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Neonatal plasmacytoid dendritic cells at the interface of innate and adaptive immunity

Neonatale plasmazytoide dendritische Zellen – an der Schnittstelle zwischen angeborener und adaptiver Immunität

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Meinen Eltern in Dankbarkeit

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Abbreviations

adult blood (AB)
ammonium chloride lysing buffer (ACK)
Becton&Dickinson (BD)
carboxy fluoroscein succinimidyl ester (CFSE)
cluster of differentiation (CD)
cord blood (CB)
cytometric bead array (CBA)
cytosine-guanosine-motif with phosphate binding (CPG)
deoxyribonucleic acid (DNA)
dimethyl sulfoxide (DMSO)
fetal calf serum (FCS)
fluorescein isothiocyanate (FITC)
forward scatter channel (FSC)
hours (h)
human immunoglobulin G (hIgG)
human peripheral mononuclear cells (PBMC)
interleukin (IL)
infrared (IR)
interferon (IFN)
kilo Dalton (kDA)
linear scale (Lin)
logarithmic scale (Log)
logarithmic scale with compensation applied (Log Comp)
major histocompatibility complex (MHC)
mean fluorescence intensity (MFI)
milliampere (mA)
minutes (min)
myeloid dendritic cells (MDC)
natural killer cells (NK)
oligodeoxynucleotides (ODN)
optical densities (OD)
p38 mitogen-activated protein kinase (p38 MAPK)
pathogen-associated molecular patterns (PAMP)

phorbol myristate acetate (PMA)

phosphate-buffered saline (PBS)

photomultiplier tube (PMT)

phycoerythrin (PE, when used in conjunction with antibody description)

plasmacytoid dendritic cells (PDC)

polyethylene (PE, when used in conjunction with laboratory material)

room temperature (RT)

Roswell Park Memorial Institute (RPMI)

side scatter channel (SSC)

supernat. = supernatant (SN)

T cell (TC)

toll-like receptor (TLR)

ultraviolet (UV)

unstim. = unstimulated

volt (V)

wash medium (WM)

β-methoxyethoxymethyl ether (MEM)

Introduction and discussion of current literature

Starting from conception, the human body is exposed to pathogen. During pregnancy, the fetus is somewhat protected by the immune system of its mother. The maternal immune system bears the brunt of pathogen defense.

Some infectious agents manage to escape maternal immunity and expose the fetus to pathogen, leading to infection *in utero*. The possibility of therapeutically influencing neonatal immunity *in utero* is small and the first vaccinations can only be given directly after birth (1).

Besides infection, the fetus can be threatened by the maternal immune system itself. Accordingly, neonatal immunity is a Janus face: *In utero*, both mother and fetus have to hold to an immunological truce so that the pregnancy is successful (2, 3). The bias of fetal and maternal immunity towards tolerance prevents the deteriorating effects of inflammatory cytokines leading to abortion (4) or premature birth. After birth maternal immunity in the newborn wanes while the newborn is confronted by a world full of microbes.

Neonatal immunity is impaired and/or depressed while favouring a T helper cell response type 2 (Th2). Th2 responses typically induce tolerance to pathogen but also favor defense against extracellular parasites and development of allergies. Juxtaposed to Th2 responses are Th1 responses. These are cytotoxic in nature, killing intracellular pathogen. The bias towards Th2 is underlined by reports that production of those cytokines associated with a cytotoxic T helper cell response (Th1) is impaired in human newborns.

Certain cytokines typical for Th1 responses (for example IL6, IL10, IL23) can nonetheless be induced by stimulation of neonatal monocytes and antigen-presenting cells (APC), actually exceeding adult levels (4), though this not true of all cytokines typically associated with Th1 responses. This bias against Th1-polarizing cytokines seems to make newborns prone to infection and inhibit full immunogenicity in answer to vaccination. Until a few years ago, it was assumed that there is an age-dependent maturation of immune response (4). This thesis is being challenged in favor of the hypothesis of age-specific response (5).

Furthermore, prenatal and postnatal exposure to environmental microbial products appear to activate innate immunity and thus might accelerate the change in the nature of the immune response. This is the case when exposure is recurrent, thereby diminishing Th2 polarization and/or enhancing Th1 polarization and in this manner potentially reducing allergy and atopy in accord with the hygiene hypothesis (4).

To deal with infection, the immune system has two large, interacting and interdependent response systems which are commonly labeled innate immunity versus adaptive immunity.

The adaptive immune system is highly specialized and is made up of antigen-specific B and T cells. Somatic recombination, positive selection, clonal expansion and affinity maturation ensure a selective cellular immune response to pathogens. The adaptive immune system takes a few days to mount a specific response to pathogen. Immunological memory is maintained by specialized memory T and B cells.

Innate immunity has multiple lines of defense, such as epithelial barriers, humoral factors like antimicrobial proteins, the complement system and the cellular innate immune system. This consists of macrophages, dendritic cells (DC) and natural killer (NK)-cells. In innate as in adaptive immunity, a specialized immune response early on is important in order to direct defense to a specific target. This is illustrated by the induction of eosinophils in order to kill extracellular parasites or activation of NK cells to control viral infection until cytotoxic T cells can take over.

The evolution of the immune system has favored the development of mechanisms that recognize pathogen-associated molecular patterns (PAMP). These refer to components in or on the surface of pathogens that microbes need to survive and which can therefore not easily be varied. PAMP receptors ensure a specialized immune response in the early phases of infection and constitute part of the innate form of immune “memory”.

One group of cells that have developed PAMP receptors are the antigen-presenting cells. Macrophages, monocytes and dendritic cells belong to this group. Activation of

these sensors or receptors induces a fast immunological response, in contrast to adaptive immunity which takes longer to react.
Dendritic cells are special in this context as they demonstrate the highest capacity of all cell types to present antigen, thereby inducing a primary immune response and permit the formation of immunological memory (6). Dendritic cells therefore constitute the main interface between innate and adaptive immunity.

DC are not a homogenous population but differ in origin, differentiation, function and migratory profile. In humans, at least two major DC subsets were identified in peripheral blood – the $CD11c^{+}$ myeloid dendritic cell and the $CD11c^{-}$ plasmacytoid dendritic cell (PDC). The latter owe their name to their distinctive endoplasmatic reticulum reminiscent of plasma cells (7).
PDC have been identified for some time, being referred to as $CD11c^{-}$ $IL3Ra^{+}$ (CD123) plasmacytoid T cells (8) or interferon-α (IFNα) producing cells (6) and conclusively as plasmacytoid dendritic cells (9). PDC can be identified by the cell surface marker CD304 (BDCA-4/Neuropilin-1). PDC bearing that marker are characterized as being $CD11c^{-}$, $CD123^{high}$, $CD4^{+}$, $CD45RA^{+}$ (7), CD303 (BDCA-2)$^{+}$, CD141 (BDCA-3)^{dim}, CD1c (BDCA-1)$^{-}$, and $CD2^{-}$.
They lack expression of lineage markers ($lineage^{-}$) and express neither myeloid markers nor Fc receptors (receptor binding the crystallizable fragment of antibody). Their plasmacytoid shape and the expression of the pre-T cell receptor α-chain indicate their lymphoid origin. Their interferon-α producing function was pin-pointed in numerous studies (10-13).

Survival of PDC depends on their expression the IL3 receptor-α-chain (IL3Rα). In accordance with the importance of the IL3Rα we established protocols to exploit its survival-promoting capabilities *in vitro.* Upon addition of CD40 ligand (CD40L) and IL3, PDC mature and adopt a distinctive dendritic morphology and seem to be able to encourage T cell proliferation (7).
Having been identified as the main IFNα producing cell type, it became clear that because of the pleiotropic effects of IFNα on different cell types PDC influence monocytes, blood monocyte-derived dendritic cells, B cells and T cells. Beside fighting viral and bacterial infection, PDC play an important role in the pathogenesis of certain autoimmune disorders such as lupus erythematodes (14), Sjögren's

syndrome, inflammatory arthritis, psoriasis (15), drug hypersensitivity reaction (16) and chronic viral infections such as HIV (15, 17).

Since PDC produce large amounts of cytokines, in particular type I interferons they not only control inflammation and play a critical role linking innate to adaptive immunity but also assist in the development of natural regulatory T cells (18).

