Neonatal human autologous dendritic cells pulsed with recombinant protein antigen prime the generation of non-polarized CD4 T-cell effectors

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Abstract

The functional capability of human neonatal CD4 T cells to respond to vaccine antigens is frequently described as Th2 biased, but whether this is due to defective T-cell or antigen-presenting cell (APC) function is unclear. In this study, we used purified T cells and autologous monocyte-derived dendritic cells (MDDCs) as APCs to model primary and secondary neonatal CD4 T-cell responses in vitro to BBG2Na, a recombinant protein subunit vaccine candidate against respiratory syncytial virus (RSV). Neonatal MDDCs were phenotypically and functionally comparable to adult-derived MDDCs in terms of stimulatory capacity, longevity and ability to direct Th1 differentiation. When pulsed with BBG2Na, they induced antigen-specific neonatal CD4 T-cell proliferation. Analysis of cytokine production by quantitative real-time PCR showed significant production of IFN-γ and IL-13 mRNA, analogous to the non-polarized primary cytokine mRNA response exhibited by both neonatal and adult naive CD4 T cells when primed by keyhole limpet haemocyanin. This contrasts with BBG2Na-activated adult CD45R0⁺ve memory CD4 T-cell responses, originally primed by natural RSV infection, which demonstrated a polarized Th1 cytokine profile. Importantly, on secondary stimulation, BBG2Na-primed neonatal CD4 T cells exhibited a 4-fold increase in antigen-specific proliferation and a 5-fold increase in IFN-γ production. These data suggest that early life human CD4 T cells in vitro are intrinsically functionally capable of being primed by subunit vaccine candidate antigens such as BBG2Na, and differentiate into non-polarized rather than Th2 effectors.

Introduction

Adaptive immunity to viral and bacterial infections is mediated by subsets of antigen-specific memory T cells that can kill infected cells, secrete cytokines, provide help to B cells and boost antigen-presenting cell (APC) function (1). Human newborns, like other neonatal mammals, usually have little endogenous adaptive T-cell-mediated immunity and are thus much more vulnerable to infectious diseases than adults. Whether this is primarily due to uniquely intrinsic defects or antigenic naivety is unclear (2).

Since neonatal T cells are naive, optimal antigenic priming of T-cell memory needs stimulation by mature dendritic cells (DCs) expressing high levels of adhesion, co-stimulatory and antigen-presenting molecules (3). Different groups have described human neonatal monocyte-derived dendritic cells (MDDCs) as having low levels of expression of MHC and co-stimulatory molecules, and a defect in IL-12 p70 production in response to maturation with LPS in vitro (4, 5), consistent with reports of diminished neonatal Th1 responses and a bias towards Th2 cytokine production compared with adults (6). On the other hand, more recent studies show that neonatal human MDDCs are essentially adult like in terms of phenotype and function (7, 8), similar to neonatal murine splenic DCs in vivo (9). In the T-cell compartment, neonatal mice develop adult-like virus-specific CD8 T-cell responses to viral antigens in vivo (10). In humans, ex vivo studies of cord blood (CB) from neonates congenitally infected with...
Trypanosoma cruzi or cytomegalovirus show that competent adult-like CD8 T-cell responses are also generated in utero (11, 12). Furthermore, recent data suggest that neonatal CD4 T cells, which have greater stimulatory requirements than CD8 T cells (13), can generate adult-type effector responses after priming by neonatal BCG vaccination (14, 15). However, while neonatal T cells may be primed by the complex and powerful adjuvant-like nature of BCG and infectious pathogens, no data exist as to whether naive neonatal human T cells can be adequately primed by modern protein subunit vaccine antigens.

A major target for neonatal vaccination is respiratory syncytial virus (RSV), the main cause of severe viral pneumonia and bronchiolitis in neonates and infants (16, 17). No effective vaccine has been developed since trials in infants with alum-precipitated formalin-inactivated respiratory syncytial virus (Fl-RSV) promoted fatal inflammatory lung disease in some Fl-RSV recipients following natural RSV infection (18, 19). In mice, the native G-attachment protein of RSV primes the generation of pathogenic T,2 effector cells that cause pulmonary eosinophilia upon challenge with RSV (20, 21). However, immunization of both adult and neonatal mice with BBG2Na, a recombinant RSV-G protein subunit vaccine candidate, generates protection against RSV through T,1 and T,2 effector cells, but without promoting pathology upon exposure to live RSV (22).

