Differential IFN-γ production by adult and neonatal blood CD56⁺ natural killer (NK) and NK-like-T cells in response to *Trypanosoma cruzi* and IL-15

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SUMMARY

Early interferon-gamma (IFN-γ) release by innate cells is critical to direct type 1 immune response able to control intracellular pathogens like Trypanosoma cruzi. *Although CD56ᵇʳⁱᵍʰᵗ natural killer (NK) cells are reported to be potent early IFN-γ producers, other CD56⁺ cells like CD56ᵈⁱᵐ NK cells and NK-like T cells have recently been shown to also release IFN-γ. We have here studied the contribution of each CD56⁺ lymphocyte populations in early IFN-γ production in both adults and neonates. On this purpose, we analysed the kinetics of IFN-γ production by RT-PCR, ELISA and flow cytometry from 2 h onwards after* T. cruzi *and IL-15 stimulation and sought for the responding CD56⁺ cells. CD56ᵇʳⁱᵍʰᵗ and CD56ᵈⁱᵐCD16⁻ NK cells were the more potent IFN-γ early producers in response to IL-15 and parasites in adults and neonates. In both age groups, the majority of IFN-γ producing cells were NK cells. However, on the contrary to neonates, CD3⁺CD56⁺ NK-like T cells and CD3⁺CD56⁻ classical T cells also contributed to early IFN-γ production in adults. Altogether, our results support that whereas NK cells responded almost similarly in neonates and adults, cord blood innate CD56⁺ and CD56⁻ T cells displayed major quantitative and qualitative defects that could contribute to the well-known neonatal immune immaturity.*

Keywords *human neonate and adult, innate immunity, Interferon-gamma, natural killer cell, T cell, Trypanosoma cruzi*

INTRODUCTION

Early production of interferon-gamma (IFN-γ) by innate cells is known to be essential to rapidly contain infections with intracellular pathogens such as *Trypanosoma cruzi*, the agent of Chagas disease (1). Indeed, this cytokine activates microbicidal properties of phagocytes and favours maturation of dendritic cells (DCs) endowed to drive the adaptive immune response towards a protective type 1 phenotype (2). Natural killer (NK) cells are known to be an important early source of IFN-γ during infections, whereas T cells are the predominant source later when adaptive responses have developed (3, 4). In addition, other innate cells than NK cells are also able to rapidly produce IFN-γ, such as NKT cells (5), γδ T cells (6), NK-like T cells (7), type 1 innate lymphocytes cells (8) and, to a lower extent, monocytes/macrophages and dendritic cells (9).

Two main subpopulations of NK cells, defined by differential expression of CD56 and CD16, are generally considered as follows: the CD56ᵇʳⁱᵍʰᵗCD16⁻/ᵈⁱᵐ and the CD56ᵈⁱᵐCD16⁺ NK cell subsets, the first population mainly producing cytokines and the second in charge of cytotoxicity (10). IFN-γ release by CD56ᵇʳⁱᵍʰᵗ NK cells becomes usually patent after 18–24 h of activation (11, 12). Recent studies have, however, shown that CD56ᵈⁱᵐ NK cells may transiently produce IFN-γ earlier (from the first hours of stimulation) (11, 13). The less-studied CD56ᵈⁱᵐCD16⁻ NK cell subset has also recently been identified as able to produce IFN-γ (14). This latter subset shares properties with CD56ᵇʳⁱᵍʰᵗCD16⁻/ᵈⁱᵐ NK cells (high proliferative rate, cytokine production, weak cytotoxic capacity, absence of KIR receptors) and expresses lower levels of some major activating NK receptors such as NKp46 and NKG2D, present on most of CD56ᵇʳⁱᵍʰᵗCD16⁻/ᵈⁱᵐ and CD56ᵈⁱᵐCD16⁺ NK cells (14–16). NK-like T cells is a minor T cell subset characterized by the coexpression of a TCR/CD3 and of NK markers like CD56 and NKG2D (17). They display both NK

functions as well as TCR-mediated-specific cytotoxicity. Like NK cells, they respond quickly to activating stimuli like cytokines and pathogens and have recently been shown able to produce cytokines, amongst which IFN-γ (7, 17, 18).

Previous studies have underlined early NK cell- and innate T cell-dependent IFN-γ production and its protective effect in *T. cruzi*-infected mice (1, 19, 20). Data on innate sources of IFN-γ in human Chagas disease are scarce. Sathler-Avelar *et al.* (21) have detected IFN-γ-positive NK cells in chronically infected individuals. We have observed, in the context of congenital *T. cruzi* infection, phenotypic and functional alterations of cord blood NK cells that are suggestive of a previous *in utero* activation of these cells by the parasite, and showed, in line with this hypothesis, that *T. cruzi* was able to induce *in vitro* IFN-γ release by neonatal cytokine-sensitized CD56^bright NK cells (22). To consider the potential of other, recently described (see above), innate sources of IFN-γ in response to *T. cruzi*, we have here studied *in vitro* the kinetics of IFN-γ production by cord and adult blood mononuclear cells (BMC) from 2 to 48 h of stimulation, focusing our analysis on the different CD56^+ (NK and NK-like T) cell populations.

MATERIALS AND METHODS

Patients and blood collection

Umbilical cord blood samples from full-term healthy newborns and peripheral blood samples from healthy adult volunteers were harvested in endotoxin-free heparinized tubes [Becton Dickinson (BD), Erembodegem, Belgium] at the maternity ward of Erasmus Hospital (U.L.B., Brussels, Belgium). The ethical committee of U.L.B. has approved this study, and we obtained informed written consent from volunteers and mothers.

Trypanosoma cruzi parasites

Live *T. cruzi* trypomastigotes [TcVI genotype, Tulahuen strain (23)] were obtained from supernatants of infected fibroblasts as previously described (24). Parasites were verified to be *Mycoplasma*-free by PCR (VenorGeM-Mycoplasma detection Kit; Lucron Bioproducts, Sint Martens-Latem, Belgium).