Myeloid/monocytic dendritic cells (MDC) isolated from peripheral blood do not express CD304 (BDCA-4/Neuropilin-1) but can be made to do so upon culturing (9, 19). Neuropilin-1, as indicated by its name, is expressed on numerous non-hematopoietic cell types, e.g. neurons, endothelial and tumor cells. However, in peripheral blood BDCA-4 expression is limited to PDC.
Binding of antibodies to BDCA-2 has effects on cell function. Binding of antibodies to BDCA-4 was not reported to have substantial effects on IFN type I production in PDC after viral stimulation (17, 19, 20).

The immune system is able to fight tumors after localized bacterial infection (21). This concept of immune stimulation by bacteria was later narrowed down to bacterial DNA using palindromic sequences (16, 22, 23) that could be synthesized in the lab and be used to induce IFNα in human peripheral blood lymphocytes (PBL) (24). Krieg et al. recognized that the stimulatory sequences contained a central cytosine-guanosine-motif (CPG, P representing the phosphate binding) (25).

To date, we know that lectin receptors, scavenger receptors and toll-like receptors (TLR) belong to the group of PAMP receptors. CPG motifs, which have a high frequency in viral and bacterial DNA but a low frequency in mammalian DNA are recognized by TLR9 (26, 27).
TLR9 conveys responsiveness via the adaptor protein MyD88 which transduces the signal via a cascade of protein kinases, including p38 mitogen-activated protein kinase (p38 MAPK) in a phosphorylated state. PDC also express TLR7 (which responds to guanosine analogs and imidazoquinolines), TLR6 and TLR8 (28, 29) and other non-TLR pattern recognition receptors (15). This study focused exemplarily on TLR9, leaving investigation of other PAMP for future studies.

CPG motifs can activate cells of both the innate and the adaptive immune system, adding to their therapeutic value. In the adult immune system, B cells and dendritic cells (DC) react with a cascade of signaling mechanisms that induce expression of co-stimulatory cell surface molecules such as maturation markers, co-stimulatory molecules CD80, CD83, CD86 and CD40 (30) and start cytokine production (31).
Co-stimulatory molecules are important for T cell activation in the context of the two-signal-hypothesis. This hypothesis states that T cell activation works best if antigen presentation via the major histocompatibility complex (MHC) receptor is accompanied by co-stimulation. If the second signal is weak, tolerance or anergy towards the antigen are induced. The ligation of CD40 on PDC by T cells is the reciprocal activating feedback mechanism (32). The nature of primary allogeneic T cell responses can be evaluated by co-culture with activated PDC (33). Distinct DC subsets elicit distinct T helper cell responses (6). These divergent T cell responses induce different types of immune response appropriate to eliminate given pathogens. PDC can generate a Th2 response (33) as well as a Th1 response (34).

In the human system, two types of CPG-oligonucleotides (CPG ODN) can be differentiated based on their effect.
CPG ODN type A (for example ODN 2216, referred to in this study as CPG A) is characterized by poly-G tails around a central palindromic sequence containing a CPG motif and a phosphothioate backbone. This motif leads to high IFNα production (35) and is therefore often cited as a prototype for the simulation of viral infection.
CPG ODN type B (for example ODN 2006, referred to as CPG B) does not have poly-G tails but possesses a phosphorothioate backbone. It leads to a potent B cell response and to maturation and activation of PDC but only to low levels of IFNα production and weak activation of NK cells. As these effects correspond to bacterial infection, CPG B is referred to as a prototype for bacterial infection.
CPG are potent activators of immune responses in primates (36-39) and humans (37, 39) and are therefore considered potential adjuvants to enhance vaccine response in cell-mediated immunity (38). The specifity of CPG motifs may be a factor for rate of uptake of DNA into PDC and other cells (15).

Neonatal immune response, weakened by increasing loss of maternal antibodies gives the impression of being impaired not only by lack of experience but also by lack

of function. Neonatal APC have been shown to promote a Th2 response instead of Th1 (1, 40). CPG used as an adjuvant in mouse newborn vaccinations was able to overrule Th2 polarization and allow Th1 response to vaccine antigens (41).
Nonetheless, murine and human DC subsets differ greatly in TLR expression and responsiveness to CPG (29, 35, 39). Immunization trials of human adults with vaccines using CPG as an adjuvant are ongoing. A thorough *in vitro* investigation of the human neonatal immune response is needed before adjuvant-aided immunization can progress to *in vivo* trials in older children and later to trials in newborns.

Circumventing induction of tolerance to pathogens (the risk of which is actually quite low (1)), avoiding neonatal excess reactogenicity as well as the challenges of developing ethical and regulatory considerations in this vulnerable population are the great challenges ahead.

The role of PDC in adults has been studied extensively. Some research has been done on comparing their function in neonates to their function in adults, although most studies, like that of De Wit et al. (42) have focused on whole blood assays that use mixed cell populations. These assays mimic *in vivo* situations better than single cell populations but limit the possibilities of a differentiated analysis (42) of the effect of CPG on PDC. Further studies by Hartmann's group demonstrated that CPG indeed have a direct effect on PDC (43, 44) without help from other cell lines in whole blood.

Newborns fail to mount a sufficient immune response to viral and bacterial pathogens and physicians have so far failed to provide adequate solutions to this need. This study aims to aid vaccine development for newborns by establishing the physiological reactions of plasmacytoid dendritic cells to CPG.

We therefore decided to ask the following questions:

1) How do isolated human cord blood PDC react to stimulation with CPG A and CPG B in terms of cell surface activation and maturation markers, cytokine production and intracellular phosphorylation?
2) Do these PDC primed with CPG A, CPG B and CD40L promote division of autologous T cells?
3) Does priming of PDC with CPG A or CPG B induce a bias in T helper cell response towards Th1 or Th2?
4) On what level do neonatal PDC produce these effects compared to adult PDC?

Materials

1.1 Chemicals

Methanol	Roth, Karlsruhe
HCl	Roth, Karlsruhe
BSA	Sigma-Aldrich, Munich
PBS	Invitrogen, Karlsruhe
Isopropanol	Hedinger, Stuttgart
Benzonase	Merck, Darmstadt
NH_4Cl	Merck, Darmstadt
$KHCO_3$	Merck, Darmstadt
Sodium azide	Merck, Darmstadt
Na_2EDTA	Sigma-Aldrich, Munich
Paraformaldehyde	Sigma-Aldrich, Munich

Table 1 Chemicals

1.2 Devices

Pipetus	Hirschmann, Eberstadt
Bench-top centrifuge MiniSpin	Eppendorf, Wesseling
ELISA Reader	MRXII, Dynex Technologies
Eppendorf Pipette Research 1000 µl	Eppendorf, Wesseling
Eppendorf Pipette Research 200 µl	Eppendorf, Wesseling
Eppendorf Pipette Research 100 µl	Eppendorf, Wesseling
Eppendorf Pipette Research 20 µl	Eppendorf, Wesseling
Eppendorf Pipette Research 10 µl	Eppendorf, Wesseling
Eppendorf Pipette Research multi-channel	Eppendorf, Wesseling
Water bath DC10	Haake, Karlsruhe
VarioMACS	Miltenyi, Bergisch-Gladbach
MiniMACS	Miltenyi, Bergisch-Gladbach
pH-meter WTW pH538	WTW, Weilheim
Ice machine	Ziegra, Isernhagen
FACS Canto	BD, Heidelberg
Incubator B5060	Heraeus, Hanau
Scale Kern770	Kern, Balingen-Frommern
Water deionisation system MilliQ Plus	Millipore, Schwalbach
Laminar Flow, Class II, Series ‚SE' Modell 51424/2, Microflow	Nunc GmbH, Wiesbaden
Centrifuge Jouan CR 3.22	Jouan, Fernwald
Microscope	Zeiss, Jena
Sterile working bench Holten LaminAir	Holten, Allerod, Dänemark

Table 2 Devices

1.3 Consumables

Disposable	**Manufacturer**

Parafilm	American National Can Company, USA
6-well plates	Nunc, Wiesbaden
24-well plates	Nunc, Wiesbaden
96-well plates	Nunc, Wiesbaden
1.8 ml CryoTubes	Nunc, Wiesbaden
Cryo 1°C PE Freezing container	Nalge, Neerijse, Belgium
Sterile filter 0.2 µm 500 ml	Nalge, Neerijse, Belgium
LS Separation columns and tubes	Miltenyi, Bergisch-Gladbach
MS Separation columns and tubes	Miltenyi, Bergisch-Gladbach
TruCount tubes	BD, Heidelberg
FACS tubes	BD, Heidelberg
Serological pipette 5 ml	Greiner, Frickenhausen
Serological pipette 10 ml	Greiner, Frickenhausen
Serological pipette 25 ml	Greiner, Frickenhausen
Pipette tips 1000 µl	Sarstedt, Nümbrecht
Pipette tips 200 µl	Sarstedt, Nümbrecht
Pipette tips 0.5-20 µl	Eppendorf, Wesseling
1.5 ml reaction container	Eppendorf, Wesseling
Falcon conical tube 15 ml, PE	BD, Heidelberg
Falcon conical tube 50 ml, PE	BD, Heidelberg
SemperCare latex gloves M	Lohmann & Rauscher, Neuwied