The protective effects of BBG2Na are mediated in part by CD4 T cells in mice (23), and BBG2Na is immunogenic in RSV-experienced human adults (24). As T,2 T cells are clearly implicated in immunopathogenesis in a mouse model of Fl-RSV-induced enhanced disease, it was of particular importance to determine whether the neonatal human CD4 T-cell response to this recombinant RSV-protein antigen would have a T,2 bias. However, given their disastrous history (18, 19), there are clear ethical concerns about the safety of inactivated/subunit RSV vaccine candidates, which to date preclude their use in clinical trials in RSV-naive recipients—especially infants. We addressed this using a simple in vitro model of T-cell priming with purified CD4 T cells and autologous vaccine-pulsed MDDCs. We report here that neonatal human MDDCs matured by autologous monocyte-conditioned media (MCM) (25–27) are fully capable of priming neonatal human CD4 T cells in vitro. BBG2Na-primed neonatal human CD4 T cells generated effectors producing IFN-γ and IL-13 analogous to those of keyhole limpet haemocyanin (KLH)-primed neonatal and adult naive human CD4 T cells. This was in stark contrast to the polarized type 1 recall response of BBG2Na-stimulated adult-derived RSV memory CD4 T cells. However, secondary exposure of BBG2Na-primed neonatal CD4 T cells to cognate antigen-induced rapid and substantial production of IFN-γ. These data suggest that human neonatal CD4 T cells show little sign of intrinsic defects upon stimulation by autologous MDDCs and that the cytokine responses to protein antigens are predominantly T,0 rather than showing T,2 bias.

Methods

Cells and culture

All media components were supplied by Life Technologies (Paisley, UK) unless stated otherwise. All cytokines were obtained from R&D Systems Europe (Oxford, UK). Other chemicals were obtained from Sigma Aldrich (Poole, UK) unless stated otherwise.

T-cell isolation and purification

Adult peripheral blood (AB) was obtained from healthy volunteers. Umbilical cord blood (CB) from full-term neonates was supplied by the Coombe Women's Hospital (following parental permission and local ethical committee approval). Adult and neonatal CD3 and CD4 T, cells were purified by negative selection (StemSep, Vancouver, Canada) from cryopreserved Ficoll–Hypaque separated AB and CB mononuclear cell (MNC) samples. CD3 and CD4 T-cell purity was routinely >95%. Memory or naive adult T cells were enriched by negative/positive selection with CD45RA microbeads, respectively (Miltenyi-Biotec, Bergisch Gladbach, Germany). The purity of these fractions was routinely >90%.

Generation of MDDCs

Monocytes were purified from freshly obtained CB or AB MNC by positive selection using CD14 microbeads (Miltenyi-Biotec). Monocyte purity was routinely >90%. DCs were differentiated as previously described (25–27). Briefly, CD14 monocytes were cultured at a density of $3 \times 10^5$ per ml in 24-well plates (Corning Costar, High Wycombe, UK) for 6 days in medium (RPMI 1640 containing 1% heat-inactivated autologous plasma and 25 mM HEPES) plus GM-CSF (70 ng ml$^{-1}$) and IL-4 (35 ng ml$^{-1}$) and IL-4 (35 ng ml$^{-1}$). Fresh media and cytokines were added every 2 days. Aliquots of 25 μg of KLH (Calbiochem, Notts, UK) or a dose range of 5–20 μg of BBG2Na (a recombinant fusion vaccine against RSV consisting of the BB streptococcal-G protein albumin binding domain fused with residues 130–230 of the G-attachment protein from human Long strain RSV-A, (Pierre Fabre Medicament, France) were added to immature DCs at day 5 and incubated overnight (28). In some experiments, separate preparations of BB and G2Na were added. DC maturation was induced at day 6 by adding 500 μl of MCM from immobilized IgG-stimulated MNC, as previously described (25–27) to requisite wells. Mature unpulsed and antigen-loaded DCs were harvested at day 8. The maturation status of DCs was assessed by immunophenotyping. Briefly, aliquots of $2 \times 10^4$ DCs were stained for 40 min on ice with pre-titrated aliquots of FITC- or PE-labelled CD14, CD40, CD80, CD83, CD86 and HLA-DR (all from Becton-Pharmingen, Oxford, UK). Conjugated iso-type controls were used to determine the specificity of staining. DC longevity was assessed by flow cytometry using Annexin-V/PI staining (Nexins BV, The Netherlands) to monitor spontaneous apoptosis after transfer of mature DCs to complete media in the absence of cytokines (27). Flow cytometry was performed with a Becton Dickinson FACScan cytometer using standard CellQuest acquisition software (Becton Dickinson, Mountain view, CA). All samples were gated using forward and side scatter to exclude dead cells.

