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The Contribution of Non-Conventional T Cells and NK Cells in the Mycobacterial-Specific IFN γ Response in Bacille Calmette-Guérin (BCG)-Immunized Infants

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Abstract

Background: The *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccine is given to >120 million infants each year worldwide. Most studies investigating the immune response to BCG have focused on adaptive immunity. However the importance of TCR-gamma/delta ($\gamma\delta$) T cells and NK cells in the mycobacterial-specific immune response is of increasing interest.

Methods: Participants in four age-groups were BCG-immunized. Ten weeks later, in vitro BCG-stimulated blood was analyzed for NK and T cell markers, and intracellular IFN γ (IFN γ) by flow cytometry. Total functional IFN γ response was calculated using integrated median fluorescence intensity (iMFI).

Results: In infants and children, CD4 and CD4-CD8- (double-negative (DN)) T cells were the main IFN γ -expressing cells representing 43-56% and 27-37% of total CD3+ IFN γ + T cells respectively. The iMFI was higher in DN T cells compared to CD4 T cells in all age groups, with the greatest differences seen in infants immunized at birth ($p=0.002$) or 2 months of age ($p<0.0001$). When NK cells were included in the analysis, they accounted for the majority of total IFN γ -expressing cells and, together with DN V δ 2 $\gamma\delta$ T cells, had the highest iMFI in infants immunized at birth or 2 months of age.

Conclusion: In addition to CD4 T cells, NK cells and DN T cells, including V δ 2 $\gamma\delta$ T cells, are the key populations producing IFN γ in response to BCG immunization in infants and children. This suggests that innate immunity and unconventional T cells play a greater role in the mycobacterial immune response than previously recognized and should be considered in the design and assessment of novel tuberculosis vaccines.

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Introduction

The *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccine is given to more than 120 million children worldwide each year and remains a key intervention in the prevention of tuberculosis (TB) [1]. In infants it provides approximately 80% protection against severe forms of TB [2].

Understanding the immune response to BCG immunization provides important information in the search for immunological correlates of protection against TB. Surrogate biomarkers of

protection against TB remain elusive but are important for the development of improved TB diagnostics and vaccines.

Most studies investigating the immune response to BCG and protection against TB have investigated adaptive immunity [3–5]. In recent years there has been increasing recognition of the importance of the innate immune response in early neonatal life [6–9]. T cells with a gamma-delta ($\gamma\delta$) TCR and NK cells play a key role in innate immunity. These cells increase in frequency during foetal development and represent major cell subsets in cord blood [10–12]. To date, only few

studies have investigated the innate immune response to BCG immunization in infants.

We have previously reported the CD4 and CD8 T cell responses 10 weeks after BCG immunization [3,13]. In this study we used samples from the same studies to investigate the role of CD4⁺CD8⁺ double negative (DN) T cells, V δ 2 γ δ T cells and NK cells in the mycobacterial-specific IFN γ response after BCG immunization.

Methods

Ethics Statement

The study was approved by the Human research ethics committees at the Mercy Hospital for Women (R07/16), the Royal Children's Hospital (26191) and The University of Melbourne (0828435). Written informed consent was obtained from participants or parents.

Study participants

Infants were recruited at the Mercy Hospital for Women in Melbourne as part of a previous study [3]. Children aged between 10 and 24 months that needed BCG immunization for travel to high TB-prevalence countries were recruited at the Royal Children's Hospital, Melbourne [13]. Adult volunteers were recruited from University of Melbourne medical students aged between 22 and 27 years who planned to work during their elective overseas in high TB-prevalence countries [13].

BCG vaccine

BCG Denmark, SSI-1331 (Statens Serum Institute, Copenhagen, Denmark) was used to immunize infants in the first week of life or at 2 months of age [3]. BCG Connaught (Sanofi Pasteur, Toronto, Canada) was used to immunize children older than 2 months and adult participant [13]. BCG vaccine was administered intradermally in the left deltoid region.

Whole blood assay

Blood was obtained 10 weeks after immunization for *in vitro* assays. To measure cytokine production, whole blood was stimulated with BCG (1.6×10^6 CFU/ml of the same BCG vaccine strain used for immunization reconstituted with Roswell Park Memorial Institute medium) for 7 hours at 37°C in the presence of co-stimulatory antibodies CD49d and CD28 (1 μ g/ml each; both from BD Biosciences, San Jose, USA) or left unstimulated (nil control). After addition of brefeldin A (Sigma-Aldrich, St. Louis, USA) at a concentration of 10 μ g/ml cells were incubated for 5 additional hours, harvested with 2 mM EDTA (Sigma-Aldrich) then fixed with FACS lysing solution (BD Biosciences) and stored at -80 °C.