Table 3 Consumables

1.4 Antibodies, dyes and microbeads

Name	**Specificity**	**Clone**	**Isotype**	**Fluorescence**	**Concentration used**	**Manufacturer**
Anti-phospo-Stat1 (Y701):PE	Stat1	4a	$MsIgG_{2a}$	PE	2 µl/ $1x10^5$ cells	BD, Heidelberg
Anti-phospo-p38 MAPK (T180/Y182):PE	p38 MAPK	36	$MsIgG_1$	PE	2 µl/ $1x10^5$ cells	BD, Heidelberg
Anti-CD40	CD40	5C3	$MsIgG_1{}^{K}$	FITC	5 µl/ $1x10^6$ cells	BD, Heidelberg
Anti-CD80	CD80	L307.4	$MsIgG_1{}^{K}$	PE	5 µl/ $1x10^6$ cells	BD, Heidelberg
Anti-CD83	CD83	HB15e	$MsIgG_1{}^{K}$	APC	5 µl/ $1x10^6$ cells	BD, Heidelberg
Anti-CD86	CD86	2331	$MsIgG_1{}^{K}$	PE	5 µl/	BD,

		FUN-1			$1x10^6$ cells	Heidel-berg
Polyglobin N®	none	none	MsIgG	none	0.05 g/ml	Bayer Vital, Lever-kusen
CFSE	none	none	None	PE	1000:1	Sigma-Aldrich, Munich

Table 4 Antibodies and dyes

Name	Specifi-city	Volume used	Conjugation	Manufacturer
Anti-CD304 (BDCA-4)	CD304	80 µl / $1x10^6$ cells	Superparamagnetic microbead	Miltenyi Biotec, Bergisch-Gladbach
Anti-CD4 MicroBeads	CD4	20 µl / $1x10^7$ cells	Superparamagnetic microbead	Miltenyi Biotec, Bergisch-Gladbach
Anti-CD45RA MicroBeads	CD45 RA	20 µl / $1x10^7$ cells	Superparamagnetic microbead	Miltenyi Biotec, Bergisch-Gladbach
Dynabeads® CD3/CD28 T cell expander	CD3/ CD28	50 µl / $1x10^6$ cells (2 beads/cell)	Superparamagnetic bead	Invitrogen, Karlsruhe

Table 5 Cell isolation reagents ("beads")

1.5 Kits and standards

Name	Specificity	Manufacturer
Human Th1/Th2 Cytokine Kit (BD CBA) Including: - Human IL2 Capture Beads: 1 vial, 0.8 ml - Human IL4 Capture Beads: 1 vial, 0.8 ml - Human IL5 Capture Beads: 1 vial, 0.8 ml - Human IL10 Capture Beads: 1 vial, 0.8 ml - Human TNF Capture Beads: 1 vial, 0.8 ml - Human IFNγ Capture Beads: 1 vial, 0.8 ml - Human Th1/Th2 PE Detection Reagent: 1 vial, 4 ml - Human Th1/Th2 Cytokine Standards: 2 vials, 0.2 ml lyophilized - Cytometer Setup Beads: 1 vial, 1.5 ml - PE Positive Control Detector: 1 vial, 0.5 ml - FITC Positive Control Detector: 1 vial, 0.5 ml - Wash Buffer: 1 bottle, 130 ml - Assay Diluent: 1 bottle, 30 ml - Serum Enhancement Buffer: 1 bottle, 10 ml	IL2, IL4, IL5, IL10, TNF, IFNγ	BD, Heidelberg

Human Inflammation Kit (BD CBA) Including: α) Human IL8 Capture Beads: 1 vial, 0.8 ml β) Human IL1β Capture Beads: 1 vial, 0.8 ml χ) Human IL6 Capture Beads: 1 vial, 0.8 ml δ) Human IL10 Capture Beads: 1 vial, 0.8 ml ε) Human TNF Capture Beads: 1 vial, 0.8 ml ϕ) Human IL12p70 Capture Beads: 1 vial, 0.8 ml γ) Human Inflammation PE Detection Reagent: 1 vial, 4ml η) Human Inflammation Standards: 2 vials, 0.2 ml, lyophilized ι) Cytometer Setup Beads: 1 vial, 1.5 ml φ) PE Positive Control Detector: 1 vial, 0.5 ml κ) FITC Positive Control Detector: 1 vial, 0.5 ml λ) Wash Buffer: 1 bottle, 260 ml μ) Assay Diluent: 1 bottle, 30 ml ν) Serum Enhancement Buffer: 1 bottle, 10 ml	IL8, IL1β, IL6, IL10, TNF, IL12p70	BD, Heidelberg
Human IFNα ELISA Kit	IFNαA, α2, αA/D, αD,	PBL Interferon

Including: • Pre-coated microtiter plates • Plate sealers • Wash Solution Concentrate • Human IFNα Standard • Dilution Buffer • Antibody Concentrate • HRP Conjugate Concentrate • HRP Conjugate Diluent • TMB Substrate • Stop Solution	αK, and α4b.	Source, Piscataway, New Jersey, USA

Table 6 Kits and standards

1.6 Cell culture reagents and CPG oligodenucleotides

Reagent	Manufacturer
β-Mercaptoethanol (2-Mercaptoethanol) Component: β-Mercaptoethanol	Invitrogen, Karlsruhe
RPMI Medium 1640 (1X) liquid *containing no L-glutamine*	Invitrogen, Karlsruhe
Ficoll Composition: Ficoll 400	Invitrogen, Karlsruhe
Penicillin/streptomycin Composition: 5000 units/ml Penicillin and 5000 µg/ml Streptomycin utilizing penicillin G (sodium salt) and streptomycin sulphate, prepared in normal saline	Invitrogen, Karlsruhe
L-Glutamine Composition: L-Glutamine 200 mM (29.2 mg/ml)	Invitrogen, Karlsruhe
PBS (phosphate-buffered saline) Composition: Monobasic potassium phosphate, sodium chloride and dibasic sodium phosphate.	Invitrogen, Karlsruhe
MEM amino acids Composition in mg/ml L-Arginine 6300 — L-Methionine 750 L-Cystine 1200 — L-Phenylalanine 1600 L-Histidine 2100 — L-Threonine 2400 L-Isoleucine 2600 — L-Tryptophan 500 L-Leucine 2600 — L-Valine 2300 L-Lysine 3500 — L-Tyrosine 1800	Biochrom, Berlin
Sodium pyruvate Composition: Sodium pyruvate (100 mM)	Biochrom, Berlin
Stain buffer (FBS) Composition: Dulbecco's phosphate-buffered saline (DPBS) with 2% (w/v) heat-inactivated FBS and 0.09% (w/v) sodium azide, pH 7.4 (0.2 µm-pore filtered)	BD, Heidelberg
Human immunoglobulin G (hIgG) Composition: Purified recombinant human immunoglobulin G	Bayer, Leverkusen
Human sCD40L Composition: Purified recombinant human CD40Ligand (CD154, TRAP)	Strathmann Biotec, Hannover
Human IL2	Strathmann

Composition: Purified recombinant human interleukin-2	Biotec, Hannover
Human IL3 Composition: Purified recombinant human interleukin-3	Strathmann Biotec, Hannover
Human IFNγ Composition: Purified recombinant human interferon-gamma	Strathmann Biotec, Hannover
PMA Composition: Phorbol myristate acetate	Sigma-Aldrich, Munich
DMSO Composition: Dimethyl sulfoxide	Serva, Heidelberg

Table 7 Cell culture reagents

Name	Character	Length	Sequence from 5’ end to 3’ end	Manufacturer
ODN 2216	Stimulatory oligo-nucleotide with human-specific type A CPG	20-mer	5’-ggGGGACGA:T CGT Cgggggg-3’ Note: Bases shown in capital letters are phosphodiester, and those in lower case are phosphorothioate (nuclease resistant). Palindrome is underlined.	InvivoGen, San Diego, USA
ODN 2006	Stimulatory oligo-nucleotide with human-specific type B CPG	24mer	5’-tcgtcgttttgtcgttttgtcgtt-3’ Note: Bases are phosphorothioate (nuclease resistant).	InvivoGen, San Diego, USA

Table 8 CPG oligonucleotides

All CPG oligonucleotides used were endotoxin-free and were used, if not expressly noted otherwise, at a concentration of 50 µg/ml. CD40L was used, if not noted expressly otherwise at a concentration of 10 ng/ml. Dilution to stock solution were made with sterile endotoxin-free water, aliquots were prepared in sufficient size for each experiment.