T-cell proliferation assays

All proliferation assays were performed in round-bottomed 96-well plates containing 200 μl of complete medium (RPMI
1640 medium containing 10% heat-inactivated autologous plasma, 2 mM L-glutamine and 25 mM HEPES) per well. T-cell proliferation was assessed by methyl-[\(^3\)H] thymidine incorporation (1 \(\mu\)Ci per well; Amersham Life Science, Bucks, UK), which was added for the last 16 h of culture. Thymidine incorporation was measured by liquid scintillation counting using a microplate reader (Wallac, Turku, Finland). The stimulatory capacity of neonatal MDDCs was compared with adult MDDCs using the allogeneic-mixed lymphocyte DC reaction (allo-MLR). Aliquots of 1 \times 10^5 neonatal CD3 T cells were cultured with allogeneic DCs at a ratio of 20:1 to 1000:1 for 5 days. To assess adult recall T-cell responses elicited by BBG2Na, aliquots of 2 \times 10^5 adult PBMC were treated with BBG2Na (dose range of 1–20 \(\mu\)g ml\(^{-1}\)) in a 7-day proliferation assay.

To measure primary proliferative responses of T cells primed to antigen, aliquots of 2 \times 10^5 neonatal CD4 T cells or adult CD4CD45RA T cells were cultured with mature autologous MDDCs, pulsed with or without antigen, at a ratio of 10:1 for 5 days. For secondary proliferative responses, neonatal CD4 T cells were cultured in complete medium in 24-well plates at a density of 1 \times 10^5 per ml with autologous MDDCs, pulsed with or without antigen, at a ratio of 10:1. After 5 days of stimulation, IL-2 (5 U ml\(^{-1}\)) was added before culture for a further 4 days. At day 9, MDDC-primed T cells were harvested, washed and counted. Aliquots of 5 \times 10^5 primed T cells were cultured in complete media in 96 U-well plates with thawed mature control unpulsed and antigen-pulsed MDDCs at a ratio of 20:1 for 3 days (cryopreserved at the time of DC harvest).

**Real-time PCR assay for quantification of cytokine mRNA production**

Cytokine mRNA production by neonatal and adult CD4 T cells was assessed by real-time quantitative reverse transcription-PCR (Q-RT-PCR). Aliquots of 5 \times 10^5 CD4 T cells were primed with autologous or antigen-loaded DCs at a ratio of 10:1 for 4 days before harvesting. For the secondary response, aliquots of 1 \times 10^5 neonatal-primed CD4 cells were mixed with thawed DCs at a ratio of 20:1 and cultured for 20 h before harvesting. Cells were pelleted and total RNA was extracted using the Qiagen RNAeasy kit according to the manufacturer’s conditions and reverse transcribed into cDNA using a Prostar First strand generation kit (Stratagene, La Jolla, USA). Aliquots of 2 \(\mu\)l of cDNA template were subjected to Q-RT-PCR using FAM-labelled scorpion primers (29; ATDBio Ltd, Southampton, UK).