Flow cytometry

Stored blood samples were thawed at 37 °C, permeabilized with Perm 2 buffer for 10 minutes (BD Biosciences) and stained for 30 minutes in the dark with the following anti-human antibodies: CD4-allophycocyanin-eFluor 780 (clone SK3; eBioscience, San Diego, USA), CD8-Qdot605 (3B5; Invitrogen,

Carlsbad, USA), CD3-Pacific blue (UCHT1), V δ 2 TCR-PE (B6), CD56-allophycocyanin (NCAM 16.2), IFN γ -AlexaFluor 700 (B27) (all BD Biosciences). Cells were acquired using LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo 8.8 (TreeStar, Ashland, USA) and Prism 5 (GraphPad Software, La Jolla, USA). Cytometer setup and tracking beads (BD Biosciences) were used to define LSRII baseline and run daily measurements. CompBeads set anti-mouse Igk (BD Biosciences) was used to optimize fluorescence compensation settings. For each sample, a minimum of 10^6 cells was acquired. Proportions of BCG-induced cytokine producing cells were analyzed after background correction by subtracting the nil control sample values. Median fluorescence intensity (MFI) was calculated using FlowJo. MFI of BCG-stimulated samples was background corrected by subtracting the MFI of nil control samples. The total functional response of a cell population producing IFN γ is expressed as the integrated MFI (iMFI) and was calculated by multiplying the frequency of IFN γ -expressing T cells by the related MFI as described previously [14].

Results shown in Figures 1 and 2 depict re-analyzed data from samples used in previous studies [3,13]. The following conjugated anti-human antibodies were used in these samples: CD3 PerCP-Cyanin5.5 (SK7), CD4 FITC (RPA-T4), CD8 AlexaFluor-700 (RPA-T8) and IFN γ PE-Cyanin7 (4S.B3) (all BD Biosciences). A hierarchical gating strategy was used to determine proportions of CD4, CD8, DN and double positive (DP) cells within the CD3⁺IFN γ ⁺ population (Figure S1). Only samples with more than 100 cells detected in the CD3⁺IFN γ ⁺ gate (Figure 1) or in the combined NK and CD3 IFN γ ⁺ gate (Figure 3) were included in the analysis. Only samples with more than 10 cells detected in the CD3⁺CD4⁺CD8⁺IFN γ ⁺ gate were included in Figures 2 and 4.

Statistical analysis

A Kruskal-Wallis test and Dunn's multiple comparison tests were used to compare groups. If the p-value was less than 0.05, a Wilcoxon signed rank test was done to compare two pairs. Graphs were generated and statistics calculated using Prism 5 (GraphPad Software, La Jolla, USA).

Results

Participants in four age-groups were immunized with BCG. After 10 weeks, blood samples from participants were stimulated with BCG or left unstimulated (nil control), and the mycobacterium-specific immune response was measured by flow cytometry. In children below two years of age, DN T cells represented between 3.4% (n=28) and 7.8% (n=26) of CD3 T cells (Table 1). Despite their small proportion, this subset was responsible for a large share of mycobacterial-specific IFN γ -expressing cells (Figure 1), comparable with the contribution from CD4 T cells. Notably, in contrast to the response observed in children, CD8 T cells were the major contributor of IFN γ -expressing cells in adults (n=5) (Figure 1).

DN T cells more frequently expressed IFN γ than CD4 T cells following BCG immunization. At birth (n=28), 1.69% (interquartile range (IQR) 0.8-2.4%) of DN T cells expressed IFN γ compared to 0.08% (IQR 0.04-0.18%) of CD4 T cells,