1.7 Solutions

Wash medium (WM)

500 ml RPMI 1640 (1x)

100 U/ml penicillin

100 µg/ml streptomycin

ACK lysing buffer (ACK)

4.145 g NH_4Cl

0.5 g $KHCO_3$

17.6 mg Na_2EDTA, dissolved in 400 ml Aqua bidestillata

pH adjusted with 1 N HCl to 7.2-7.4,

filled up with Aqua bidestillata to 500 ml, filtered through a 0.2 µm filter, stored at RT.

Freezing medium

FCS with 10% dimethyl sulfoxide

MACS buffer (Qiagen™)

500 ml PBS (pH 7.2)

0.5% BSA

2 mM EDTA

Buffer was kept cold (4−8 °C) and worked with on ice.

Culture medium

500 ml RPMI 1640 (1x)

100 U/ml penicillin

100 µg/ml streptomycin

2 mM L-glutamine

5 ml MEM amino acids

2 mg/ml sodium pyruvate

50 ml FCS (inactivated*)

500 µl β-Mercaptoethanol

**FCS was inactivated at 56°C for 30 min prior to use. At this temperature, the molecules of the complement system are destroyed. The serum was aliquoted into portions of 50 ml and frozen at -20°C.*

FACS wash buffer

500 ml PBS

2.5 g BSA (0.5%)

1.25 g sodium azide (0.025%)

Dynal buffer

100 ml PBS

1 mg BSA (0.1%)

Wash Buffer for IFNα-ELISA

50 ml Wash Solution Concentrate (Human IFNα ELISA kit)

950 ml deionised water

Stop Solution for IFNα-ELISA

100 ml 2M H_2SO_4

1.8 Software

Software	***Manufacturer***
Excel™	Microsoft
FACS Diva™	BD, Heidelberg
FACS Comp™	BD, Heidelberg
BD CBA Software	BD, Heidelberg
Revelation™	Dynex Technologies GmbH, Berlin

Table 9 Software

Methods

All procedures were, if not stated otherwise, performed under sterile conditions under a class II sterile hood. Containers for reactions, storage, glass pipettes and pipette tips were sterilized by 30-minute autoclaving at 121°C. Solutions, wash and culture mediums used were sterile. Cell culture was done in a gas incubator at 37°C under 100% air humidity and 5% CO_2. If not stated otherwise, the culture medium described above was used.

1.9 Samples

All experiments were done with human peripheral mononuclear cells (PBMC). In this study, neonatal PBMC from umbilical cord blood (CB) were the main subject of investigation while adult PBMC were used as control. All PBMC were separated from whole blood directly after sample taking and used directly or stored at -80°C (cf. 1.12).

Cord blood was used as a donor of large quantities of neonatal blood that could be used without inducing anemia in the newborn. Cord blood was gained under sterile conditions from the umbilical cord of neonates born through caesarean section free from infections and pregnancy complications. Adult blood was gained from healthy adult volunteers.

1.10 Separation of PBMC from whole blood

To inhibit coagulation, cord blood was mixed with a heparinized solution (Na-heparin 40 IU/ml whole blood) directly after sample taking, adult blood was mixed with 20 IU/ml whole blood) and processed directly for further use. Separation of PBMC was done by Ficoll density gradient centrifugation. Ficoll is a branched non-ionic saccharose polymer with a molecular weight of around 400 kDA. The solution used has a density of 1.077 G/ml at room temperature (RT). Depending on the density of the cells they can either pass through the Ficoll phase (erythrocytes and granulocytes) or be held back on top of the phase (lymphocytes and monocytes), so that PBMC are enriched in the interphase between Ficoll and plasma. Thrombocytes are retained together with plasma in the layer above the Ficoll phase.

In a 15 or 50 ml polyethylene tube an amount of Ficoll matching the blood volume sample was prepared and carefully topped with whole blood without mixing Ficoll and whole blood. If the sample volume surmounted the maximum available volume, the

sample was split into multiple tubes and PBMC from this sample only were reunited after separation (autologous recombination). To avoid cell losses and mixing of phases, Ficoll and whole blood were used at the same temperature of 20°C. The two phase system was centrifuged for 30 minutes (min) with 600 xg at 20°C at lowest acceleration and without braking. After centrifugation, the PBMC fraction formed an interphase between Ficoll and plasma. This was carefully removed with a sterile pipette without removal of Ficoll as Ficoll tends to have negative impact on cell viability. Because of the high frequency of erythrocyte precursors in cord blood, the interphase in cord blood samples consisting of PBMC is contaminated with erythroblasts. Thus, a further step is needed to obtain a clean cord blood PBMC sample. Adult PBMC do not pose this problem. The process of PBMC isolation is depicted in Fig 1.

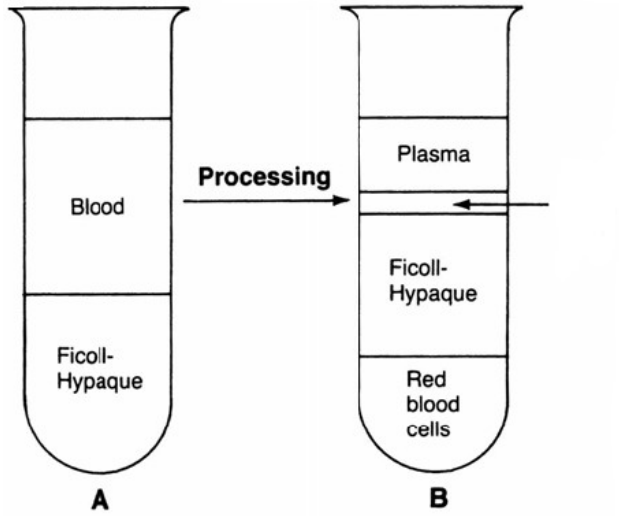


Fig 1 Phases during lymphocyte isolation
through Ficoll density gradient centrifugation before and after centrifugation.

Lymphocyte separation medium is topped up with the same amount of whole blood and centrifuged. After centrifugation PBMC are found in the interphase between Ficoll and plasma where they can be aspirated with a pipette.

1.10.1 Adult blood

To remove Ficoll and cell detritus from the samples, adult PBMC were washed twice with wash medium (WM). For washing the cells were suspended in 12-40 ml wash medium and centrifuged for 10 min with 360 xg at 4°C. After sedimentation the cell pellet was homogenously resuspended in 1 ml of the medium used in the further experimental course and cell count was determined (cf. 1.11).

1.10.2 Cord blood

To separate PBMC from the contaminating erythroblasts, the interphase was removed to a new 50 ml PE tube and centrifuged at 600 xg for 10 min at RT. The pellet was resuspended in 5 ml hypotonic ammonium chloride lysing buffer (ACK) per 10 ml whole blood sample volume and incubated at RT for 5 min. The resulting suspension was then mixed with 25 ml phosphate-buffered saline (PBS) and centrifuged for 15 min with 300 xg at RT. The resulting pellet was resuspended with 30 ml PBS and centrifuged again for 15 min at 300 xg at RT. After this second wash cycle the pellet was suspended in 1 ml PBS and cell density was determined (cf. 1.11).

1.11 Cell count determination

Prior to all cell culture experiments, cell count was determined to obtain a reference figure for cell culture growth rate results. To do this, cell count was either performed with a Neubauer-counting chamber or with the TruCount® method.

The Neubauer chamber is engraved with a counting grid consisting of 4 x 16 quadrants on a flat polished glass bottom. If a coverslip is fitted on the chamber, a defined volume of 0.4 µl is formed. The cells suspended in 1 ml medium were diluted in PBS 1:50. An aliquot of this dilution was pipetted into the chamber and the cells were counted in all 4 x 16 quadrants under a microscope at 20 x magnification. The cell count in 1 ml undiluted cell suspension was calculated as follows:

(counted cells / chamber volume) x dilution factor x suspension volume
= (counted cells / 0.4 µl) x 50 x 1000 = Cell count / ml

Cell count determination under the microscope was performed under nonsterile conditions. PBS dilution and aliquot were discarded.

