, Fig. 1B). Since granulocytes express high levels of HLA DR and low levels of CD14 and CD11c, exclusion of granulocytes was required for accurate identification of CD14⁺HLA DR⁺CD11c⁺ mDC and HLA DR⁺CD14⁺ monocytes. Upon granulocyte exclusion the frequency of cells falling into the HLA DR⁺ gate decreased from 61% (IQR, 58–72%) to 9% (IQR, 7–13%, Fig. 1C–E). Similarly, the proportion of HLA DR⁺CD14⁺ cells expressing CD11c amongst all leucocytes decreased from 55% (IQR, 51–58%) to 1% (IQR, 0.7–1.5%) upon exclusion of CD66a/c/e⁺ cells (Fig. 1C–E).

3.2. BCG-activated monocytes downregulate CD14 expression

We investigated whether innate cell activation affects expression levels of innate lineage markers and flow cytometric delineation of monocytes, mDC and granulocytes. Lower frequencies of CD14⁺ monocytes were detected upon BCG stimulation, compared with unstimulated samples. This was observed when expression of CD14 was measured by flow cytometric staining with QDot605 or Pacific Blue conjugated anti-CD14 antibodies (Fig. 2A and B). A decrease in median fluorescence intensity of CD14 was also observed upon BCG stimulation, albeit only when QDot605-conjugated anti-CD14 was used. A recent study reported that the HLA DR⁺CD11c⁺CD14⁺/dim cell population may also contain CD14⁺CD16⁺ monocytes (Cros et al., 2010). We could not delineate these monocyte sub-populations, as we did not measure CD16 expression in our analyses.

No difference in the frequency of CD66a/c/e⁺ granulocytes or CD11c⁺ mDC among HLA DR⁺CD14⁺ cells was observed upon BCG stimulation, and CD11c fluorescence was only moderately decreased (p=0.03, Fig. 2A and data not shown). Similar results were obtained when whole blood was incubated with LPS (data not shown).

The downregulation of CD14 and CD11c necessitated optimal blood processing and antibody staining conditions to identify these key lineage markers after incubation of whole blood with BCG or LPS. Because monocytes are adhesive cells, we tested whether EDTA treatment after stimulation would increase the number of CD14⁺ monocytes. Although we observed a higher proportion of CD14⁺ cells in most donors after EDTA treatment, compared with untreated samples, this difference was not significant (Supplementary Fig. 2). Regardless, EDTA treatment was included for all subsequent experiments. Using this protocol, a median (IQR) of 8,049 mDC (6,152–11,432), 27,218 monocytes (19,447–33,437) and 368,000 granulocytes (273,500–412,500) were acquired for analysis.

3.3. QDot-conjugates are sensitive to staining buffer

Given the difference in staining performance between the QDot and Pacific Blue conjugated anti-CD14 antibodies, we further optimized staining conditions for QDot conjugates. QDots are fluorescent nanocrystals commonly used for imaging and flow cytometric analysis (Zarkowsky et al., 2011). Performance of these fluorochromes can be sensitive to components in staining buffers, such as heavy metals (Chen et al., 2002; Meallet-Renault et al. 2006). To optimize the antibody staining method for innate cell delineation, we tested the performance of fluorochrome-conjugated antibodies in different staining buffers. We observed low fluorescence of CD66a/c/e-QDot565 and CD14-QDot605 staining when cells were incubated with a single cocktail of all 8 antibodies in PBS (Fig. 2C). The low signal of these markers precluded reliable delineation of monocyte and DC subsets, especially after BCG or LPS stimulation. By contrast, cell staining with the single antibody cocktail in BD Perm/Wash buffer resulted in higher fluorescence of the QDot-conjugates, allowing more precise gating of cell subsets. When cells were first stained with non-QDot-conjugated antibodies in PBS, followed by a second staining step with anti-CD66a/c/e-QDot565 and anti-CD14-QDot605 in BD Perm/Wash buffer, the fluorescence of CD14-QDot605 was even brighter. However, this did not enhance CD66a/c/e-QDot565 fluorescence markedly. The fluorescence of non QDot-conjugated antibodies did not change when PBS or BD Perm/Wash was used as staining buffer (data not shown).

1mM EDTA in staining buffer has been shown to improve QDot fluorescence (Zarkowsky et al. 2011). We did not observe any improvement in fluorescence of QDot-conjugated antibodies when cells were stained in PBS containing 1mM EDTA (data not shown). We therefore proceeded with single-step staining in BD Perm/Wash buffer for all subsequent experiments.