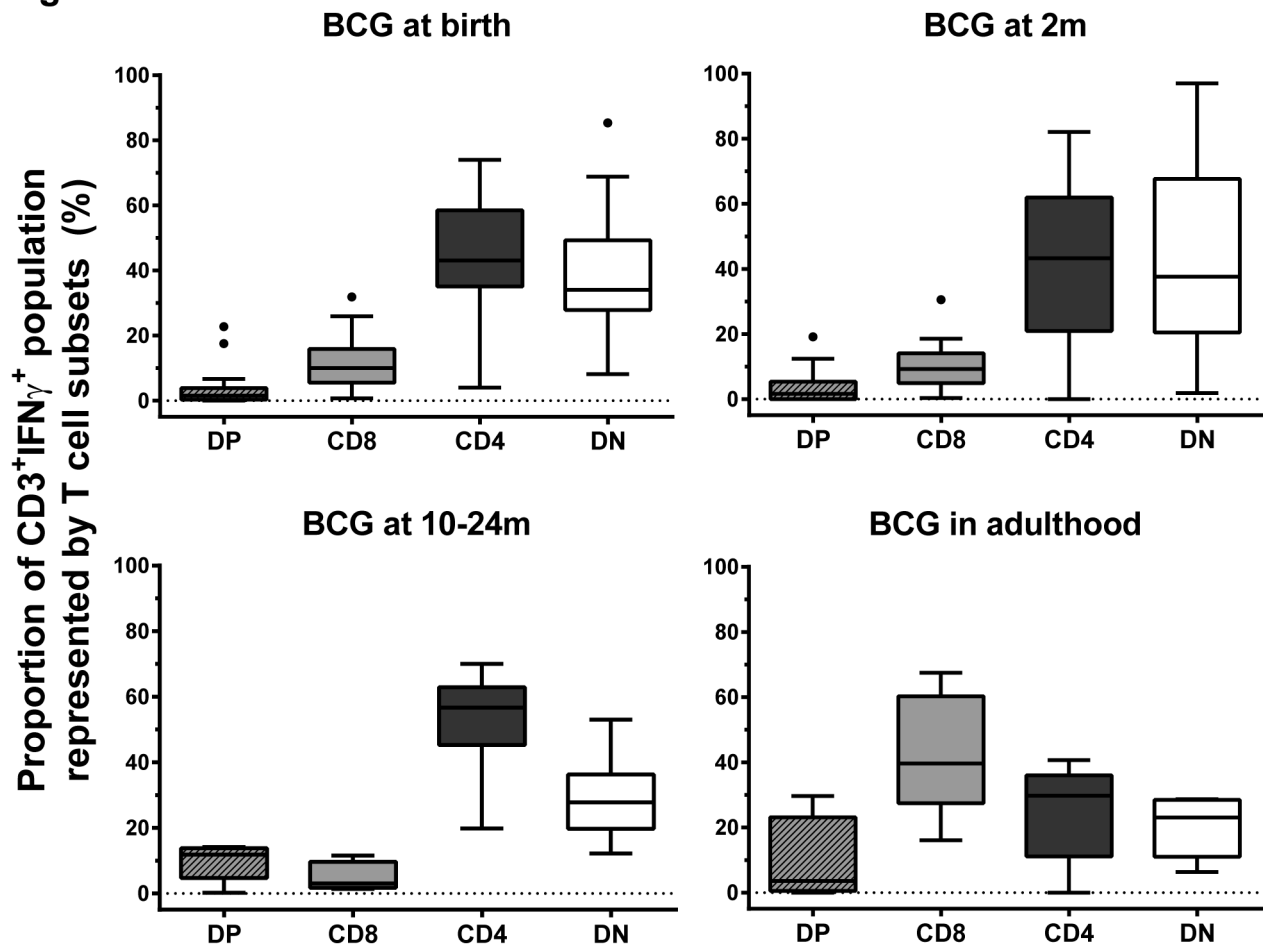
Figure 1

Figure 1. CD4 and DN (CD4⁺CD8⁻) T cells are the main IFN γ -expressing subsets in blood taken from infants 10 weeks after BCG immunization. Box plots (with lower quartile, median and upper quartile, Tukey whiskers) of the proportion of DP, CD8, CD4, DN T cell subsets within the IFN γ ⁺ expressing cells in individuals given BCG at birth (n=28), at 2 months of age (2m; n=27), between 10 and 24 months of age (10-24m; n=7) and in adulthood (n=5). DN: CD4⁺CD8⁻ double negative T cells. DP: CD4⁺CD8⁺ double positive T cells.

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$p < 0.0001$. Similarly at two months of age (n=26), 3% (IQR 0.7-6.2%) of DN T cells expressed IFN γ compared to 0.1% (IQR 0.04-0.16%) of CD4 T cells, $p < 0.0001$ (Figure 2A). Importantly, DN T cells also showed a higher IFN γ -producing capacity (median fluorescence intensity (MFI)) than CD4 T cells and the total functional IFN γ response (combining frequency of IFN γ -expressing cells and MFI) was higher in DN T cells than in CD4 T cells (Figure 2B and 2C).