For the TruCount® method, cells were resuspended in 5 ml wash medium and 50 µl were transferred to a TruCount tube with 200 µl PBS. After 15 min incubation in darkness at RT, cells were analyzed by flow cytometry (cf. 1.20).

On the basis of the defined number of beads in the TruCount tube and under consideration of the dilution factor used the number of cells in the tube and

consequently also in the original cell suspension can be determined (Kawamoto et al., 1988).

1.12 Long time storage of PBMC

With the storage of BDCA-4 negative cells (non-PDC) at -74°C for maximum of seven days and the further option of storage in liquid nitrogen (-196°C) it was possible to use cells from certain donors at a later date in the further course of the experiment for autologous co-culture. After cell count determination and separation of PDC the remaining PBMC were centrifuged for 10 min at 360 xg at 4°C. The cell pellet was resuspended in 4°C cold freezing medium at a cell density of 5 x 10^6 cells/ml. The cell suspension was transferred into 1.8 ml Cryotubes and inserted into pre-cooled (4°C) isopropanol-filled freezing containers. The containers were then stored at -74°C for up to 7 days which guaranteed a cool down rate of 1°C/min.

1.13 Thawing of frozen PBMC

Frozen samples were transferred on ice to a pre-warmed (37°C) water bath and thawed until only a small rest of frozen cell suspension was visible. The cell suspension was directly transferred to a 15 ml Falcon tube prepared with 3 ml wash medium, filled up to nominal volume with wash medium and centrifuged for 10 min at 360 xg at RT. The supernatant was removed and discarded and the pellet was carefully resuspended with wash medium to remove all rests of DMSO and FCS. After a second wash cycle with wash medium and centrifugation for 10 min at 360 xg at RT the pellet was resuspended in 1 ml of the medium used in the next step of the experiment and cell count was determined.

1.14 Magnetic separation of PBMC subpopulations

To determine the effect of stimuli on PDC and their interaction with T cells, it was necessary to gain highly pure cell populations. With the aid of magnetic activated cell sorting (MACS) it is possible to enrich cells in a sample or deplete this population from it. To do this, cells are incubated with antibodies against certain surface molecules and washed out via separation or depletion columns. Therefore the antibodies are conjugated with paramagnetic MACS micro beads. Physiologic cell function is supposedly not impeded by the beads because of their small size (50 nm diameter). The columns consist of a matrix of differently packed steel wool, which is coated with plastic for cell protection. For separation the columns are exposed to a

strong magnetic field, which retains the cells with bound magnetic beads while the unmarked fraction passes through the column. The effluent is collected in a 15 ml tube and either discarded or frozen for later use (cf. 1.12). To wash out the labeled cells the column is removed from the magnetic field and eluted into a new tube with a plunger. The purity of cells can be determined by flow cytometry (cf. 1.20).

For the enrichment of PDC a protocol developed by Miltenyi Biotec was used. For the enrichment of $CD4^+$ and $CD45R0^+$ cells, a protocol already established in the laboratory was used. MACS beads used are listed in Table 5.

1.14.1 Isolation of PDC

PDC were isolated from PBMC via MACS. For the separation of PDC a protocol for the isolation of PDC with the BDCA-4 kit developed by Miltenyi Biotech was used. BDCA-4 *(= Blood dendritic cell antigen)* is a surface molecule which is mainly expressed on plasmacytoid dendritic cells. Following the protocol, work was performed fast, cells were kept cold and pre-cooled solutions were used.

Volumes for magnetic labeling given below are for up to 10^8 total cells. When working with lower numbers of cells, the same volume as indicated was used. For higher cell numbers, volumes were scaled up accordingly. First, cell number was determined and PBMC were washed as described above. PBMC were then incubated for 15 min with the paramagnetically marked anti-BDCA-4 antibody (BDCA-4 micro particle) and the Fc receptor-blocking agent (Polyglobin N®) at 8°C. The Fc receptor blocker was used to inhibit unspecific binding of antibodies to the Fc receptor. The cell suspension was then topped up to 15 ml and centrifuged for 10 min at 300 xg at 4°C. The resulting pellet was resuspended in 300 µl and applied to the rinsed selection column placed in a strong magnetic field. Through the paramagnetic labeling the PDC were retained in the column. Unlabeled cells passed through the column and were either discarded in the experiments where only PDC were needed or frozen (cf. 1.12) for storage when T cells were needed for the proliferation experiments. The purity of PDC obtained via this method when measured by flow cytometry was, depending on the donor, between 70 and 100%, median 93%.

For all isolation steps, a special buffer (MACS buffer) was used. After isolation, the tube containing PDC was topped up with wash medium and centrifuged for 10 min at 360 xg at 4°C. The cell pellet was resuspended in culture medium.

1.14.2 Isolation of naïve T cells

Isolation of naïve T cells was performed analogously to isolation of PDC. As markers for naïve T cells, CD4 and CD45RA were used. As noted above (cf. ??), PDC have been shown to be $CD4^+$ and $CD45RA^+$. Therefore, isolation of naïve T cells from the same samples used for the isolation of PDC had to be performed after isolation of PDC. PDC were thus eliminated from the pool of $CD4^+$ cells. For the separation of naïve T cells a protocol already established in the laboratory (successive isolation of $CD4^+$ and $CD45RA^+$ cells) was used. Following the protocol, isolation was done by first isolating of $CD4^+$ cells from the flow-through collected during PDC isolation through MACS. Work was performed fast, cells were kept cold and pre-cooled solutions were used.

Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer numbers, volume was used as indicated. For higher cell numbers, volumes were scaled up accordingly. First, cell number was determined and PBMC were washed as described above. PBMC were then incubated for 15 min with the paramagnetically marked anti-CD4 antibody at 8°C. The cell suspension was then topped up to 15 ml and centrifuged for 10 min at 300 xg at 4°C. The resulting pellet was resuspended in 500 µl per 10^8 cells and applied to the rinsed selection column placed in a strong magnetic field. Through the paramagnetic marking the $CD4^+$ cells were retained in the column. Unmarked cells passed through the column and were discarded.

For all isolation steps, MACS buffer was used. After isolation, the tube containing PDC was topped up with wash medium and centrifuged for 10 min at 360 xg at 4°C. The cell pellet was resuspended in culture medium.

To furthermore select only naïve T cells from the $CD4^+$ cells isolated, the flow-through containing the anti-CD4 microbead-marked cells was used to isolate $CD4^+$ $CD45RA^+$ cells. The same technique as described above was employed. The purity of $CD4^+$ $CD45RA^+$ cells obtained via this method when measured by flow cytometry was, depending on the donor between 70 and 100%.

1.15 PDC phenotype determination

Specific cell surface proteins, the CD antigens ("*cluster of differentiation*") make it possible to distinguish different lymphocyte subpopulations. With the aid of monoclonal antibodies to which fluorochromes (fluorescent dyes) are conjugated and

which bind to cell surface proteins, cells are stained (labeled) and assigned to the different populations (cf. 1.20).

Prior to staining, cells had to be washed. For this purpose, cells from culture well plates were resuspended in PBS and transferred to 15 ml Falcon tubes. The cell solutions were topped up with PBS to nominal tube volume, centrifuged at 360 xg for 10 min at 4°C and transferred to individual FACS tubes. To block Fc receptors and unspecific binding sites on the cell surface, Polyglobin N® was added equal in volume (0.05 g/ml) to the cell solution and incubated for 15 min in darkness. To each tube, staining antibodies were added (in the concentration given in Table 4) and incubated for 20 min in the dark at RT. After incubation, cells were washed with FACS wash buffer at 4°C. Supernatant was decanted and the cell pellet was resuspended in 200 µl PBS. FACS analysis (cf. 1.20) was performed directly afterwards.

1.16 Lymphocyte culture

For in vitro proliferation and differentiation inactive lymphocytes need an effective activation by mitogens or antigen. Furthermore, in the experiments on cytokine secretion, different time points of cell culture were used to determine highest cytokine production over entire culture period. Cells were incubated for 48 h to 120 h. Cell stimulation and incubation was done either in polystyrene round bottom-culture plates with 24 to 96 impressions (“wells”) or in Falcon tubes. As control, unstimulated cells incubated in plain culture medium were used. Cell cultures were incubated at 37°C, 5 % CO_2 and 100 % humidity.

1.17 Maturation of PDC

The focus of this study was the maturation capacity of neonatal PDC. Therefore, PDC maturation was used in all sets of experiments and is therefore described extensively here.