Cell sample isolation and culture

Cord blood and peripheral blood mononuclear cells (CBMC and PBMC, respectively) were isolated by Nycoprep density gradient centrifugation (Nycomed Pharma AS, Oslo, Norway). Cells (5×10^5) were distributed in round-bottom polypropylene tubes in a volume of 1 mL RPMI1640 containing 10% heat-inactivated FCS, 100 U/mL penicillin G, 100 µg/mL streptomycin, 1 mM non essential amino acids and 1 mM Na pyruvate (all from Lonza, Verviers, Belgium). They were incubated with recombinant human (h)IL-15 (20 ng/mL; R&D Systems Europe, Abingdon, UK) and/or live *T. cruzi* trypomastigotes in a parasite-to-cell ratio 1 : 1 for 2 to 48h at 37°C in 5% CO_2 atmosphere. Cells incubated in medium alone were used as control. The protein-secretion-inhibitor brefeldin A (10 µg/mL; Sigma-Aldrich, Diegem, Belgium) was added for the last 4 h in cultures planned to detect intracellular IFN-γ. After stimulation, the cell cultures were centrifuged at 750 g for 5 min at room temperature (RT). The supernatant was removed and kept at −70°C for IFN-γ quantification. Cells were further processed for flow cytometry analyses or quantitative RT-PCR.

Quantitative RT-PCR

Total RNA was isolated using High Pure RNA Isolation Kit (Roche Applied Science, Brussels, Belgium). Briefly, the cell pellet obtained after 2–48 h stimulation (0.5×10^6 cells) was resuspended in 200 µL of PBS. Cells were then lysed by adding 400 µL of lysis buffer and kept at −20°C for maximum a month, after which total RNA was extracted following the manufacturers instructions. The amount of RNA was determined at 260 nm using the Nano-Drop1000 Spectrophotometer (Thermo Fisher Scientific, Tournai, Belgium). Its purity was checked by measurement of the A260/280nm ratio, which was routinely in the range of 1·6–2·0. Subsequent RT-PCR process using 400 ng of RNA from each sample has been performed on Mastercycler ep gradient (Eppendorf, Hamburg, Germany) using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) with oligo-dT primers following the manufacturers instructions. The retro-transcription reaction was then processed on the Mastercycler during 30 min at 55°C. The transcriptor reverse transcriptase was finally inactivated by heating to 85°C for 5 min and the reaction stopped by descending at 4°C. Reverse-transcribed RNA samples were then half-diluted and processed by real-time PCR on the Lightcycler480 (Roche Applied Science) using SYBR-Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA) and the following gene-specific primer pairs (purchased from Invitrogen, Merelbeke, Belgium), available in the public database RTPrimerDB (http://medgen.ugent.be/rtprimerdb/) under the following entry code: GAPDH (3539) and IFNG (3027). Amplification protocol consisted in a denaturation phase at 95°C for 5′ (Ramp Rate 4·40°C/s) and 50 cycles of amplification [95°C 3′, (Ramp Rate 4·40°C/s), 60°C 1′ (Ramp Rate 2·20°C/s)]. Fluorescence emission was measured at the end of each elongation

step. A melting curve phase programme was finally applied with a continuous fluorescence measurement between 50 and 95°C (Ramp Rate 0·11°C/s). Lightcycler 480 software was used to determine the cycle number at which fluorescence emission crossed the determined C_t value. Melting curve analysis was used to assess the specificity of the assay, and fold change was calculated using the $2^{-\Delta\Delta C_T}$ method with GAPDH as housekeeping gene and unstimulated cells cultured for the same time as control. Each sample was tested in duplicate.

IFN-γ assay

Interferon-gamma levels were measured in culture supernatants by ELISA using antibody pairs and standard (Invitrogen) following the instructions of the manufacturer. The limit of detection was 2 pg/mL. Quantifications were conducted in duplicate.

Staining of cells and flow cytometry analysis

Intracellular staining has been performed as followed: a surface staining was first performed with 5 μL of anti-hCD3-peridinin chlorophyll protein (BD), anti-hCD56-allophycocyanin (Miltenyi Biotec, Leiden, the Netherlands) and anti-hCD16-phycoerythrin (BD) mAb or their matched control isotypes after resuspending the cell pellet in 100 μL of PBS containing 1% BSA. After 20 min incubation at RT, remaining red cells were lysed during 8 min with FACS lysing solution. After washing with cell wash, cells were fixed and permeabilized for 10 min in the dark at RT with permeabilization II solution (BD) prior to incubation in the dark at 4°C for 30 min in 50 μL of 0·85% sodium chloride containing 30% BSA (Sigma-Aldrich) and 5 μL of anti-hIFN-γ-FITC (BD) or its matched control isotype. We finally washed them with FACS cell wash and resuspended them in fix solution (all solutions from BD Biosciences).

Data acquisition and analysis were performed using a four-colour BD FACSCalibur flow cytometer and CELL-QUEST software (version 6.0; BD). Analyses were made on lymphoid CD3$^+$ and CD56$^+$ cells, encompassing classical T cells, NK cells and NK-like T cells, defined as CD56$^-$CD3$^+$, CD56$^+$CD3$^-$ and CD56$^+$CD3$^+$ cells, respectively. In NK cells, we analysed separately the CD56^{bright}CD16$^{-/low}$, CD56^{dim}CD16$^+$ and CD56^{dim}CD16$^-$ subsets. These populations were identified by the following gating strategy: (i) a large gate was determined in the FSC-SSC graph encompassing lymphocytes and monocytes (R1); (ii) the CD3$^-$ cells (R2), the CD56$^+$CD3$^+$ NK-like T cells and the CD56$^-$CD3$^+$ classical T cells were gated within R1 (Figures S1 and S2); (iii) the CD56^{bright}CD16$^{-/low}$, CD56^{dim}CD16$^+$

and CD56^{dim}CD16$^-$ subpopulations were selected amongst cells present in both R1 and R2. Limits for the quadrant markers were based on negative populations and isotype controls. Additionally, we gated IFN-γ$^+$ cells in R1 and determined the percentage of each foregoing subsets, considering these subsets as a whole.

Expression of results and statistical analysis

Results are expressed as means ± SEM or box-and-whisker plots (showing medians, quartiles and minimum and maximum values). Differences between unstimulated and stimulated cells were tested for significance using Wilcoxon matched paired test. Comparisons between adults and neonates were made using Mann–Whitney U-test. Statistical significance was accepted if $P < 0·05$. All statistical analyses were performed using the GRAPHPAD PRISM software 5.02 (GRAPHPAD Software Inc., San Diego, CA, USA).