The PCR primer sequences used were (where 6 = 6-carboxyfluorescein and 7 = methyl red-quencher) GAPDH: forward: 5’-CCGCCAAGACTCATGACCAACAGCCCGCGG76GGGCCATCCAGCTTCTCTCTC-3’; reverse: 5’-GCCCTCTGCA-CACCAACTG-3’; IL-2: forward: 5’-CCGCCGAGTGGTGGGATTTCTGATTCCAGG76GATTTACTCAGTCTGTAAGGA-3’; reverse: 5’-AGGTTTGAAGCTTCTGTTTCTCTGC-3’; IL-4: forward: 5’-CCGCCGAGTGGTGGGATTTCTGATTCCAGG76GATTTACTCAGTCTGTAAGGA-3’; reverse: 5’-AGGTTTGAAGCTTCTGTTTCTCTTG-3’; IL-5: forward: 5’-CCGCCGAGTGGTGGGATTTCTGATTCCAGG76GATTTACTCAGTCTGTAAGGA-3’; reverse: 5’-AGGTTTGAAGCTTCTGTTTCTCTTC-3’; IL-13: forward: 5’-CCGCCGAGTGGTGGGATTTCTGATTCCAGG76GATTTACTCAGTCTGTAAGGA-3’; reverse: 5’-AGGTTTGAAGCTTCTGTTTCTCTTC-3’.

**Intracellular cytokine staining**

The cytokine profile of allogeneic mature MDDC-stimulated CD3 T-cell cultures was assessed by intracellular cytokine staining. Briefly, cells were harvested at day 5 and re-stimulated for 5 h with phorbol myristate acetate (PMA) (50 ng ml\(^{-1}\)) and ionomycin (1 \(\mu\)g ml\(^{-1}\)) in the presence of Brefeldin A (10 \(\mu\)g ml\(^{-1}\)). Cells were surface stained with anti-CD3-Cy5, fixed and permeabilized with PermFix™ (Becton-Pharmingen), and stained with PE- and FITC-labelled antibodies to IL-4 and IFN-\(\gamma\), respectively (Becton-Pharmingen), or equivalent isotype control antibodies.

**Statistical analysis**

Comparisons between groups were made using the Mann-Whitney rank sum test for non-parametric data and by the paired \(t\)-test for parametric data. A value of \(P<0.05\) was considered significant.

**Results**

**Mature adult and neonatal MDDCs are phenotypically and functionally equivalent**

In order to determine the functionality of neonatal human T cells in response to antigens \(ex\;vivo\), it was first essential to assess whether the neonatal MDDCs to be used as APCs could differentiate into a competent and mature state capable of priming naive T cells. Immature adult or CB MDDC generated over 6 days in culture with GM-CSF and IL-4 in 1% autologous plasma were stimulated with the TLR-4 agonist LPS for 24 h or differentiated with MCM for 48 h (Fig. 1a and b). Neonatal MDDC showed similar levels of maturation...
to adult MDDCs as indicated by similar levels of upregulation of surface expression of the co-stimulatory molecules CD40, CD80, CD86, the antigen-presenting molecule HLA-DR and the marker of terminal DC differentiation CD83 (Fig. 1a and b). Extended culture of these MDDCs in the absence of cytokines showed that neonatal DC longevity—a further functional indicator of DC maturity—was similar to adult MDDCs with 50% surviving after a 3-day culture period (Fig. 1c). Neonatal and adult MDDCs were equipotent in the allo-MLR, both stimulating substantial neonatal CD3 T-cell proliferation at T:DC ratios as high as 1000:1 (Fig. 1d). Further, in the allo-MLR, neonatal MDDCs stimulated the generation of type 1 effectors from neonatal CD3 T cells as effectively as adult MDDCs (Fig. 1f and g). Intracellular cytokine staining of PMA- and ionomycin-stimulated alloantigen-primed neonatal T cells showed that neonatal MDDCs have an equivalent capacity to direct T_{h1} effector differentiation from naive precursors (Fig. 1e). These data suggest that neonatal MDDCs are phenotypically and functionally equivalent to adult MDDCs.