Thawed or freshly isolated PBMC were transferred to a 15 ml Falcon tube. Benzonase® was added (10 µl at a concentration of 250 U/µl) to digest free DNA in order to avoid unwanted stimulation of PDC by DNA of apoptotic cells. The tube was topped up with wash medium and washed twice at 360 xg for 10 min, 4°C. Cell count was determined either with the Neubauer-counting chamber or via TruCount (cf.

1.11). PDC were isolated with the positive selection method using MACS microbeads specific for CD304 (cf. 1.14.1).

After isolation, cells were counted once more to determine percentage of PDC contained in original blood sample of PBMC. Resuspended in culture medium, cells were plated on 24- or 96-well plates at a concentration of 2 x 10^5 cells/100 µl. Depending on total PDC number available three to six experimental conditions were set, as described in *Table 10*.

Nr.	PDC	Culture medium	Addition of stimuli				
	PDC in culture medium (100 µl)	Additional culture medium (equal to cell suspension volume)	CPG A 50 µg/ml	CPG B 50 µg/ml	CD40L (10ng/ml)	PMA (50ng/ml)	IFNγ (50ng/ml)
1	X	X					
2	X	X	**X**				
3	X	X		**X**			
4	X	X			**X**		
5	X	X	**X**	**X**			
6	X	X		**X**	**X**		
7	X	X				**X**	
8	X	X					**X**

(If stimuli were added, their volume was subtracted from additional culture medium volume added)

Table 10 PDC stimulation

1.18 Proliferation cultures of PDC and T cells

For proliferation cultures, isolated PDC were pre-incubated with IL3 at a concentration of 10 ng/ml for 60 h, then divided into six distinct populations, stimulated according to experimental conditions given above (cf. Table 10, 1-6) and incubated for 6 h. During this interval, autologous T cells were prepared for co-incubation.

Naïve T cells which had been isolated from PBMC by MACS on the day of sampling (cf. 1.14.2) and had been frozen until preparation (cf. 1.12) were thawed (cf. 1.13) and suspended in culture medium.

PBS was slowly heated to 37°C in a 50 ml Falcon and CFSE stock solution was added (1000:1) for labeling of naïve T cells. Label mix was incubated for 15 min and turned over twice during incubation to insure good mixing. The mix was then centrifuged at 400 xg for 10 min at RT. The cell pellet was resuspended in ice-cold PBS and washed twice with ice-cold PBS at 400 xg for 10 min at RT. Between

washing steps, cell count was performed with the TruCount™ method (cf. 1.11) and number of wells needed was calculated. According to experimental conditions, a 96-well round bottom-plate was labeled as shown in Table 11.

	Added stimuli							
Nr. of condition			**0**	**1**	**2**	**3**	**4**	**5**
Cells	No PDC	No PDC	PDC unstim. control	PDC + CPG A	PDC +CPG B	PDC +CD40L	PDC +CPG A +CD40L	PDC +CPG B +CD40L
T cells+ Dyna-beads		**X**	**X**	**X**	**X**	**X**	**X**	**X**
T cells	**X**							
Nr. of condition			0	1	2	3	4	5
Cells	No PDC	No PDC	PDC unstim. control supernat	PDC + CPG A supernat	PDC +CPG B supernat	PDC +CD40L supernat	PDC +CPG A +CD40L supernat	PDC +CPG B +CD40L supernat
T cells+ Dyna-beads		**X**	**X**	**X**	**X**	**X**	**X**	**X**
T cells	**X**							

supernat = supernatant, unstim = unstimulated

Table 11 PDC and T cell co-incubation

After washing, T cells were resuspended in culture medium and plated at a concentration of 2 x 10^6 cells/ml. CD3/CD28 T cell-expander beads (Dynabeads®) were washed once in special buffer (Dynal buffer) and added at a concentration of 10 µl/100 µl T cell-suspension with a ratio of 2 beads/cell. After stimulation with T cell expander, PDC were removed from the incubator. First supernatant from stimulated PDC was added to the wells containing T cells and Dynabeads designated for stimulation by PDC supernatant following labeling for their different experimental conditions. Next, PDC were resuspended in the wells they were incubated in and transferred to the other T cell-containing wells which had not received any supernatant yet (according to the experimental conditions cited in Table 11). Cells were co-incubated for 120 h.

For use in ELISA (cf. 1.22) or CBA (cf. 1.21) analysis, supernatant from all wells (PDC + T cells /Dynabeads or Supernatant from PDC + T cells/Dynabeads) was carefully

removed from each well after 72 h and 120h and transferred into a new 96 well-plate according to the experimental layout used for the first 96 well-plate and stored at -20°C. After removal of supernatant, culture medium with IL2 was added at a concentration of 2 µl/ml culture medium.

1.19 Phosphorylation of signal kinases

PBMC were isolated from fresh adult and cord blood samples and PDC were isolated (cf. 1.14.1). Adult PDC were stimulated according to experimental conditions (cf. 1.17) 1-3 and 7-8 to determine the best incubation time (10, 20 or 30 min). Incubation time of 10 min was established as delivering best stimulatory results with lowest cell loss. Stimulation was performed according to the following protocol:

PDC were washed with culture medium without FCS at 360 xg for 10 min at 4°C then resuspended in 10 ml culture medium and 1 ml aliquots were distributed to 10 Falcon tubes. Experimental conditions were set as shown in Table 12.

Antibody used	**Added stimuli**				
	unstimulated control	PMA (50 ng/ml)	IFNγ (50 ng/ml)	CPG A 50 µg/ml	CPG B 50 µg/ml
Anti-phospo-Stat1 (Y701):PE 2 µl/1x10^5 PDC	**X**	**X**	**X**	**X**	**X**
Anti-phospo-p38 MAPK (T180/Y182):PE 2 µl/1x10^5 PDC	**X**	**X**	**X**	**X**	**X**

Table 12 PDC stimulation and labeling for phosphorylation analysis

As both antibodies (anti-STAT1 and anti-p38 MAPK) were PE-labeled, the same experimental condition was established in two different tubes to avoid overlap in fluorescence.

The different stimuli were added and PDC were incubated for 10 min at 37°C in the water bath. Directly after incubation, 1 ml paraformaldehyde was added to each tube and PDC were again incubated for 10 min at 37°C for fixation. Directly after fixation, PDC were permeabilized with 2 ml ice-cold methanol (90% methanol, 10% aqua dest.) and kept at -20°C for 30 min. PDC were then washed once with Stain Buffer at 360 xg for 10 min at 4°C and resuspended in 100 µl Stain Buffer. Anti-phospo-Stat1 PE and Anti-phospo-p38 MAPK PE-antibodies were added at a concentration of

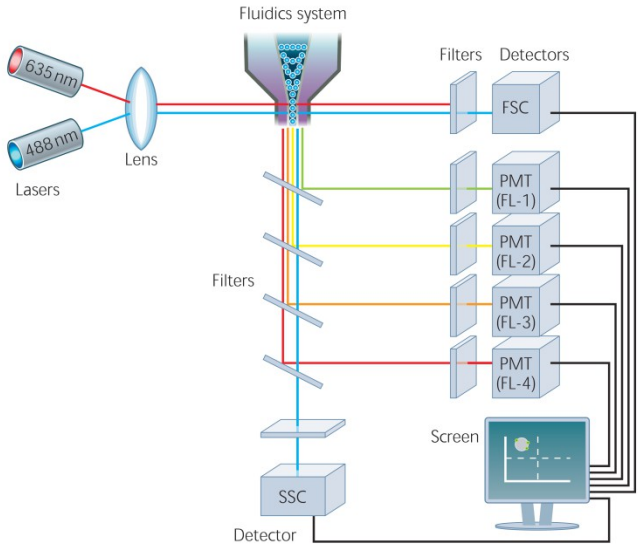
2 µl/1x10^5 cells. PDC were then incubated for 60 min in darkness. After incubation, PDC were washed once with Stain Buffer at 360 xg for 10 min at 4°C and resuspended in 500 µl Stain Buffer and analyzed by FACS.

1.20 FACS

The fundamental principle of flow cytometry is the ability to measure properties of individual particles. For analysis, the sample is streamlined by the fluidics system. This creates a single file of particles and is called hydrodynamic focusing. This applies not only to cells, but also to smaller particles such as cytokines that, once bound to plastic beads, undergo a similar analysis as cells in the flow cytometer.