RESULTS

Kinetics of IFN-γ transcript levels and IFN-γ release in response to *Trypanosoma cruzi* and IL-15

We first studied the evolution of IFN-γ mRNA levels in adult and CBMC throughout time, from 2 to 48 h, in response to IL-15 and/or *T. cruzi* live trypomastigotes. Parasites alone induced a slight accumulation of IFN-γ transcripts in PBMC and CBMC, which started earlier (12 vs. 24 h) and was significantly superior in adult than in neonatal cells (Figure 1a,b). Meanwhile, IL-15 alone induced earlier accumulation of IFN-γ transcripts than parasites in adult and cord cells, without significant differences between adult and cord responses (Figure 1c,d). Furthermore, *T. cruzi* significantly potentiated the effect of IL-15. This synergy became statistically significant from 8 h of culture onwards in both adult and cord cells. Maximal increases of mRNA levels were reached earlier (18 vs. 24 h) and were significantly higher ($\times1522$ vs. $\times400$, $P < 0·05$) in adult than in cord cells (Figure 1e,f).

No IFN-γ was released when cells were cultured in the absence of any stimulant. In the presence of parasites alone, significant IFN-γ amount was detected in supernatants of PBMC from 18 h onwards, whereas very low levels of IFN-γ were detected in supernatants from CBMC at 48 h of culture only (Figure 1a,b). In response to IL-15, a small secretion of IFN-γ by both PBMC and CBMC started at 18 h and regularly increased afterwards (Figure 1c,d). The addition of parasites to IL-15-stimulated cells induced a faster and much higher IFN-γ secretion in both adult and cord cells (Figure 1e,f). The synergy between IL-15 and *T. cruzi* was observed for both
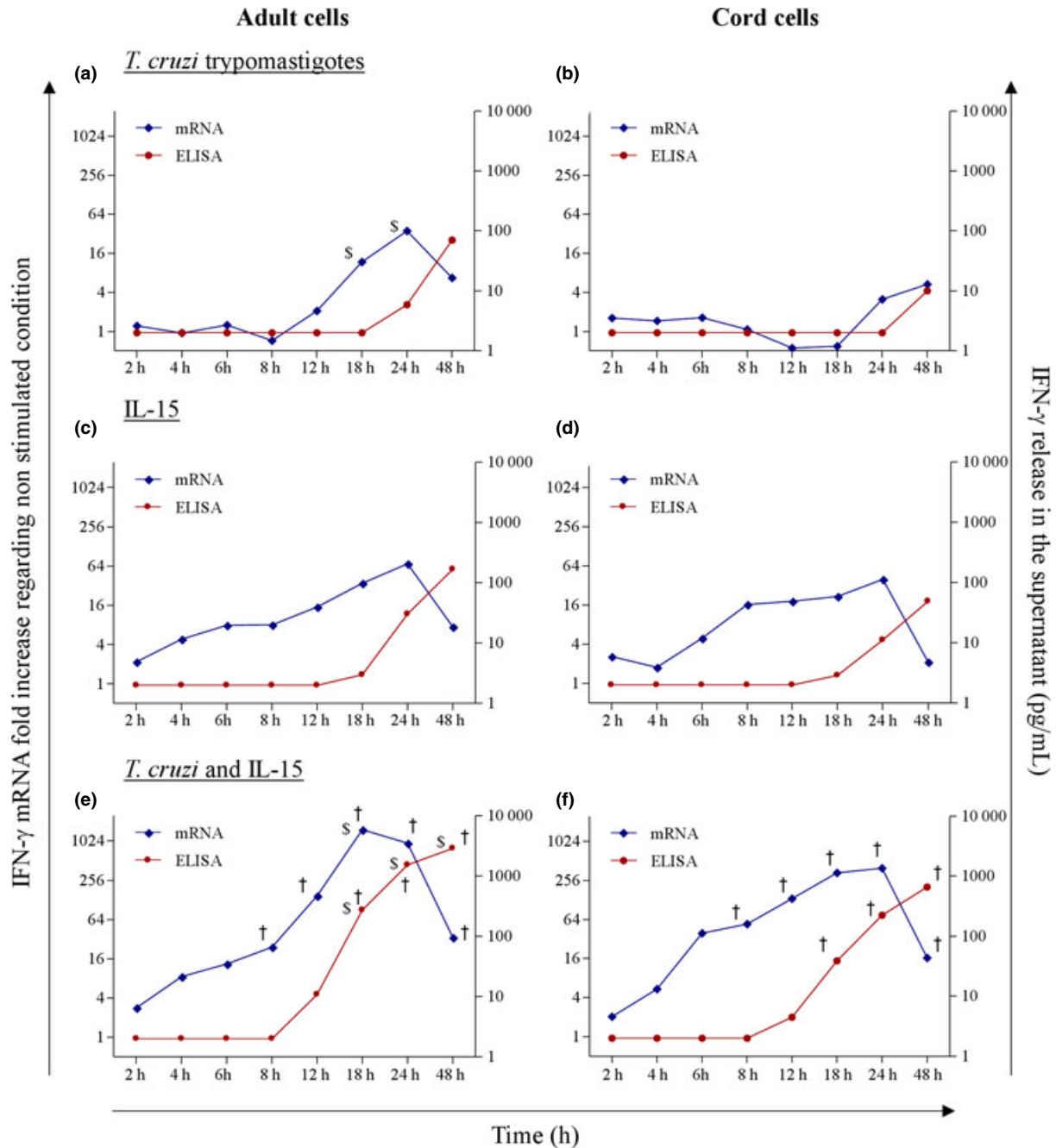
Figure 1 Kinetics of IFN-γ mRNA accumulation and protein secretion in/by cord and adult cells. Kinetics of IFN-γ mRNA accumulation and protein secretion following stimulation of PBMC (a,c,e) or CBMC (b,d,f) with *Trypanosoma cruzi* trypomastigotes at a ratio of 1 parasite per cell (a,b), IL-15 (20 ng/mL) (c,d) or both (e,f). mRNA amounts were calculated by the method of $2^{\wedge}\Delta\Delta CT$ in relation to unstimulated cells cultured for the same time and GAPDH. Results are shown as median of 5–6 independent experiments. $\dagger P < 0.05$ as compared with the effect of IL-15 alone (Wilcoxon paired test), $P < 0.05$ as compared with cord blood cells (Mann–Whitney *U*-test). CBMC, cord blood mononuclear cells; PBMC, peripheral blood mononuclear cells.

PBMC and CBMC from 12 h of culture onwards, but adult cells released significantly higher levels of IFN-γ in regard to cord cells (2879 vs. 661 pg/mL at 48 h, respectively, $P < 0.05$).

Interferon-gamma release in supernatants logically occurred later than the increase in transcript levels. We may, however, notice that the delay between the increase in transcript levels and the protein secretion was shortened

when parasites and IL-15 were combined as compared with the response to IL-15 alone in both adult and cord blood cells (Figure 1c–f).