**BBG2Na stimulates neonatal CD4 T-cell primary proliferative responses**

As virtually all adults have had at least one infection with RSV, and neonates have had none, we used adult PBMCs to determine whether such predicted RSV memory T cells primed by natural RSV infection would be stimulated by recall to antigens in the BBG2Na preparation. Figure 2a shows that BBG2Na was immunogenic ex vivo and induced adult recall PBMC proliferation in a dose range of 2–20 μg ml⁻¹.
Generation of neonatal effector T-cell responses

Fig. 2. The stimulation of adult and neonatal T cells by BBG2Na or KLH. (A) Dose-dependent recall induced proliferation of adult PBMC (2 × 10^5 per well) stimulated with BBG2Na in a 7-day proliferation assay. The data are the means and standard deviation (SD) of triplicate determinations and are representative of three experiments. (B) The dose response of neonatal CD4 T cells to BBG2Na-pulsed autologous MDDCs in a 5-day proliferation assay (2 × 10^5 T cells per well with 2 × 10^5 MDDCs). The data are the means of antigen-specific proliferation (defined as values corrected for proliferation induced by unpulsed MDDCs) and SD of triplicate determinations and are representative of four experiments. (C) The antigen-specific primary proliferative response of neonatal CD4 T cells to BBG2Na is dependent on mature MDDCs. Aliquots of 2 × 10^5 neonatal CD4 T cells were co-cultured for 5 days with 2 × 10^4 immature or mature MDDCs pulsed with BBG2Na (10 μg ml^-1). Data shown are the means and SD of triplicate determinations and are representative of three independent experiments. (D) The antigen-specific primary proliferative response of neonatal CD4 T cells to KLH antigen is comparable to adult CD4CD45RA naive T cells. Aliquots of 2 × 10^5 neonatal or adult CD4CD45RA T cells were co-cultured for 5 days with 2 × 10^4 immature or mature autologous MDDCs pulsed with or without KLH (25 μg ml^-1). Data shown are the means and SD of triplicate determinations and are representative of three independent experiments.

with maximal responses at 10 μg ml^-1. We next investigated to what extent BBG2Na stimulated primary neonatal T-cell proliferation. Figure 2b shows that BBG2Na-pulsed MDDCs strongly induced antigen-specific neonatal CD4 T-cell proliferation with maximal stimulation at 5–10 μg ml^-1. To determine whether BBG2Na-induced neonatal T-cell proliferation might be from pre-existing memory T cells rather than in vitro priming, neonatal CD4 T cells were stimulated with immature or mature MDDCs pulsed with BBG2Na or the control KLH neoantigen. Immature DCs efficiently stimulated memory T cells but are poor stimulators of naive T cells (3). Figure 2c shows that only mature neonatal DCs could significantly stimulate BBG2Na-specific neonatal CD4 T-cell proliferation, suggesting that BBG2Na responses were by naive CD4 T cells rather than cross-reactive or maternal memory cells. Similarly, Fig. 2d shows that only mature DCs elicited substantial primary antigen-specific neonatal and naive adult T-cell proliferative responses to the neoantigen KLH. Stimulation by unpulsed autologous mature, but not immature, MDDCs induced substantial levels of background proliferation by both neonatal CD4 T cells and naive adult CD4CD45RA T cells (data not shown). The comparable levels of primary antigen-specific CD4 T-cell proliferation exhibited by KLH-stimulated neonatal and naive adult cells suggest they have similar functional responsiveness and that mature BBG2Na-pulsed human neonatal MDDCs are as functionally capable as adult MDDCs of processing and presenting antigen.

Antigen-specific secondary proliferative response of primed neonatal CD4 T cells to BBG2Na and its components

We next investigated whether neonatal CD4 T cells were capable of exhibiting memory responses through antigen-specific secondary proliferation. Following 5 days of priming with KLH or BBG2Na, cultures were expanded for 4 days with low dose IL-2 (5 U ml^-1) before harvesting for secondary proliferation assays. Lymphocyte proliferation greater than background was only observed when KLH- and BBG2Na-primed T cells were re-stimulated with their respective DCs (5 × 10^5 primed CD4 T cells at a T:DC ratio of 20:1) (Fig. 3a). Antigen-specific secondary proliferative responses were induced faster and were proportionately 3- to 4-fold greater than antigen-specific primary proliferative responses indicating that some neonatal T cells had undergone antigen-specific priming.