In a next step, we analyzed the IFN γ expression of NK cells and the phenotypic subgroups of DN T cells (for this, only samples from infants BCG-immunized at birth and two months of age were available [3]). As shown in the gating strategy (Figure S2), NK cells were chosen from the CD56⁺CD3⁻ population and DN T cells were selected from the CD56⁻CD3⁺CD4⁺CD8⁻ population and then analyzed for their V δ 2 TCR γ δ expression. The proportions of V δ 2 γ δ T cells within the

DN T cell population were 12.6% (IQR 6.5-19.7%) and 7.9% (IQR 4.8-12.3%) in blood taken from infants immunized with BCG at birth (n=21) and at two month of age (n=25) respectively. NK cells, DN V δ 2 TCR γ δ ⁺ and DN V δ 2 TCR γ δ ⁻ T cells represented a substantial proportion of IFN γ -expressing cells, with NK cells alone contributing to more than half the measured total IFN γ -expressing cells in both age groups (Figure 3).

Up to 23% of NK cells and 11% of DN V δ 2 TCR γ δ ⁺ T cells expressed IFN γ compared to less than 1% of double positive (DP), CD8, CD4 and DN V δ 2 TCR γ δ ⁻ T cells expressing IFN γ in infants BCG-immunized at birth (n=21) and at 2 months of age (n=25) (Figure 4A). The IFN γ -expressing capacity was comparable in all subsets with the exception of DP T cells, which had a lower IFN γ MFI in infants immunized at two months of age (Figure 4B). Consequently, the greatest IFN γ

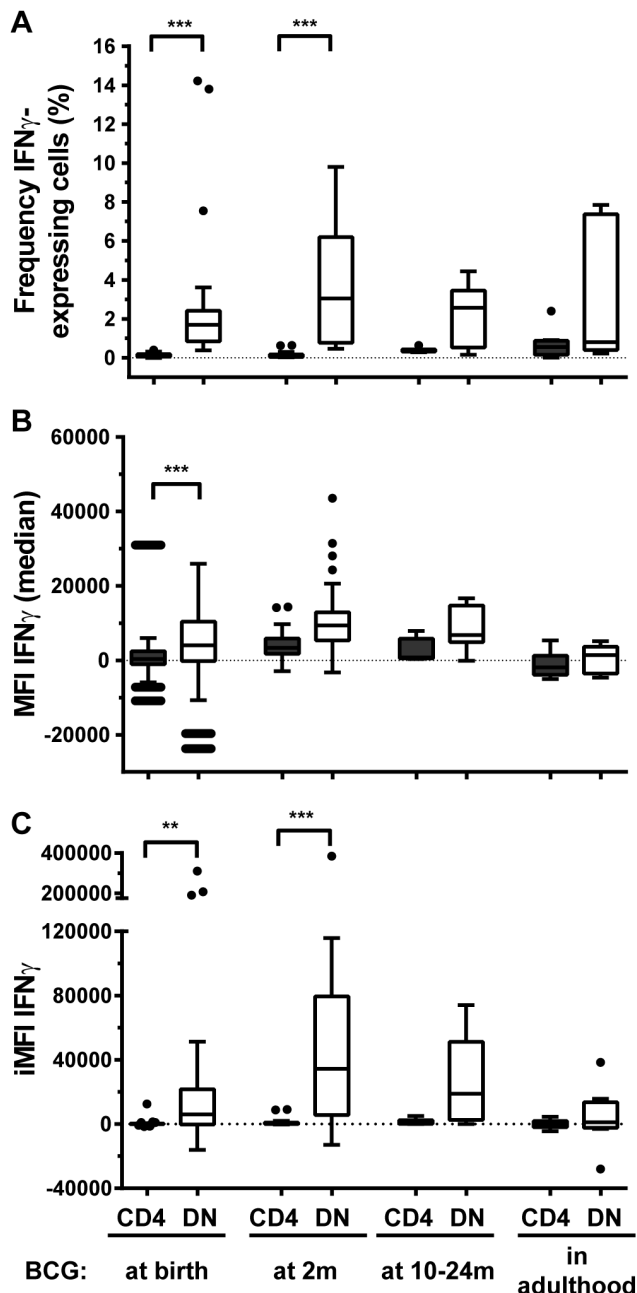
Figure 2

Figure 2. DN (CD4⁻CD8⁻) T cells have a higher IFN γ functional response than CD4 T cells in blood taken from infants 10 weeks after BCG immunization. (A) Frequency and (B) median fluorescence intensity (MFI) of IFN γ -expressing CD4 (grey bars) and DN T cells (white bars), and (C) IFN γ total functional response (iMFI) in individuals given BCG at birth (n=28), at 2 months of age (2m; n=26), between 10 and 24 months of age (10-24m; n=7) and in adulthood (n=9). Box plots with lower quartile, median, upper quartile and Tukey whiskers are shown. **: p<0.001, ***: p<0.0001. DN: CD4⁻CD8⁻ double negative T cells.