These results clearly show the strong potentiating effect of *T. cruzi* on IFN-γ production in response to IL-15 in both PBMC and CBMC, with an earlier and more pronounced effect in adults.

CD56^{bright} and CD56^{dim}CD16^{−} NK cells are the more potent IFN-γ producing cells in both adult and neonatal BMC in response to *Trypanosoma cruzi* combined with IL-15

We next determined by flow cytometry the IFN-γ producing capacities of the different CD56^{+} and/or CD3^{+} cell subsets in adult and cord cells. IFN-γ was not found in unstimulated cells while <0·6% (PBMC) and 0·15% (CBMC) of classical CD3^{+}CD56^{−} T cells contained IFN-γ in cultures with parasites and IL-15 (data not shown).

Figures 2 and 3 show that, whatever the stimulus (parasites combined or not with IL-15) and the cell subset, no intracellular IFN-γ (or only traces) was detected before 8 h of culture. Parasites associated with IL-15 induced the highest proportions of IFN-γ positive cells amongst CD56^{bright} and CD56^{dim}CD16^{−} NK cells in both adult (Figure 2i,k) and cord cells (Figure 3i,k). Responses of these two NK cell subsets peaked at 24 h at similar levels and were significantly higher in adults than in neonates, although the difference is at most of a factor 2. Indeed, in PBMC and CBMC, respectively, 32% and 18% of CD56^{bright} and 27% and 14% of CD56^{dim}CD16^{−} NK cells were IFN-γ^{+} after 24 h coculture with parasites and IL-15. CD56^{dim}CD16^{+} NK cells also participated in IFN-γ production, although to a lesser extent (4·3% and 2·4% in adult and cord, respectively, Figures 2j and 3j). Finally, a low proportion of adult but not cord blood NK-like T cells synthesized IFN-γ when parasites were combined with IL-15 (4·3% of IFN-γ positive NK-like T cells at 24 h, Figure 2l).
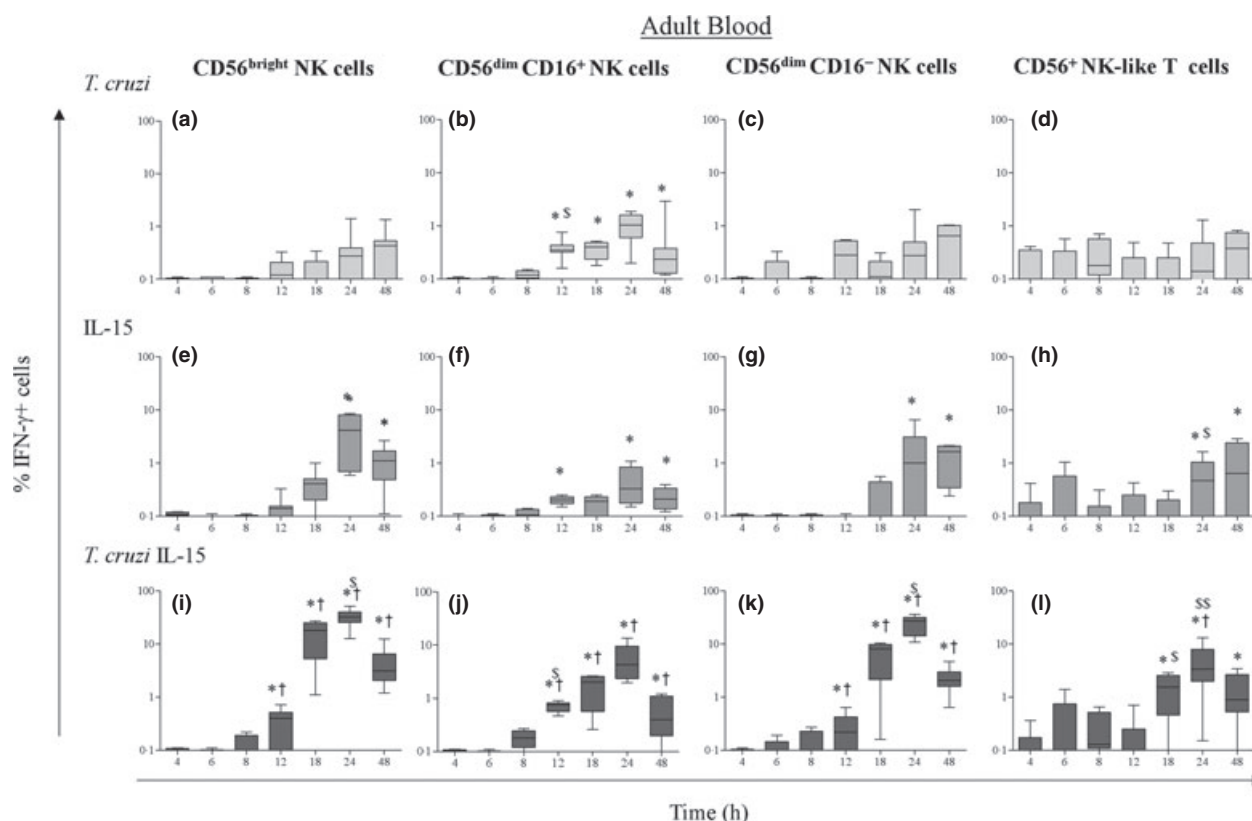