3.4. Green fluorescent protein-expressing BCG (BCG-GFP) allows measurement of BCG binding and/or internalization by innate cells

Upon pathogen recognition, activated innate cells may bind to and/or phagocytose the microbe and express cytokines and chemokines (Gille et al., 2009). Alternatively, activation and cytokine expression may occur in the absence of pathogen binding or phagocytosis. We used a GFP-expressing BCG to allow detection of innate cells that have bound and/or internalized BCG, by flow cytometry (Fig. 3A). The concentration of BCG inoculum was an important determinant of innate cell response to BCG. The proportion of GFP⁺ monocytes and granulocytes reached a plateau at an inoculum concentration of 3.5×10^5 CFU/mL of whole blood, whereas the proportion of GFP⁺ mDC appeared to reach a maximum at a lower inoculum dose of 1.7×10^5 CFU/mL (Fig. 3B, upper panel). Surprisingly, expression of the pro-inflammatory cytokine IL-6 was remarkably sensitive to the mycobacterial inoculum concentration (Fig. 3A and B). IL-6 expression by monocytes, mDC and granulocytes peaked at a mycobacterial inoculum of 3.5×10^5 CFU/mL and markedly decreased at higher doses (Fig. 3B). The dose of 3.5×10^5 CFU/mL was chosen for subsequent experiments.

3.5. Different frequencies of innate cells directly bind or internalize BCG

We next determined the proportion of GFP⁺ cells among each innate cell subset, as well as the proportion of each subset among GFP⁺ cells. A median proportion of 45% (IQR, 37–50%) of granulocytes were GFP⁺ (Fig. 4A). A lower proportion of monocytes were GFP⁺ (Median, 36%; IQR, 31–38%) while mDC displayed the lowest proportion of GFP⁺ cells (median, 26%; IQR, 23–30%) (Fig. 4A). A similar, but markedly more pronounced picture was observed when we assessed the relative proportions of these innate cell subsets among GFP⁺ cells. Granulocytes comprised 87% (IQR, 82–89), monocytes comprised 7% (IQR, 5–9%), while mDC contributed 1% (IQR, 0.8–1.4%) of all GFP⁺ innate cells (Fig. 4B).

Granulocytes were thus the peripheral blood innate cell subset with the highest capacity for binding and/or internalization of BCG.

3.6. Freezing medium is not a critical determinant of assay performance

Batch thawing of samples cryopreserved after stimulation and fixation allows more standardized antibody staining for flow cytometry. To determine the effect of cryopreservation on assay performance, we compared the frequencies of monocytes and mDC when cells were stained immediately after culture without cryopreservation, with cells that were cryopreserved. Prior cryopreservation did not significantly affect the frequencies of mDC or monocyte subsets in unstimulated or BCG-stimulated blood (Supplementary Fig. 3A, right). Choice of cryopreservation medium may also affect cellular proteins and thus antibody staining. We evaluated the use of either FACS Lysing Solution (FLS) or 10% DMSO in FCS as cryopreservation media. Frequencies of CD14⁺ and CD11c⁺ cells in unstimulated or BCG-stimulated whole blood were also not significantly affected by the choice of cryopreservation medium (Supplementary Fig. 3B). Although cryopreservation did appear to result in lower frequencies of IL-6 expressing monocytes and mDC in 4 donors, this difference was not significant (Supplementary Fig. 3C). Similarly, frequencies of IL-6-expressing monocytes appeared to be higher when FACS Lysing solution was used, compared with DMSO in FCS, but this was not significant (Supplementary Fig. 3C). Cryopreservation medium did not influence frequencies of IL-6-expressing mDC after incubation of whole blood with BCG. Subsequent experiments were performed with 10% DMSO as freezing medium.

3.7. Cytokine expression by BCG-GFP-negative cells

Finally, we applied the optimized whole blood innate ICS assay to compare cytokine expression between innate cells that bound and/or internalized BCG (GFP⁺) and GFP-negative cells. A substantial proportion of GFP⁻ monocytes and mDC expressed the pro-inflammatory cytokines IL-6, IL-12/23p40 and/or TNF- α , in various combinations, albeit at lower frequencies than GFP⁺ monocytes and mDC (Fig. 5A). Notably, monocytes expressing IL-12/23p40 alone were observed at a higher frequency in GFP⁻, compared with GFP⁺ cells (Fig. 5A). Monocytes expressing IL-6 alone and mDC co-expressing IL-6 and TNF- α were the dominant cytokine-expressing subsets in both GFP⁺ and GFP⁻ cells (Fig. 5A and B).