As BBG2Na is a fusion protein composed of one-third of the RSV-G protein fragment (G2Na) and two-thirds of the streptococcal-G protein albumin-binding domain (BB), we asked to what extent each component was immunogenic in our antigen priming system. This was important since if priming was primarily to the BB component this might translate as little RSV-specific immunity in vivo upon immunization. Figure 3b shows that BBG2Na-primed neonatal CD4 T cells generated significant antigen-specific secondary proliferative responses to BBG2Na-pulsed DCs (P < 0.01) and G2Na-pulsed DCs (P < 0.002) but not to BB-pulsed DCs. Indeed, BB-pulsed DCs failed to elicit any antigen-specific secondary proliferation from BB-primed neonatal CD4 T cells. In contrast, G2Na-primed neonatal CD4 T cells showed highly significant antigen-specific secondary
Cytokine responses of adult and neonatal CD4 T cells stimulated by BBG2Na or KLH

Cytokine production by antigen-primed naive T cells represents a differentiation or effector phase of these cells in response to antigen. Q-RT-PCR analysis showed that freshly isolated PMA and ionomycin-stimulated naive adult CD4 T cells had little ability to produce T_{h}1 or T_{h}2 cytokine mRNA other than IL-2 (data not shown). Adult memory CD4CD45RA^{ve} naive T cells stimulated by recall with BBG2Na-pulsed DCs showed significant levels of IFN-γ mRNA and minimal IL-4, IL-5 or IL-13 mRNA production relative to stimulation by unpulsed mature MDDCs (P < 0.04; Fig. 4a) indicating a strong T_{h}1 polarized recall response to natural exposure to RSV in vivo. In contrast, priming of adult CD4CD45RA^{ve} naive T cells with KLH-pulsed MDDCs induced significant production of IL-13 as well as IFN-γ mRNA (P < 0.04; Fig. 4b) and higher mean levels of IL-5 compared with stimulation by unpulsed mature MDDCs, although the latter did not reach statistical significance, indicating a T_{h}2 cytokine response to neoantigen-pulsed MDDCs had been primed.

Primary stimulation of neonatal CD4 T cells with either KLH or BBG2Na also induced significant levels of IFN-γ (P < 0.04) and IL-13 mRNA (P < 0.04) (Fig. 4c). Antigen-induced neonatal and naive adult CD4 T cell IL-5 and IL-13 mRNA production was highly variable between individuals in both groups. These data suggest that the in vitro antigen-induced primary cytokine responses of neonatal CD4 T cells to BBG2Na and KLH, and of naive adult CD4 T cells to KLH, are non-polarized T_{h}0.