Figure 2 Kinetics of intracellular IFN-γ production in different CD56^{+} adult cells. Kinetics of IFN-γ production by CD56^{+} cells following stimulation of PBMC with *Trypanosoma cruzi* trypomastigotes at a ratio of 1 parasite per cell (a–d), IL-15 (20 ng/mL) (e–h) or both (i–l). Results are shown as box-and-whisker plots of proportions of blood CD56^{bright} (a, e, i), CD56^{dim} CD16^{+} (b, f, j), CD56^{dim} CD16^{−} (c, g, k) natural killer (NK) cells and CD3^{+}CD56^{+} (d, h, l) NK-like T cells (*n* = 5). *$P < 0·05$ as compared with unstimulated cells, †$P < 0·05$ as compared with the effect of IL-15 alone (Wilcoxon paired test), $$P < 0·05$ as compared with cord blood cells (Mann–Whitney *U*-test). PBMC, peripheral blood mononuclear cells.
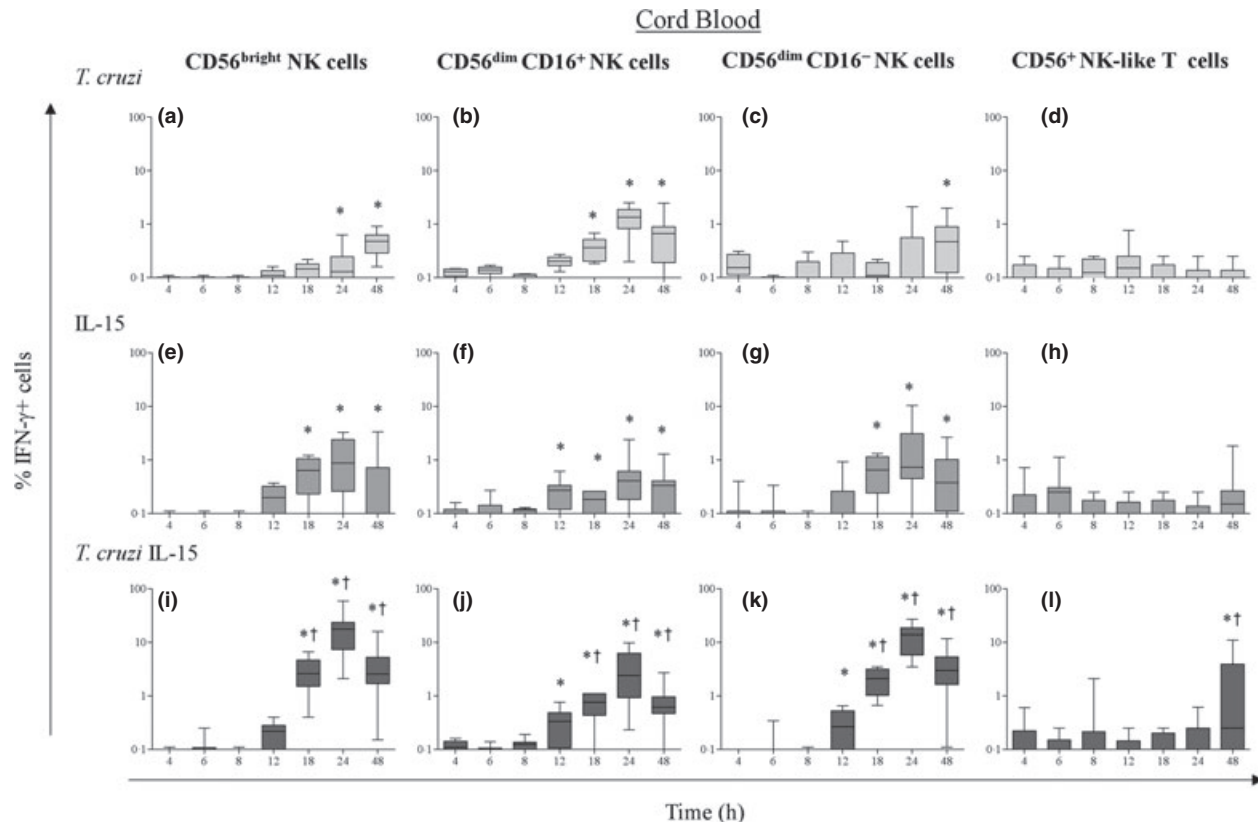
Figure 3 Kinetics of intracellular IFN-γ production in different CD56+ cord cells. Kinetics of IFN-γ production by CD56+ cells following stimulation of CBMC with *Trypanosoma cruzi* trypomastigotes at a ratio of 1 parasite per cell (a–d), IL-15 (20 ng/mL) (e–h) or both (i–l). Results are shown as box-and-whisker plots of proportions of blood CD56bright (a, e, i), CD56dim CD16+ (b, f, j), CD56dim CD16− (c, g, k) natural killer (NK) cells and CD3+CD56+ (d, h, l) NK-like T cells (*n* = 6). *$P < 0.05$ as compared with unstimulated cells, †$P < 0.05$ as compared with the effect of IL-15 alone (Wilcoxon paired test). CBMC, cord blood mononuclear cells.

This study also reveals different kinetics of the IFN-γ response according to the stimulus. Indeed, *T. cruzi* alone induced earlier and better IFN-γ synthesis in CD56dimCD16+ NK cells than in CD56bright or CD56dimCD16− subsets (Figures 2a–c and 3a–c). On the contrary, these two later subsets responded better to IL-15, in the presence or not of parasites, than does the CD56dimCD16+ subset (Figures 2e–g and 3e–g). These features were observed for both adult and cord cells.

We also studied the IFN-γ response of cord blood NK cells to lower IL-15 concentrations (Figure 4). This shows that even at 1 ng/mL of IL-15, which did not trigger IFN-γ production by NK cells (or hardly in CD56bright NK cells, but this is not statistically significant), the addition of parasites already induces a clear synergic response in CD56bright and in CD56dimCD16− NK cells (Figure 4a,c) and not in CD56dimCD16+ ones (Figure 4b).

Our results indicate that amongst mononuclear cells, CD56bright and CD56dimCD16− NK cell subsets are the most potent IFN-γ producers in response to *T. cruzi* associated with IL-15, both in adult and neonates. Interestingly, responses of these two subsets were of similar amplitude, allowing considering the CD56dimCD16− NK cells as effective as CD56bright NK cells in producing IFN-γ in response to *T. cruzi* and IL-15.

Contribution of CD56+ and/or CD3+ cell subsets to global IFN-γ production in relation to their relative abundance