Upon LPS stimulation, monocytes expressing IL-6 alone also comprised the dominant subset, while cells co-expressing IL-6 and TNF- α were prominent (Fig. 5C). By contrast, similar frequencies mDC expressed IL-6 alone or co-expressed IL-6 and TNF- α (Fig. 5D).

IL-10-expressing cells were detected at very low frequencies compared with IL-6, TNF- α and IL-12/23p40, and IL-10 was not co-expressed with these pro-inflammatory cytokines (data not shown). Again, IL-10-expressing monocytes and mDC were observed at higher proportions amongst GFP⁺ cells, compared with GFP⁻ cells (Fig. 5E and F). LPS stimulation induced higher frequencies of IL-10-expressing monocytes, compared with BCG (Fig. 5E). BCG binding or internalization induced a higher frequency of cells expressing cytokines.

4. DISCUSSION

We developed and optimized an assay for measuring intracellular cytokine expression by peripheral blood innate cells in response to viable mycobacteria using very small volumes of blood. Our method presents a number of important variables for optimal performance of this innate cell ICS assay: 1. Exclusion of granulocytes is required for reliable flow cytometric

delineation of myeloid DCs and monocytes in whole blood; 2. Anti-CD66a/c/e antibody staining allows flow cytometric identification and analysis of granulocytes among activated innate cells; this was not possible using forward and side scatter parameters; 3. Innate cells that have not bound or phagocytosed mycobacteria express cytokines, and this cytokine expression is exquisitely sensitive to the dose of mycobacterial inoculum; 4. Fluorescent antibody staining buffer and cell activation are important determinants of performance and outcomes of the ICS assay.

We show that the increase in monocyte and mDC granularity, and decrease in granulocyte granularity, after whole blood stimulation with BCG or LPS precludes discernment of granulocytes from monocytes and mDCs. Further, granulocyte expression of the mDC lineage marker, CD11c, necessitated exclusion of granulocytes to identify peripheral blood mDCs. We show that anti-CD66a/c/e antibody allows identification and exclusion of granulocytes, and subsequent identification of monocytes and mDC using key lineage markers. Our results highlight that innate assays should take into account the marked changes that occur upon innate cell activation. In our hands, the changes in granularity ruled out identification of granulocytes by side and forward scatter parameters, which is the most common method for phenotyping innate cells *ex vivo* (Autissier et al., 2010; Fung et al., 2010).

Our results also underscore an important consideration when identifying DC subsets in whole blood, since no single marker is expressed exclusively by all DC subsets. The most common DC identification methods enumerate HLA DR⁺ and CD11c⁺ DCs after excluding T cells (CD3), B cells (CD19 or CD20), NK cells (CD56 or CD16), and monocytes (CD14) using lineage markers (Autissier et al., 2010; Ida et al., 2006; Wang et al., 2006; Wang et al., 2009). However, exclusion gating of lineage marker-positive cells may lead to exclusion of immature DC, which express low levels of CD14 and CD16 (Wang et al. 2006). We propose a combination of markers that allows identification of unstimulated or activated granulocytes, monocytes and mDC without these confounders, while allowing simultaneous analysis of cytokine expression patterns of these cells using a single antibody cocktail. Wang et al. (2006) also showed that CD66a/c/d/e antibody-containing lineage cocktails allowed detection of higher frequencies of DCs, compared with cocktails containing anti-CD14 antibodies (Wang et al. 2006).

Incubation of whole blood with BCG or LPS led to markedly lower frequencies of CD14⁺ monocytes. Our results that EDTA treatment did not change CD14⁺ cell frequencies significantly suggest that greater adherence of monocytes upon activation was an unlikely contributor to this finding. Downregulation of CD14 was previously reported upon stimulation with high doses of TLR7/8 or TLR4 ligands (Jansen et al., 2008). CD14 downregulation is also described in response to histamines in monocytes (Takahashi et al., 2003), LPS and *E. coli* in rabbit alveolar macrophages (Lin et al., 2004). However, *M.tb* infection has been shown to upregulate the expression of CD14 on monocytes (Shams et al., 2003). Given the role of CD14 as the TLR4 co-receptor for LPS binding, the downregulation is likely due to macropinocytosis-mediated internalization upon TLR4 stimulation (Mollen et al., 2008; Poussin et al., 1998). Lower frequencies of CD14⁺ cells after stimulation may also be due to shedding of CD14 or monocyte death. A spontaneous decrease in CD14 without stimulation has been reported, which could be due to internalization of membrane-bound CD14 followed by processing and secretion of soluble CD14, or the rapid recycling of CD14-TLR4-MD2 complexes between the plasma membrane and the Golgi apparatus (Bosshart and Heinzelmann, 2011). Incubation of PBMC with *E. coli* or Group B streptococcus also leads to a reduction in viable monocytes (Gille et al., 2009). Similarly, monocytes are known to rapidly die through either classical apoptosis or alternative cell death processes after phagocytosis of mycobacteria (Webster et al., 2010).