Discussion

Many studies in mice and humans have concluded that the neonatal adaptive immune system is developmentally immature and constitutively skewed towards a T_{h}2-type effector T-cell response (reviewed in 30). However, neonatal T cells are capable of adult-type functional responses if stimulated and primed by adult DCs (31, 32) but it is unclear whether neonatal DCs have sufficient functional capability to do the same. In this paper, we investigated the priming of human neonatal naive CD4 T cells with a candidate vaccine antigen and have characterized their proliferative and cytokine responses following primary and secondary stimulation. We found that these responses were analogous to the non-polarized T_{h}0 cytokine responses of KLH-primed neonatal CD4
T cells, which were not significantly different from those of KLH-primed naive adult CD4 T cells. From these similarities in responsiveness, we conclude firstly, that ex vivo-generated neonatal and adult MDDCs are functionally equivalent and provide similar T-cell priming conditions. Secondly, we conclude that neonatal T-cell functionality is comparable to naive adult T-cell responses in terms of antigen-induced proliferative capacity and extent of cytokine mRNA production. Thirdly, we conclude that neonatal human CD4 T cells are functionally capable of being primed by BBG2Na, an RSV-G protein fragment subunit vaccine candidate, and that the primary cytokine responses to this antigen were broadly similar to those generated through priming by KLH. Finally, they generate significantly more IFN-γ on secondary stimulation. We base these conclusions on the following observations. Neonatal T cells require optimal stimulation by mature DCs and this could be delivered by CB-derived MDDCs—the ex vivo equivalent of DCs differentiated in vivo from a subset of CD2+ve monocytes which home to inflamed tissues (33). Our first question was whether autologous neonatal MCM could mature neonatal MDDCs to the same extent as adult MDDCs were matured by adult MCM or whether there was some functional deficiency. However, on finding that neonatal MCM had similar potency to adult MCM in inducing full DC maturation, we continued to use MCM rather than conventional LPS because it induces full DC maturation without DC polarization (25–27). Thus, the cytokine data presented here describe T-cell differentiation in response to priming and re-stimulation by protein antigens and subsequent T-cell–DC interactions without the effect of cytokines secreted by activated DCs upon exposure to LPS. In addition to our allo-MLR studies, in which we show that neonatal and adult MDDCs have equivalent stimulatory capacity for naive CD3 T cells (Fig. 1), neonatal MDDCs were capable of stimulating KLH-primed autologous CD4 T cells to proliferate as effectively as naive adult CD4 T cells which had been primed by KLH-pulsed autologous MDDCs. This suggests an equal ability to process, present and respond to exogenous antigen. Thus, in contrast to previous data describing human neonatal DC deficiency in vitro (4, 5), in this study, we conclude that neonatal T-cell functionality is comparable to naive adult T-cell responses in terms of antigen-induced proliferative capacity and extent of cytokine mRNA production. Thirdly, we conclude that neonatal human CD4 T cells are functionally capable of being primed by BBG2Na, an RSV-G protein fragment subunit vaccine candidate, and that the primary cytokine responses to this antigen were broadly similar to those generated through priming by KLH. Finally, they generate significantly more IFN-γ on secondary stimulation. We base these conclusions on the following observations. Neonatal T cells require optimal stimulation by mature DCs and this could be delivered by CB-derived MDDCs—the ex vivo equivalent of DCs differentiated in vivo from a subset of CD2+ve monocytes which home to inflamed tissues (33). Our first question was whether autologous neonatal MCM could mature neonatal MDDCs to the same extent as adult MDDCs were matured by adult MCM or whether there was some functional deficiency. However, on finding that neonatal MCM had similar potency to adult MCM in inducing full DC maturation, we continued to use MCM rather than conventional LPS because it induces full DC maturation without DC polarization (25–27). Thus, the cytokine data presented here describe T-cell differentiation in response to priming and re-stimulation by protein antigens and subsequent T-cell–DC interactions without the effect of cytokines secreted by activated DCs upon exposure to LPS. In addition to our allo-MLR studies, in which we show that neonatal and adult MDDCs have equivalent stimulatory capacity for naive CD3 T cells (Fig. 1), neonatal MDDCs were capable of stimulating KLH-primed autologous CD4 T cells to proliferate as effectively as naive adult CD4 T cells which had been primed by KLH-pulsed autologous MDDCs. This suggests an equal ability to process, present and respond to exogenous antigen. Thus, in contrast to previous data describing human neonatal DC deficiency in vitro (4, 5), in this study,
neonatal human MDDCs matured with MCM resemble neonatal murine splenic DCs, which, strikingly, have the same capacity to upregulate MHC and co-stimulatory molecules as adult-derived DCs (9, 34). Whereas proliferative T-cell responses to either adult or neonatal allogeneic DCs could be induced at T:DC ratios as high as 1000:1, low T:DC ratios of 10:1 were essential for consistent detection of primary antigen-specific responses by both naive adult and neonatal CD4 T cells. We interpret this to mean that the greater the number of mature MDDCs in contact with naive CD4 T cells, which have a diverse TCR repertoire, the greater the chance of antigen-antibody recognition by the predicted small number of T-cell precursors with an appropriate range of TCR avidity. That we were able to detect antigen-induced primary responses in relatively small samples of 2 × 10^5 naive CD4 T cells supports the paradigm that naive T cells may be highly cross-reactive to antigen (35), thus greatly increasing the frequency of T cells that can be recruited in a primary protective immune response to a pathogen, where speed rather than high specificity may be needed (35). Dose of antigen is also an important factor in directing T-cell differentiation as well as the level of stimulation, since high-dose antigen with strong co-stimulation promotes Th2 effector development rather than Th1, whereas high-dose antigen with low co-stimulation promotes Th2 cells (36, 37). In our study, adult and neonatal MDDCs, at the same T:DC ratio, pulsed with the same dose of KLH (25 μg ml^-1), induced similar levels of T-cell proliferation and significantly increased production of IL-13 and IFN-γ mRNA by both naive adult and neonatal CD4 T cells. BBG2Na-primed CD4 T cells showed a qualitatively similar cytokine mRNA profile, suggesting that in response to primary antigenic stimulation by different proteins under similar conditions of antigen presentation and co-stimulation, neonatal CD4 T cells behave similarly to their naive adult counterparts. IL-13 appears to be a major cytokine produced by antigen-primed neonatal and naive adult CD4 T cells, a cytokine generally associated with type 2-mediated immune diseases such as atopy, allergy and RSV-induced pulmonary eosinophilia (38). However, IL-13 is a non-typical type 2 cytokine as, in contrast to IL-4 or IL-5, it is also secreted by naive adult CD4 T cells as well as by memory CD4 and CD8 T cells (39). In further contrast to IL-4, the level of IL-13 production is largely independent of T:DC ratio and has fast kinetics (40).