In order to evaluate the contribution of each lymphoid subset to IFN-γ production, we analysed their relative proportions within mononuclear cells expressing CD3 and/or CD56 as well as their proportion amongst IFN-γ-producing cells. Figure 5(a,b) shows that, at 24 h of culture in the presence of parasites and IL-15, proportions of CD3+CD56− T cells and of the three NK cell subsets were quite similar in adult and cord cells on the contrary to the
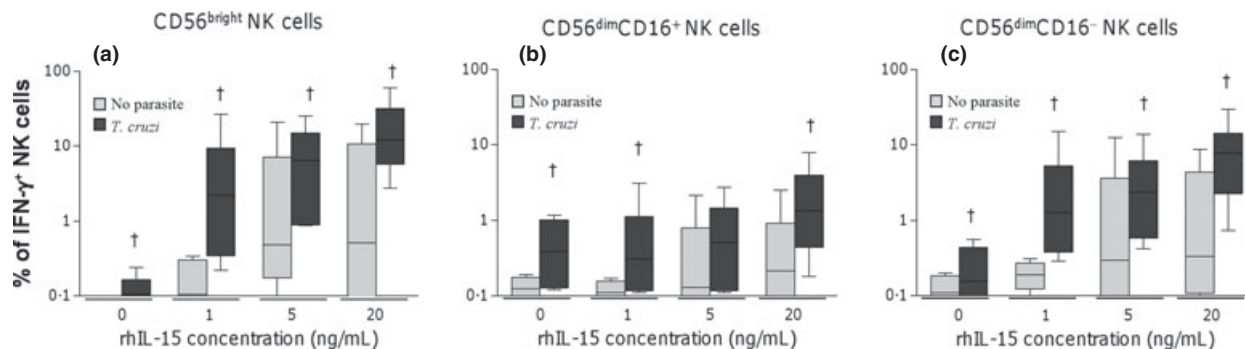
Figure 4 IL-15 dose-dependent IFN-γ response by different cord blood natural killer (NK) cell subsets. IFN-γ production by NK cells following 24 h stimulation of CBMC with different concentrations of rhIL-15 (0–20 ng/mL) in the presence or absence of *Trypanosoma cruzi* trypomastigotes at a ratio of 1 parasite per cell. Results are shown as box-and-whisker plots of proportions of IFN-γ positive cells amongst blood CD56^{bright} (a), CD56^{dim} CD16$^+$ (b) and CD56^{dim} CD16$^-$ (C) NK cells ($n = 6$). †$P < 0.05$ as compared with the condition without parasite at the same concentration of IL-15 (Wilcoxon paired test). CBMC, cord blood mononuclear cells.

NK-like T cell subset that was largely more represented in adult cells. These proportions barely changed throughout time with the different stimuli except some shifts within the NK cell subsets (Tables S1 and S2). Indeed, we can observe in PBMC as well as CBMC cultures that on the one hand, *T. cruzi* or IL-15 alone slightly increased the proportion of CD56^{bright} NK cells and decreased in parallel the proportion of CD56^{dim}CD16$^+$ NK cells. On the other hand, the proportion of CD56^{dim}CD16$^-$ NK cells lately increased at 48 h in IL-15-stimulated conditions (Table S2).

Besides, the vast majority of IFN-γ producing cells are NK cells in both adult and neonatal BMC (Figure 5c,d). However, even if only a low proportion of classical CD3$^+$CD56$^-$ T cells produce IFN-γ in our model, their predominance in BMC explain that 29% of total IFN-γ-producing cells are actually T cells in adults (Figure 5c). This percentage is markedly lower in cord blood cells (5%, Figure 5d). Because the mean fluorescence intensity of IFN-γ production is rather similar between IFN-γ responding subsets (Figures S1 and S2), we may assume that the proportion of IFN-γ-positive cells reflects their contribution to total IFN-γ production. This let us to consider that NK cells are the major source of early IFN-γ in both ages. Furthermore, within NK cells, CD56^{bright} subset is the major IFN-γ source in both age groups, followed by CD56^{dim}CD16$^-$ and CD56^{dim}CD16$^+$ subsets that contribute equally to IFN-γ production (Figure 5c,d). In addition, 4% of IFN-γ producing cells are NK-like T cells in adults, while this subset hardly contributes to IFN-γ production in cord blood. We have also detected few IFN-γ-positive cells amongst CD3$^-$CD56$^-$ cells (non-T non-NK), both in adult and cord blood samples. We have currently not further characterized these cells neither their contribution to the IFN-γ response, but may presume that they most likely belong to

innate lymphocytes now called type 1 innate lymphocytes [ILC1, (8, 25)].

Altogether, these results indicate that early IFN-γ is almost exclusively produced by NK cells in neonates while, in adults, even if NK cells remain predominant, CD56$^+$ and CD56$^-$ T cells also contribute to IFN-γ production.

DISCUSSION

The importance of CD56^{bright} NK cells as early source of IFN-γ during infections has been intensively documented (3, 10). Likewise, we here show the ability of the protozoan *T. cruzi* to rapidly drive IFN-γ by IL-15-sensitized cord and adult blood CD56^{bright} NK cells, which confirms our previous results (22). We in addition show that other innate cells are involved in the IFN-γ response to *T. cruzi*, such as the minor CD56^{dim}CD16$^-$ NK cell subset, the major CD56^{dim}CD16$^+$ NK cell subset and NK-like CD56$^+$ T cells.

The present work identifies CD56^{bright} and CD56^{dim}CD16$^-$ NK cell subsets as the most potent IFN-γ producers in response to IL-15 and *T. cruzi* in both cord and adult cells. If the role of CD56^{bright} NK cells in IFN-γ production is well known, our study is the first to our knowledge to point out a role of CD56^{dim}CD16$^-$ NK cells in response to a pathogen. Our results are furthermore in line with the study of Takahashi *et al.* (14) showing a strong IFN-γ production by this subset in response to an association of several cytokines known to activate NK cells. Only little information is currently available about this CD56^{dim}CD16$^-$ NK cell population and its role and ontogeny are still discussed. Indeed, CD56^{dim}CD16$^-$ NK cells have been described as precursors of CD56^{bright} NK cells (14) or at opposite as recently activated CD56^{dim}CD16$^+$ NK cells that have down-regulated CD16 expression upon interaction
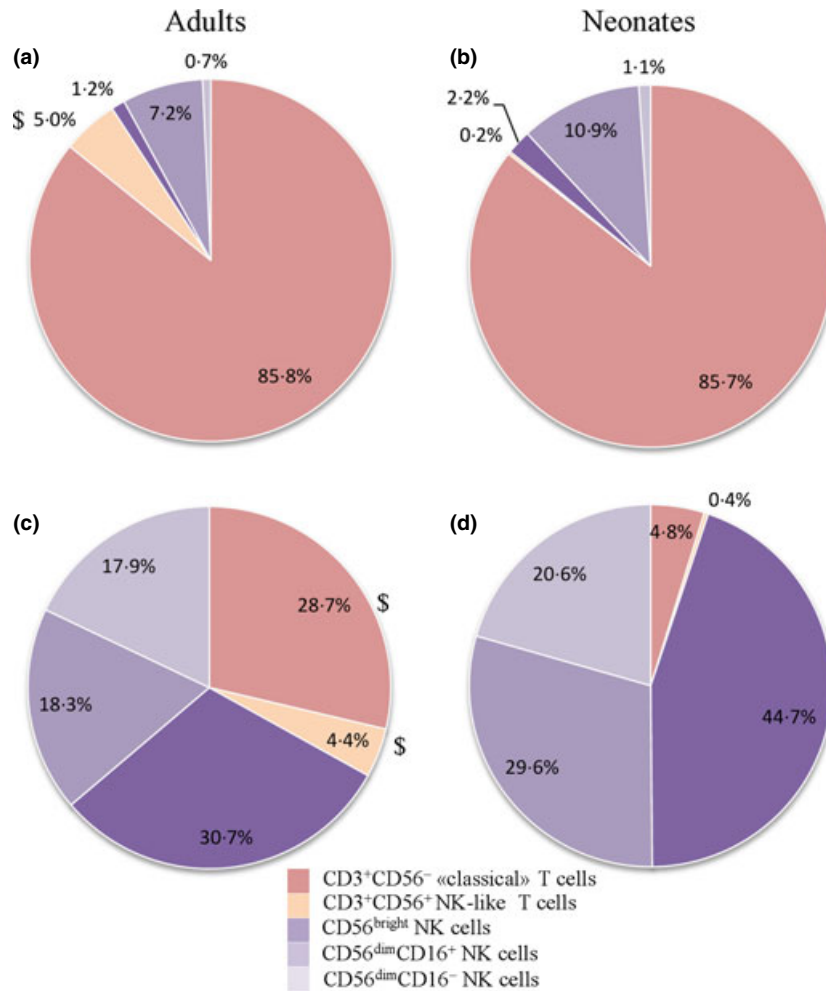
Figure 5 Relative proportions of different natural killer (NK) subsets, NK-like T cells and classical T cells in total or IFN-γ^+ mononuclear cells in adult and cord cells. Relative proportions of NK and T populations in total (a,b) or IFN-γ^+ (c,d) adult (a,c) or neonatal (b,d) CD56$^+$ and/or CD3$^+$ mononuclear cells after 24 h stimulation with IL-15 and *Trypanosoma cruzi*. We determined the proportion of classical T cells (CD3$^+$CD56$^-$), natural killer (NK)-like T cells (CD56$^+$CD3$^+$) and the three subpopulations of NK cells (CD56$^+$CD3$^-$): CD56^{bright} CD16$^{-/low}$ cells, CD56^{dim} CD16$^+$ cells and CD56^{dim} CD16$^-$ cells. Data are shown as medians of six donors. $P < 0.05$ to neonates (Mann–Whitney *U*-test).