Notably, HLA DR⁺CD11c⁺CD14⁻/_{dim} cell population may also contain CD14⁻CD16⁺ monocytes (Cros et al., 2010). Peripheral blood frequencies of CD16⁺ monocytes were reported to increase during *M.tb* infection, but these cells were more susceptible to apoptosis, and, unlike CD14⁺ monocytes, did not differentiate *in vitro* into monocyte-derived-macrophages (Castano et al., 2011). We could not delineate these monocyte sub-populations, as we did not measure CD16 expression in our analyses.

The reduction in CD14⁺ frequency and fluorescence necessitated optimization of staining conditions for detection of monocytes. QDot-conjugated antibodies (CD14 and CD66a/c/e) performed best when antibody staining was performed in BD Perm/Wash buffer. Fluorescence of QDot nanocrystals is sensitive to staining buffers, and depends on concentrations of heavy metals (Chen et al., 2002; Meallet-Renault et al., 2006). Low concentrations of cupric ions were recently shown to eliminate QDot fluorescence (Zarkowsky et al. 2011). The latter study showed that 1mM EDTA completely protected the fluorescent properties of these nanocrystals. However, in our hands staining in 1mM EDTA/PBS did not result in enhanced QDot fluorescence.

Infection of innate cells with GFP-expressing BCG permits evaluation of the proportion of cells that have phagocytosed BCG, and allows comparison of cytokine expression by BCG-containing cells with those that have not internalized BCG. Importantly, our GFP-based assay system did not allow discrimination between cells that have phagocytosed BCG and cells with surface-bound mycobacteria. Although not investigated here, we anticipate that the proportion of cells with internalized BCG markedly exceeds those with surface-bound BCG, as was previously shown for human epithelial cells (de Boer et al., 1996). Interestingly, while the proportion of GFP⁺ cells increased with greater bacterial inocula, the proportion of functional, IL-6 expressing cells peaked at an inoculum of 3.5×10^5 CFU/mL. IL-6 expression of these cells dropped rapidly at higher bacterial loads. Our data highlight that titrating the mycobacterial inoculum when measuring innate cell cytokine expression is an important optimization step. The exact mechanism for the lower cytokine expression at high bacterial inocula is not clear, but may be related to the known inhibitory effect of polar lipids, such as phenolic glycolipids (PGL), found in the cell wall of BCG (Reed et al., 2004; Vergne and Daffe, 1998). PGL derived from *M.tb* H37Rv or BCG was shown to inhibit the production of TNF- α and IL-6 by murine bone marrow derived macrophages (BMM) in a dose-dependent manner (Reed et al., 2004). It is unknown whether this sensitivity of *in vitro* innate cell cytokine expression to mycobacterial dose applies to innate cell behavior *in vivo*. Such sensitivity would imply that infection with high doses of pathogen might lead to suboptimal inflammatory responses.

We observed cytokine expression by a considerable proportion of GFP⁻ cells. A higher frequency of GFP⁻ monocytes expressed IL-12/23p40 alone, compared with GFP⁺ monocytes. The significance of this observation is unknown. Cytokine expression by GFP⁻ innate cells is likely due to bystander activation by cytokines secreted by phagocytic cells, or other cells that can directly recognize mycobacteria, such as NK or $\gamma\delta$ T cells.

Granulocytes were the major peripheral blood phagocytes of BCG, although little or no cytokine response was detected in these cells. This finding is consistent with a recent study showing that neutrophils were the predominant *M.tb*-infected cell subset in sputum and bronchoalveolar lavage from patients with multidrug resistant TB (Eum et al., 2010). However, these data do not accord with several murine studies that invariably showed alveolar macrophages and DCs to be the predominant populations of BCG-infected lung cells *in vivo* (Humphreys et al., 2006; Pecora et al., 2008). Despite being present at high numbers in lungs of infected mice, granulocytes were not infected to the same extent (Humphreys et al., 2006). These differences may simply be due to distinct immune

characteristics or presentation of TB infection/disease in mice and humans (Eum et al., 2010). The different markers and methods for identification of cell subsets may also underlie the discrepant outcomes; for example, we identified granulocytes as CD66a/c/e⁺ while Humphreys et al. (2006) identified these cells as CD11b^{+/Hi}CD11c⁻.