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functional response was measured in NK cells and DN V δ 2 TCR $\gamma\delta^+$ T cells (Figure 4C). Notably, CD4 T cells were not major contributors to the total IFN γ functional response (Figure 4C).

Discussion

Our study is the first to investigate in detail the importance of NK cells, $\gamma\delta$ T cells and DN T cells in the mycobacterial-specific IFN γ response following BCG immunization in infants. We found that the key populations producing IFN γ in response to BCG in infants and children were NK cells and DN T cells, including V δ 2 $\gamma\delta$ T cells, rather than CD4 T cells. This highlights the potential importance of the innate immune response and unconventional T cells in the immunoprotective response to BCG.

Previous studies of the immune response to BCG have largely focused on cell-mediated immunity. A CD4 T cell (Th1-type) response associated with IFN γ expression and cytotoxic activity is observed in infants and children after BCG immunization [3,13,15–19]. BCG also induces dendritic cell maturation and production of IL-12 that leads to Th1 differentiation [20–22]. Activation of CD8 T cells producing IFN γ , TNF α and perforin has also been demonstrated [3,23]. In our study, we found that in BCG-immunized adults, in contrast to infants, CD8 T cells were the main IFN γ -producing cells. This suggests that this subset is a crucial player in the immune response to TB in adults as previously proposed [23]. Another recent study in adults shows that CD4 T cells expressed lower IFN γ level than CD8 and DN T cells in TB patients [24] consistent with our results. Although it has been suggested that non-conventional T cells and innate immunity play a role in the response to BCG immunization [25], this aspect of TB immunity has been less well investigated.

Our results show that while DN T cells represent only a small proportion of T cells, this subset makes a considerable contribution to the IFN γ response in infants immunized with BCG that is greater than that made by CD4 T cells. These findings are consistent with a previous study in humans showing that DN T cells represent approximately 4% of T cells in PBMC and express 3 to 4 times more IFN γ than CD4 T cells [26]. It has been suggested that DN T cells play an immunoregulatory role as they can express perforin and suppress cytotoxic CD8 T cells [26]. In humans, DN T cells suppress CD4 and CD8 T cell responses [27]. Similarly in mice, DN T cells kill CD4 T cells, B cells and NK cells and down-regulate co-stimulatory molecules on mature dendritic cells thus contributing to immune tolerance [28]. In simian immunodeficiency virus infection, DN T cells develop CD4 T cell functions that parallel the loss of CD4 T cells and protect against viral dissemination [29]. DN T cells are also involved in the mycobacterial-specific immune response in mice [30,31] and develop a memory phenotype, potentially contributing to effective protection [30].

Within the DN T cell population, $\gamma\delta$ T cells have long been known to constitute a “first line of defense” linking innate and adaptive immunity [32,33]. Their presence is necessary for the expansion of CD4 T cells and they can also act as antigen-