After hydrodynamic focusing the particles pass through a number of light beams, emitted by lasers. Light scattering provides information about the particles' size and granularity. If a particle is fluorochrome-labeled it will emit fluorescence upon laser light beam excitation. Light scattered in the forward direction at 20° offset from the laser beams axis is called forward scatter channel (FSC), equating roughly the particles size and is used to distinguish cellular debris from living cells. Light scattered at around 90° offset is called side scatter channel (SSC) and gives information on cell granularity. FSC and SSC are unique for each particle and their combination is used to differentiate cell types in heterogeneous samples.
To measure fluorescence-labeled cell surface markers or intracellular molecules, different wavelengths are used. The flow cytometer uses fluorescence channels (FL-) to detect emitted light. The detector number usually depends on machine and manufacturer. A detector can either be a silicon photodiode or photomultiplier tubes (PMT). The former usually measure the FSC if the signal is strong, while PMT are more sensitive and generally used for less strong SSC and fluorescence reading. Detection specificity is controlled by three major absorption filter types: 'Long pass' filters block light below a cut-off wavelength, 'short pass' filters block light above a certain wavelength and 'band pass' blocks all light outside a narrow wavelength range (called a bandwidth).

If light is filtered at 45° the filter becomes a dichroic mirror: Certain wavelengths pass forward while others are deflected at a 90° angle. For simultaneous detection of multiple signals, filters must be chosen and placed with consideration (cf. Fig 2).



(Source: Rahman M (2006) Introduction to flow cytometry. Abd Serotec, Serotec Ltd, Kidlington, United Kingdom.)

Fig 2 Typical flow cytometer setup

When the beam hits the photodetector, a small current of a few mA with an associated voltage proportional to the total number of photons detected is generated. The voltage is then amplified several times by linear or logarithmic amplifiers and analogue to digital convertors into electrical signals large enough (5–10 volts) to be plotted graphically.

Fluorochromes are essentially dyes that accept energy from a laser at a given wavelength (excitation) and re-emit it at a longer wavelength (emission). Emission follows excitation within nanoseconds and is called fluorescence. This is the principle of light absorbance and emission: Light is electromagnetic energy travelling in waves. These waves are distinguished by their frequency and length. The light color is determined by wave length. The use of a fluorescent probe, such as a fluorochrome-conjugated antibody, is the direct targeting of an interesting epitope. It allows its biological and biochemical properties to be measured more easily by the flow cytometer. To analyze several sample parameters at one time, more fluorochromes can be used and the excitation wavelength can be changed.

The dyes used in this study are single dyes (in contrast to tandem dyes): fluorescein isothiocyanate (FITC), a green dye and phycoerythrin (PE), a reddish dye. PE and FITC, both excited at 488 nm can be used in the same sample because of their

different wavelength (and therefore color) emissions (FITC at 525 nm and PE at 578 nm), which were measured by separate detectors. When performing multicolor fluorescence studies, the possibility of spectral overlap must be considered. If two or more fluorochromes are used in a single experiment, their emission profiles may coincide, making measurement of their true individual fluorescence difficult. To avoid this, a mathematical process called fluorescence compensation is applied during data analysis, calculating the percentage of interference a fluorochrome has in a channel not specifically designed to measure it. This means that when the two fluorochromes are used for a dual-color experiment, the real reading for fluorochrome A in FL-1 should be = (total fluorescence measured in FL-1) - (5% of fluorochrome B's total fluorescence). Similarly, the real reading for fluorochrome B in FL-2 should be = (total fluorescence measured in FL-2) - (17% of fluorochrome A's total fluorescence). Modern flow cytometry analytical software applies fluorescence compensation mathematics automatically, although in some cases, this has to be done manually.

In flow cytometry data analysis it is important to selectively visualize the cells of interest, meanwhile eliminating results from unwanted particles like dead cells and debris. This procedure is called "gating". Traditionally, cells have been gated according to their physical characteristics, for instance size in the FSC (subcellular debris is smaller, clumps are bigger than normal cells). In contrast, dead cells have lower forward scatter and higher side scatter than living cells.
The data gained from analysis can be shown in single-parameter histograms. These display a single parameter (e.g. relative fluorescence or light scatter intensity) on the x-axis and cell count on the y-axis. The histogram type in Fig 3 is useful for evaluating total cell number expressing the markers of interest, called the positive dataset. Ideally for visualizing T cell division, a single distinct peak (if cellular CFSE content was not diluted (= no division)) will be produced that can be interpreted as the positive dataset. The single-parameter histogram was used when co-incubation of stimulated PDC/supernatant of stimulated PDC with naïve T cells was analyzed. In histograms used for division studies, the distinct peaks representing each divisional step were gated again then statistically analyzed for percentage of total number of cells of all CFSE-labeled T cells in the sample. Additional statistics about the peak

gated (e.g. mean fluorescence intensity (MFI)) is also provided automatically and was used for statistical analysis.

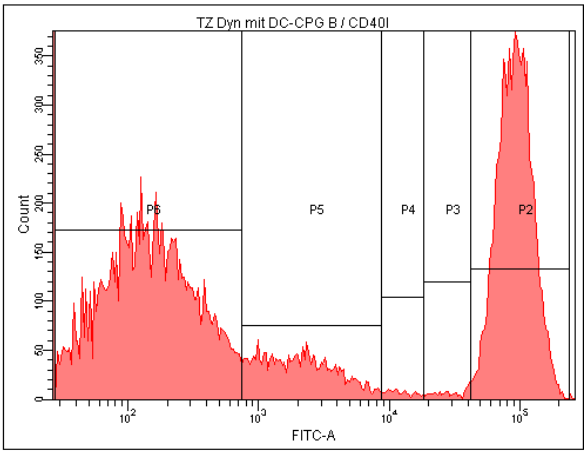


Fig 3 Single parameter histogram

Two-parameter histograms, on the other hand typically display two parameters, for example SSC, FSC and fluorescence, (one on x, one on y) and cell count again as a dot plot (density plot) or a contour map.

In Fig 4, Q1 encloses PE-labeled cells. Note the positive shift along the PE axis.

Q4 contains FITC-labeled T cells. Note here the positive shift along the FITC axis.

Q2 contains a few activated cells expressing both PE and FITC-labeled markers.

Q3 contains cells negative for both FITC and PE (no shift). Two-parameter histograms were used for analysis of CD40/CD80 and CD83/CD86 expression on activated PDC. Counts from regions Q1-Q4 were transferred from the automatic statistic analysis to an Excel® sheet before statistical analysis with SPSS®.

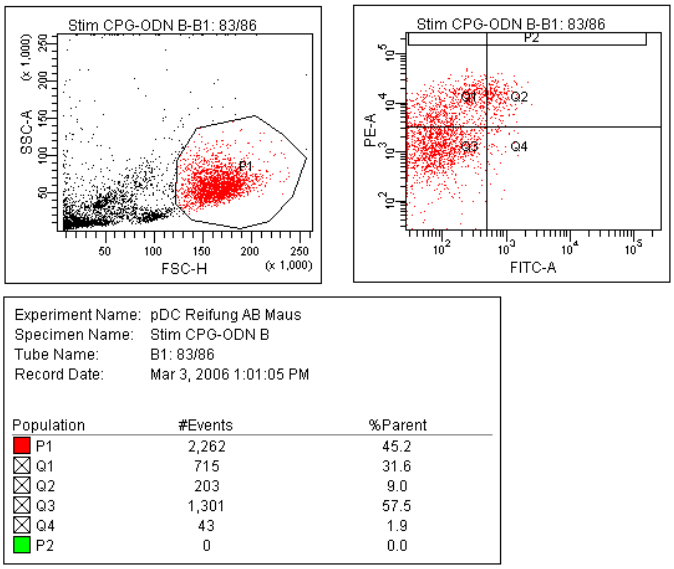


Fig 4 Two-parameter (dual-color and fluorescence) histogram

The above explains how cell surface antigens can be measured. Staining of intracellular antigens like STAT1 and MAPK3 however can prove difficult as antibody probes cannot sufficiently pass through the plasma membrane to their intracellular markers. To improve staining, cells are fixed in suspension and then permeabilized before staining. Thus, probes can access intracellular structures while leaving the morphological scatter characteristics of the cells intact. This method was employed when stimulated PDC were stained for two intracellular activation markers, STAT1 and p38 MAPK.