with target cells (15, 16). The observation that, in our experiments, the proportion of CD56^{dim}CD16$^-$ NK cells increases with time, combined to a progressive slight decrease in CD16 expression on CD56^{dim}CD16$^+$ NK cells (data not shown) supports that at least part of this population originates from activated CD56^{dim}CD16$^+$ NK cells. On the contrary, the fact that CD56^{dim}CD16$^-$ NK cells have a very similar IFN-γ response than CD56^{bright} NK cells in our conditions rather sustains the hypothesis that they are CD56^{bright} precursors. It is to note that these two hypotheses are not incompatible.

We did not find in our system very early IFN-γ synthesis by CD56^{dim} NK cells in adults nor neonates. This diverges with what has recently been described in other models (11,

13). However, in these studies, CD56^{dim} NK cells were directly activated by a combination of selected agonists, activating NK receptors such as CD16, NKG2D, NCRs, 2B4 or DNAM-1, to quickly produce IFN-γ. The absence of early response suggests that, in our conditions, these receptors are not concomitantly and rapidly engaged. We may, however, not exclude that some of them are later on triggered by accessory cells.

In accordance with previous studies [reviewed in Ref. (26)], neonatal IFN-γ response of NK cells to IL-15 was similar to that of adults, whatever the timing or the NK cell subset. Addition of *T. cruzi* significantly potentiated IFN-γ production of IL-15-primed NK cells in both adults and neonates. Interestingly, potentialization by

parasites occurred earlier and was slightly stronger on adult than in cord cells. Such differential effect of pathogens on neonatal and adult NK cells has already been described in bacterial infections (27). We do not currently know the mechanism by which *T. cruzi* would better trigger IFN-γ production in adult NK cells. Because activation of NK cells by protozoan has been shown to be mostly dependent on accessory cells (28, 29), it is tempting to speculate that it reflects differences in accessory cells indirectly involved in NK cell activation, such as impaired TLR signalling in early life (30, 31).

In addition to NK cells, both CD56^{+} NK-like T cells and classical CD56^{−} T cells contributed significantly to early IFN-γ production in adults in response to *T. cruzi* associated with IL-15. This is in line with other observations reporting IFN-γ induction in NK-like T cells by bacterial products (7, 17, 18) and in innate T cells by *T. cruzi* (19). Whereas the weight of NK-like T cells in the final amount of IFN-γ produced is limited, T cells significantly contributed to the early IFN-γ response. In neonates, however, in accordance with previous studies (27, 32), CD56^{+} NK-like T cells were particularly scarce in cord blood and neither this subset neither classical T cells have an essential impact on the global early IFN-γ production in response to *T. cruzi* and IL-15.

Altogether, our results support that the observed lower innate neonatal IFN-γ response is mainly related to quantitative/qualitative defects in CD56^{+} and CD56^{−} T cells, while NK cells react almost similarly in neonates and adults. This deficiency of innate T cell responses likely also contributes to the neonatal deficiency to mount type 1 immune responses, needed to control infections with intracellular pathogens which they are particularly susceptible to and which are responsible for an important morbi-mortality worldwide in early life. Nevertheless, lymphocyte and NK cell absolute counts are known to be around three times higher in cord than in adult blood (33). This may somewhat thwart the deficit of T cells in neonates. Furthermore, strong activation of NK cells may be one of the mechanisms allowing the parasite to overcome the immaturity of the neonatal immune system and favour a type 1 immune response. It emphasizes the need for complementary studies on cord blood NK cells activation pathways and the role of *T. cruzi* in their activation.

AUTHORS CONTRIBUTION

AG and CT designed the research study, AG performed the research, AG and CT analysed the data, and AG, YC and CT wrote the paper.

REFERENCES

1 Torrico F, Heremans H, Rivera MT, Van Marck E, Billiau A & Carlier Y. Endogenous IFN-gamma is required for resistance to acute *Trypanosoma cruzi* infection in mice. *J Immunol* 1991; **146**: 3626–3632.

2 Shtrichman R & Samuel CE. The role of gamma interferon in antimicrobial immunity. *Curr Opin Microbiol* 2001; **4**: 251–259.

3 Cooper MA, Fehniger TA & Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22**: 633–640.

4 Vitale M, Caruso A, Licenziati S, *et al.* Differential production of IFN-gamma, analyzed at the single-cell level, by specific subsets of human NK and T cells from healthy and HIV(+) subjects. *Cytometry* 2000; **39**: 189–194.