A limitation of the flow cytometric ICS assay described here was the absence of a viability marker to exclude dead cells. Stimulation with BCG or LPS was performed on fresh whole blood, which reduced the likelihood of cell death typically observed when culturing thawed cells. In line with this, we observed no or only very minor evidence of artifactual antibody staining and/or autofluorescence. Although not tested here, a staining step with a fixation-resistant viability dye may be incorporated after red cell lysis, but before cell fixation, to allow exclusion of dead cells, as we recently described for another whole blood assay (Soares et al., 2010). We also could not assess whether BCG in GFP⁺ cells was intracellular or on the surface.

To summarize, we developed an innate cell ICS assay that measures cytokine expression by flow cytometry to mycobacteria. This assay may be applicable to studying innate cell responses to any fluorescent pathogen, and can be performed on blood volumes as low as 200µL per condition, making it particularly suitable for pediatric studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Optimizing flow cytometric detection of innate cell subsets

(A) Forward scatter and side scatter properties of granulocytes and mDC (top row) or monocytes (bottom row), after incubation of whole blood with no antigen, BCG-GFP or LPS. Granulocytes were identified as CD66a/c/e⁺, monocytes as CD14⁺ and mDC as CD14⁻, HLA DR⁺ and CD11c⁺ (refer to Supplementary Figure 1C for gating strategy). (B) Identification and exclusion of granulocytes by anti-CD66a/c/e staining. (C and D). CD14 and HLA DR expression by peripheral blood innate cells (left plots), and CD11c expression by CD14⁻, HLA DR⁺ cells (right plots), before (C) or after (D) exclusion of CD66a/c/e⁺ granulocytes. (E) Frequencies of CD14⁻ HLA DR⁺ and CD11c⁺ mDC pre and post exclusion gating of CD66a/c/e⁺ granulocytes, in 25 adults. The frequencies of cell subsets

among total leukocytes falling into the CD14⁻ HLA DR⁺ or CD11c⁺ gates are shown in each plot (refer to Supplementary Figure 1C for gating strategy).

Figure 2. Cell activation downregulates CD14

Whole blood from 5 donors was incubated with no antigen or 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. (A) Representative plots showing flow cytometric detection of cells falling into the CD14⁺ and HLA DR⁺ (top plots) and CD11c⁺ (lower plots) gates. (B) Frequencies and CD14 median fluorescence intensities (MFI) of monocytes detected with anti-CD14 QDot (left panels) or anti-CD14 Pacific Blue (right panels). The Wilcoxon signed rank test was used for statistical analysis. (C) Comparison of QDot-conjugate antibody staining of whole blood from a single representative donor after single step staining in PBS or Perm/Wash or two-step staining in PBS (for non QDot-conjugated antibodies), then Perm/Wash (for QDot-conjugated antibodies).

Figure 3. Measurement of BCG uptake by innate cells

(A) Representative dot plots of IL-6 expression by GFP⁺ or GFP⁻ monocytes, mDC or granulocytes, after incubation without BCGGFP (upper row), or with 3.5×10^5 CFU/mL (middle row), or 10×10^5 CFU/mL of BCG-GFP (bottom row). The frequencies of cells falling into each of the 2 boolean gates are shown. (B) Frequencies of GFP⁺ monocytes, mDC, or granulocytes (upper row) after titration of BCG-GFP into whole blood from 5 donors. (Bottom row) Frequencies of total IL-6 expressing monocytes, mDC or granulocytes at different BCG-GFP inocula.

Figure 4. Comparison of mDC, monocyte or granulocyte phagocytosis of mycobacteria
Whole blood from 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. (A) Proportion of GFP⁺ cells among each innate cell subset. (B) Proportion of each innate cell subset among GFP⁺ cells. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range.

Figure 5. Differential cytokine expression by GFP⁺ and GFP⁻ innate cells

Whole blood from 25 donors was incubated with 3.5×10^5 CFU/mL BCG-GFP for 6 hours before flow cytometric analysis of cell subsets. Cytokine co-expression patterns in monocytes (A) or mDC (B) that have phagocytosed BCG (GFP⁺) or not (GFP⁻). Cytokine co-expression patterns in monocytes (C) or mDC (D) after LPS stimulation. Frequencies of IL-10-expressing monocytes (E) or mDC (F) after whole blood incubation with BCG-GFP or LPS. Horizontal lines represent the median, boxes represent the IQR and whiskers represent the range. Differences between GFP⁺ and GFP⁻ cells were calculated with the Wilcoxon signed rank test.