Figure 3

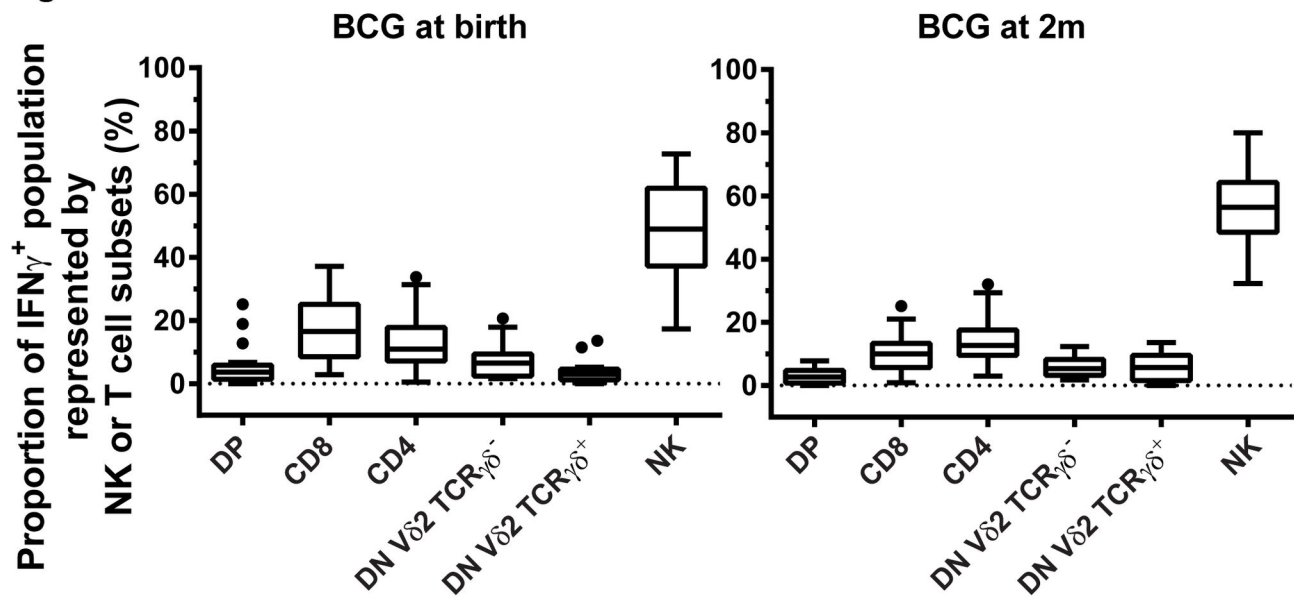


Figure 3. NK, DN V δ 2 TCR $\gamma\delta$ ⁻ and DN V δ 2 TCR $\gamma\delta$ ⁺ T cells represent two-thirds of measured IFN γ -expressing cells in blood taken from infants 10 weeks after BCG immunization given at birth (n=20) or at 2 months of age (2m; n=23). Box plots (with lower quartile, median and upper quartile, Tukey whiskers) of the proportion of DP, CD8, CD4, DN V δ 2 TCR $\gamma\delta$ ⁻, DN V δ 2 TCR $\gamma\delta$ ⁺ and NK cells within the combined NK IFN γ ⁺ and CD3⁺ IFN γ ⁺ population. DN: CD4⁻CD8⁻ double negative T cells. DP: CD4⁺CD8⁺ double positive T cells.

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presenting cells and cross-present antigen to CD8 T cells [34,35]. In the early 1990s, $\gamma\delta$ T cells were shown to be activated by phosphoantigens, which are abundant in *Mycobacterium tuberculosis* (MTB) [36,37]. In animal studies in mice and pigs immunized with attenuated MTB or BCG, $\gamma\delta$ T cells are activated, expanded and express IFN γ [38–40]. These cells have cytotoxic activity for BCG-infected macrophages and are necessary to prime antigen-specific CD8 T cell responses through the enhanced production of IL-12 by lung dendritic cells [39,40]. TCR $\gamma\delta$ T cell-deficient mice infected with BCG had markedly reduced IFN γ production, suggesting a role in immunity to BCG [39,41,42]. In neonates, when a mature TCR $\alpha\beta$ immune system is still lacking, it has been proposed that $\gamma\delta$ T cells are crucial for protection against infections [43]. Human $\gamma\delta$ T cells have been shown to produce IFN γ when BCG-stimulated *in vitro* [41] and $\gamma\delta$ T cells from BCG-immunized infants expand to comprise 60% of total T cells after *in vitro* restimulation [44]. However, the relationship between $\gamma\delta$ T cells and protection is uncertain. In infants immunized with BCG at birth, the frequency of IFN γ -producing $\gamma\delta$ T cells after immunization did not correlate with protection against TB [45]. In contrast, in patients with severe TB, the frequency of total DN T cells was increased compared to healthy donors, but the DN $\gamma\delta$ T cells frequency was reduced. However, both DN and DN $\gamma\delta$ T cells expressed IFN γ in patients with moderate disease suggesting a role in the immune response to TB [24]. TB patients with mild disease have a greater $\gamma\delta$ T cell frequency compared to patients with advanced pulmonary and

miliary TB, and therefore these cells may correlate with protective immunity [46].