5 Godfrey DI & Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 2004; **114**: 1379–1388.

6 Moens E, Brouwer M, Dimova T, Goldman M, Willems F & Vermijlen D. IL-23R and TCR signaling drives the generation of neonatal Vgamma9Vdelta2 T cells expressing high levels of cytotoxic mediators and producing IFN-gamma and IL-17. *J Leukoc Biol* 2011; **89**: 743–752.

7 van de Wetering D, de Paus RA, van Dissel JT & van de Vosse E. Salmonella induced IL-23 and IL-1beta allow for IL-12 production by monocytes and Mphi1 through induction of IFN-gamma in CD56 NK/NK-like T cells. *PLoS ONE* 2009; **4**: e8396.

8 Walker JA, Barlow JL & McKenzie AN. Innate lymphoid cells–how did we miss them? *Nat Rev Immunol* 2013; **13**: 75–87.

9 Moretto MM, Weiss LM, Combe CL & Khan IA. IFN-gamma-producing dendritic cells are important for priming of gut intraepithelial lymphocyte response against intracellular parasitic infection. *J Immunol* 2007; **179**: 2485–2492.

10 Caligiuri MA. Human natural killer cells. *Blood* 2008; **112**: 461–469.

11 De Maria A, Bozzano F, Cantoni C & Moretta L. Revisiting human natural killer cell subset function revealed cytolytic CD56 (dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. *Proc Natl Acad Sci U S A* 2011; **108**: 728–732.

12 Fehniger TA, Shah MH, Turner MJ, *et al.* Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* 1999; **162**: 4511–4520.

13 Fauriat C, Long EO, Ljunggren HG & Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010; **115**: 2167–2176.

14 Takahashi E, Kuranaga N, Satoh K, *et al.* Induction of CD16+ CD56bright NK cells with antitumour cytotoxicity not only from CD16- CD56bright NK Cells but also from CD16- CD56dim NK cells. *Scand J Immunol* 2007; **65**: 126–138.

15 Lutz CT, Karapetyan A, Al-Attar A, *et al.* Human NK cells proliferate and die in vivo more rapidly than T cells in healthy young and elderly adults. *J Immunol* 2011; **186**: 4590–4598.

16 Grzywacz B, Kataria N & Verneris MR. CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. *Leukemia* 2007; **21**: 356–359.

17 Guia S, Cognet C, de Beaucoudrey L, *et al.* A role for interleukin-12/23 in the maturation of human natural killer and CD56+ T cells in vivo. *Blood* 2008; **111**: 5008–5016.

18 Nyirenda TS, Seeley AE, Mandala WL, Drayson MT & MacLennan CA. Early interferon-gamma production in human lymphocyte subsets in response to nontyphoidal *Salmonella* demonstrates inherent capacity in innate cells. *PLoS ONE* 2010; **5**: e13667.

19 Sardinha LR, Elias RM, Mosca T, *et al.* Contribution of NK, NK T, gamma delta T, and alpha beta T cells to the gamma interferon response required for liver protection against *Trypanosoma cruzi. Infect Immun* 2006; **74**: 2031–2042.

20 Duthie MS & Kahn SJ. During acute *Trypanosoma cruzi* infection highly susceptible mice deficient in natural killer cells are protected by a single alpha-galactosylceramide treatment. *Immunology* 2006; **119**: 355–361.

21 Sathler-Avelar R, Vitelli-Avelar DM, Eloi-Santos SM, Gontijo ED, Teixeira-Carvalho A & Martins-Filho OA. Blood leukocytes from benznidazole-treated indeterminate chagas disease patients display an overall type-1-modulated cytokine profile upon short-term in vitro stimulation with *Trypanosoma cruzi* antigens. *BMC Infect Dis* 2012; **12**: 123.

22 Hermann E, Alonso-Vega C, Berthe A, *et al.* Human congenital infection with *Trypanosoma cruzi* induces phenotypic and functional modifications of cord blood NK cells. *Pediatr Res* 2006; **60**: 38–43.

23 Zingales B, Andrade SG, Briones MR, *et al.* A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. *Mem Inst Oswaldo Cruz* 2009; **104**: 1051–1054.

24 el Bouhdidi A, Truyens C, Rivera MT, Bazin H & Carlier Y. *Trypanosoma cruzi* infection in mice induces a polyisotypic hypergamma-globulinaemia and parasite-specific response involving high IgG2a concentrations and highly avid IgG1 antibodies. *Parasite Immunol* 1994; **16**: 69–76.

25 Spits H, Artis D, Colonna M, *et al.* Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol* 2013; **13**: 145–149.

26 Guilmot A, Hermann E, Braud VM, Carlier Y & Truyens C. Natural killer cell responses to infections in early life. *J Innate Immun* 2011; **3**: 280–288.

27 Peoples JD, Cheung S, Nesin M, *et al.* Neonatal cord blood subsets and cytokine response to bacterial antigens. *Am J Perinatol* 2009; **26**: 647–657.

28 Horowitz A, Stegmann KA & Riley EM. Activation of natural killer cells during microbial infections. *Front Immunol* 2011; **2**: 88.

29 Guilmot A, Bosse J, Carlier Y & Truyens C. Monocytes play an IL-12-dependent crucial role in driving cord blood NK cells to produce IFN-g in response to Trypanosoma cruzi. *PLoS Negl Trop Dis* 2013; **7**: e2291.

30 Kollmann TR, Crabtree J, Rein-Weston A, *et al.* Neonatal innate TLR-mediated responses are distinct from those of adults. *J Immunol* 2009; **183**: 7150–7160.

31 Nguyen M, Leuridan E, Zhang T, *et al.* Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. *PLoS ONE* 2010; **5**: e10407.

32 DArena G, Musto P, Cascavilla N, *et al.* Flow cytometric characterization of human umbilical cord blood lymphocytes: immunophenotypic features. *Haematologica* 1998; **83**: 197–203.

33 Walker JC, Smolders MA, Gemen EF, Antonius TA, Leuvenink J & de Vries E. Development of lymphocyte subpopulations in preterm infants. *Scand J Immunol* 2011; **73**: 53–58.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Production of IFN-γ by adult blood CD56+ cells.

Figure S2. Production of IFN-γ by cord blood CD56+ cells.

Table S1. Evolution of the relative proportions of NK cells, NK-like T cells and classical T cells within CD3+ and/or CD56+ lymphoid cells throughout time in response to *T. cruzi* and IL-15.

Table S2. Evolution of the NK subset relative proportions within NK cells throughout time in response to *T. cruzi* and IL-15.