Optimal effector-cell generation in vivo depends on an initial antigen-dependent phase followed by a period of cytokine-driven expansion and differentiation (41). To simulate this, we expanded antigen-stimulated T-cell cultures with IL-2 before harvesting for secondary assays. Analysis of secondary proliferative responses and secondary cytokine mRNA production by BBG2Na-primed neonatal CD4 T cells to the individual components of BBG2Na showed that virtually all the antigen-specific response was due to RSV-G2Na. However, this is not unique to our ex vivo system since while infant macaques immunized with BBG2Na or BB generated specific IgG antibody responses, T-cell recall responses to BB were barely detectable (42). In comparison to the non-polarized nature of the primary BBG2Na-induced cytokine mRNA response, re-stimulation with BBG2Na-pulsed MDDCs induced a 5-fold increase in IFN-γ mRNA, but no significant change in IL-5 or IL-13 mRNA. The stimulus for enhanced BBG2Na-induced secondary IFN-γ production could be occurring through endogenous IL-12 or IL-23 secretion from DCs stimulated by antigen-activated CD4 T cells through CD40–CD40L interactions (43). In addition, the more stringent conditions of secondary stimulation may have led to the selection and outgrowth of those BBG2Na memory clones with the higher levels of TCR avidity for antigen. The outcome of this in the secondary response would be to select for cells of a more Th1 phenotype. In comparison, the cytokine profile of adult memory CD4 T cells stimulated by recall to BBG2Na was dominated by IFN-γ mRNA with very low levels of IL-4, IL-5 and IL-13 cytokine mRNA production—in agreement with reports of the RSV-specific Th1 cytokine profile of healthy RSV seropositive adults and children (44). These data suggest that those RSV-G antigen-specific memory Th1 cells that had been primed and polarized by immune responses to RSV infection in vivo had retained this polarized cytokine profile, probably through secondary immune responses that had been refined through multiple rounds of infection by RSV throughout life (16).

There is little published data on memory responses of neonatal humans to vaccination. Administering vaccine directly to neonates, with all its inherent risks, has to date been the only means of gaining an insight into the nature of the immune response to such antigens. Immunization of infants with BCG (14, 15) or whole cell pertussis toxoid (45), which have factors that can directly mature and polarize DCs, primes strong adult-type Th1 responses (46). However, vaccination of infants with acellular pertussis in the presence of alum as an adjuvant induces Th2 memory responses (6, 44). Alum is an adjuvant, which, although classed as safe for human infants, stimulates the production of IL-4 from Gr1+ myeloid cells (47). If this was used in a vaccine formulation with a protein subunit vaccine such as BBG2Na, which has no intrinsic polarizing effect, this could skew neonatal T-cell differentiation in vivo since there would be no toll-receptor agonist/DC1 maturation factors to counteract alum’s potential Th2 polarizing effect. While our data show the potential of isolated neonatal human CD4 T cells to be directed towards adult-type effector functional responses to protein antigens presented by neonatal MDDCs in an in vitro model, the situation in vivo, especially with respect to optimal dos- age, DC numbers, lymphoid tissue development and choice of adjuvant for optimal polarizing conditions, is likely to be many fold more complicated. Future studies modelling neonatal vaccination are needed to address other aspects of this complexity.

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**Abbreviations**

AB adult peripheral blood

allo-MLR allogeneic-mixed lymphocyte DC reaction
References


Generation of neonatal effector T-cell responses