NK cells are major players in the innate immune response and their function during MTB infection has increasingly been investigated in the last decade. In BCG-immunized mice, NK cells play a key role in the control of bacterial replication and enhance T cell responses mediated by the secretion of IL-22 and IFN γ [47]. In addition, IFN γ produced by NK cells is crucial for the regulation of T cell-independent resistance to MTB and neutrophil recruitment in lungs of MTB-infected mice [48]. In humans, NK cells produce IFN γ , perforin and granzyme A when stimulated with BCG or PPD [49–51]. It has recently been shown that BCG induces the maturation of NK cells isolated from umbilical cord blood and enhances their cytotoxic activity against immature dendritic cells, suggesting a role in shaping adaptive immunity [52]. NK cells also play a major role in protection against TB by lysis of MTB-infected monocytes and enhancement of CD8 T cell effector functions [53]. Furthermore, in patients with active TB, NK cell activity was diminished [53].

One potential limitation of our study is that different BCG vaccine strains were used for immunization. BCG-Connaught was the licensed vaccine strain for routine immunization in Australia during the study period, while BCG-Denmark was used in the randomized study. No study has compared the *in vitro* immune response to these two vaccines in humans, but a study in mice showed comparable proportions of cytokine-

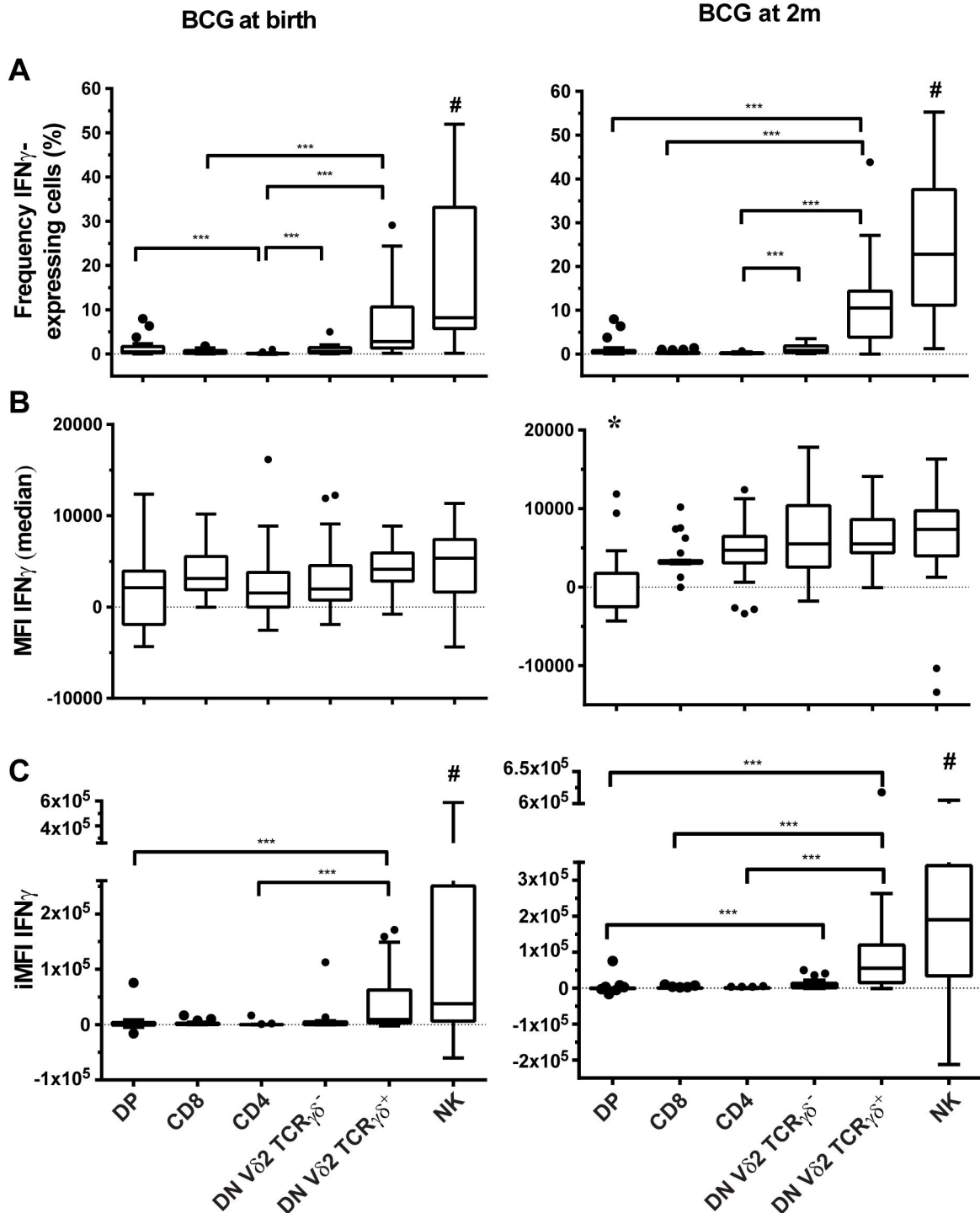
Figure 4

Figure 4. NK and DN V δ 2 TCR $\gamma\delta^+$ T cells have the highest IFN γ functional response in blood taken from infants 10 weeks after BCG immunization given at birth (n=21) or at 2 months of age (2m; n=25). (A) Frequency and (B) median fluorescence intensity (MFI) of IFN γ -expressing DP, CD8, CD4, DN V δ 2 TCR $\gamma\delta^-$, DN V δ 2 TCR $\gamma\delta^+$ T cells and NK cells, and (C) IFN γ total functional response (iMFI) in those subsets. Box plots with lower quartiles, median, upper quartiles and Tukey whiskers are shown. ***: $p < 0.0001$. #: NK cells are different from all subsets except DN V δ 2 TCR $\gamma\delta^+$ with a $p \leq 0.0012$. *: DP MFI is different from all subset MFI except CD8 with a $p \leq 0.0007$. DN: CD4 $^-$ CD8 $^-$ double negative T cells. DP: CD4 $^+$ CD8 $^+$ double positive T cells.

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Table 1. DN T cells represent approximately 3-9% of total CD3⁺ T cells.

Group given BCG at:	Median (interquartile range) proportion of T cell subset (%)			
	CD4	DN	CD8	DP
Birth (n=28)	73.2 (66.8-78.1)	3.4 (2.2-4.7)	23.7 (16.9-28.6)	0.23 (0.2-0.3)
2 months of age (n=26)	70.8 (59.1-74.7)	4.6 (3.2-5.3)	24.6 (20.5-34.2)	0.15 (0.1-0.2)
10-24 months of age (n=7)	63.6 (57.1-71.3)	7.8 (6-13.1)	22.5 (20-25.5)	0.81 (0.5-0.9)
Adulthood (n=9)	50.8 (46.6-58.5)	9.4 (7.9-12.8)	37.9 (28.7-43.8)	0.44 (0.4-0.8)

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producing CD4 and CD8 T cells in the lungs after immunization with either BCG-Connaught or Denmark [54].

The development of new improved TB vaccines is one of the *WHO Stop TB* priorities, and vaccines that rely on boosting BCG at birth are the most advanced. In a recent randomized controlled trial, the novel boosting vaccine MVA85A failed to show protection of infants despite having shown good mycobacterial-specific adaptive immune responses in previous trials [54]. This underlines the importance of investigating the effects of BCG on early life anti-mycobacterial immunity and the potential importance of other cells such as unconventional T cells and NK cells.

Our results highlight an important role for both DN V δ 2 $\gamma\delta$ T cells and NK cells in the mycobacterial-specific IFN γ response to BCG immunization in infants. Recent studies in both mice [55] and humans [24,45] suggest there is not a simple relationship between IFN γ production from T cells and

protection against TB. However, our study supports the concept that the role of the innate immune response and unconventional T cells should be considered in future investigation of the immunoprotective function of BCG and potential new TB vaccines.

Supporting Information

Figure S1. Gating strategy to select IFN γ -expressing cells within the CD3⁺ T cell population. The IFN γ positive gate was set using Nil-stimulated samples (top right panel). In BCG-stimulated samples (bottom panels), CD8 and CD4 expression was then analyzed on CD3⁺ IFN γ ⁺ cells. (TIF)

Figure S2. Gating strategy to select CD56⁺ NK cells and CD56-CD3⁺ T cells. Within the CD56-CD3⁺ cells, DN T cells were further gated into DN V δ 2 TCR $\gamma\delta$ ⁺ and DN V δ 2 TCR $\gamma\delta$ ⁻ populations. Bottom panels show IFN γ expression in NK, DN V δ 2 TCR $\gamma\delta$ ⁺ and DN V δ 2 TCR $\gamma\delta$ ⁻ cells. Note, for clarity, gating of CD4⁺, CD8⁺ and CD4⁺CD8⁺ T cells within CD56-CD3⁺ gate is not shown. (TIF)

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Author Contributions

Conceived and designed the experiments: CZ SG BD NR NC. Performed the experiments: CZ SG. Analyzed the data: CZ SG. Wrote the manuscript: CZ NR NC.

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