To obtain the best results from each analysis, specific adjustments had to be made to instrument settings. For each FACS tube containing a sample, a threshold of 50 and a maximum of 10.000 events (cells) were counted in the stopping gate. This number had shown to be adequate in the establishing of FACS analysis of PDC. For each event, on the one hand the light scatter characteristics (FSC and SSC) and on the other hand two different fluorescence types were measured. To exclude cell debris from analysis, a threshold of 70-100 was set in the FSC. All scatter signals were amplified linearly, all fluorescence signals were amplified logarithmically, whereas the voltage applied to the photo multipliers varied depending on the experiment. For the FL1 detector 505-722 V, for the FL2 detector 439-576 V and for the FL3 detector 486-674 V had to be applied. To compensate spectral overlap of the fluorescence emissions, 0.1–0.5% of FL2 signal intensity had to be subtracted from the FL1 signal

intensity, 10-30% of FL1- from FL2 signal intensity, 0-2% of FL3 from FL2 signal intensity and 38-48% of FL2 from FL3 signal intensity.

Gates were set depending on the objective of the experiment and the lymphocyte population analyzed (PDC or T cells). To calculate percentages of distinct populations in the different experiments, an unstimulated control was used as a reference for the placement of statistical markers (e.g. quadrants).

1.21 CBA

The cytometric bead array (CBA) is used to detect and quantify cytokines in a sample solution. Traditionally, the ELISA (enzyme-linked immunosorbent assay) has been used to do this, but the CBA has many advantages that make it a very useful technique to obtain fast and reliable results. This method uses amplified fluorescence detection to display soluble analytes, analogue to the ELISA. However, in CBA the capture surface is not a coated well but a coated bead which makes multiple analyses in the same solution possible – in this study, analysis of six distinct cytokines.

The soluble nature of the capture surface gives access to the advantages offered by flow cytometry – speed and flexibility - which in turn enables the CBA to use fewer sample dilutions and obtain analyte concentrations much faster than in a conventional ELISA. Furthermore, due to the nature of the test and optimizations developed previously in our laboratory, required sample quantity is only about 1/12 of the volume necessary for conventional ELISA assays because of the detection of six different analytes in a single sample. This makes it even possible to work with very small cell numbers, which is advantageous when working with the restricted number of PDC provided by a single cord blood sample.

Capture beads, detection antibodies conjugated to the fluorochrome PE and recombinantly produced cytokine standards are incubated together so that they form sandwich complexes. As the bead populations all employ the same fluorochrome, one might wonder if signals from different populations can be differentiated from one another. This is achieved through distinctions in brightness of fluorescence so that counted events can be allocated to their respective bead populations.

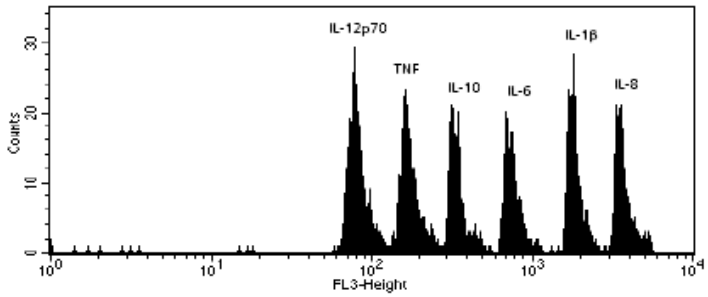
Six bead populations with different fluorescence intensities in the FL-2 channel (for exp. PE) are coated with capture antibodies specific for example in the Human Inflammation Kit, for IL8, IL1β, IL6, IL10, TNF, and IL12p70 proteins. Mixed together,

they form the CBA signals, which are measured in the FL-3 channel of a flow cytometer such as the BD FACS Canto.

In this study, two kinds of CBA kits have been used:

5) BD™ Cytometric Bead Array (CBA) Human Inflammation Kit
6) BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit

Using the Human Inflammation Kit, supernatant from PDC stimulated along experimental conditions 1-3 (cf. Table 10) was analyzed for the following cytokines: interleukin-8 (IL8), interleukin-1β (IL1β), interleukin-6 (IL6), interleukin-10 (IL10), tumor necrosis factor (TNF), and interleukin-12p70 (IL12p70). The Human Th1/Th2 Cytokine Kit (supernatants from days 3 and 5) was used to analyze cytokines produced by T cells after stimulation with PDC (experimental conditions 1-14, cf. Table 11): interleukin-2 (IL2), interleukin-4 (IL4), interleukin-5 (IL5), interleukin-10 (IL10), tumor necrosis factor (TNF) and interferon-γ (IFNγ) (cf. 1.18).



(Source: BD Cytometric Bead Array (CBA) Human Inflammation Kit Instruction Manual, BD Biosciences, San Diego, USA)

Fig 5 Exemplary diagram of distinct bead counts

1.21.1 Assay procedure

Cytometer setup was performed according to protocols previously established in the laboratory for CBA experiments. All beads were carefully resuspended by pipetting repeatedly to mix up settled beads. All mixing during the assay was done by pipetting. Wash Buffer was used for wash steps and to resuspend the washed beads for analysis. Assay diluent was used to reconstitute and dilute the standards and to dilute test samples.

Standards were reconstituted and serially diluted before mixing with the Capture Beads and the PE Detection Reagent. This was done as follows:

The standards were reconstituted with 200 µl assay diluent and allowed to equilibrate for 15 min before further dilution. 100 µl dissolved standard solution was transferred to a FACS tube with 900 µl assay diluent. This tube then contained 5000 pg/ml cytokine standard. Remaining standards stock solution was stored for later use.
11 more FACS tubes were labeled and arranged in the following order: 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312.5 pg/ml, 156 pg/ml, 78 pg/ml, 39 pg/ml, 18 pg/ml, 9 pg/ml, 4.5 pg/ml, 0 pg/ml (only Assay Diluent). These tubes received 300 µl of Assay Diluent each. A serial dilution was performed by transferring 300 µl from the top standard (1:1) to the 1:2 dilution tube (2500 pg/ml) and thorough mixing. Serial dilution was continued by transferring 300 µl from the 2500 pg/ml tube to the 1250 pg/ml tube and so on to the 4.5 pg/ml tube and thorough mixing. The last tube containing only assay diluent served as the 0 pg/ml negative control.

After preparation of standards, individually bottled capture beads were pooled before being mixed with PE detection reagent, standards and samples. For each tube to be analyzed, a 5 µl aliquot per cytokine per tube was added to an additional tube labeled “Capture Bead Mix”. Beads were mixed by pipetting then transferred to the assay tubes (25 µl of mixed Capture beads/tube) as described below.

1. 25 µl of mixed Capture Beads were added to all tubes.
2. 25 µl of Human Inflammation PE Detection Reagent were added all tubes.
3. 25 µl of the Human Inflammation Standard dilutions were added to the standard tubes.
4. 25 µl of each test sample were added to the assay tubes.
5. Tubes were incubated for at least 3 h at RT in the dark.

During this incubation time, setup tubes were prepared (see below).

6. 500 µl of Wash Buffer were added to each assay tube and centrifuged at 200 xg for 5 min.

7. The supernatant from each assay tube was carefully aspirated and discarded.
8. 150 µl of Wash Buffer were added to each assay tube to resuspend the bead pellet.
9. Samples were analyzed in FACS after being vortexed directly before analysis.

Thus, a standard curve for each protein could be constructed, covering a defined set of concentrations from 0-5000 pg/ml. To obtain best results, samples suspected of containing high cytokine levels were diluted. If those did not fit the curve, the mean fluorescence value was extrapolated on the standard curve. Test samples were thawed at RT and added to the analysis tubes. Now, reagents and test samples were transferred to the appropriate assay tubes for analysis. In order to calibrate the flow cytometer and quantify test samples correctly, it was necessary to run the Inflammation Standards and the Cytometer Setup controls in each experiment.

The FACS cytometer was set up using the BD FACS Comp™ software. BD FACS DIVA™ Software was used for analyzing samples and formatting data for subsequent analysis using the BD™ CBA Software. Instrument Setup with the Cytometer Setup Beads was done by employing the setup which had been established in the laboratory previously. Data acquisition was performed with the acquisition template stored previously containing optimized instrument settings. Resolution was set to 1024 in the Acquisition and Storage window. 2000 events were counted in gate R1 (region 1).

Number of events to be collected was set to “all events”. Saving all events collected ensured that no true bead events were lost due to incorrect gating. In setup mode, the first tube was run and using the FSC vs. SSC dot plot, the R1 region gate was placed around the single bead population. Sample acquisition was begun with the flow rate set at ‘Medium’. The negative control tube (0 pg/ml standards) was run before any of the recombinant standard tubes to avoid contamination. Control assay tubes were run before any unknown test assay tubes. After measuring all samples, standard curves were constructed for each analyte.

1.21.2 CBA for human Th1/Th2 cytokines

Analysis of supernatants from T cell cultures was done using the same procedure, but using the BD™ CBA Human Th1/Th2 Cytokine Kit. The kit used tested quantitatively for the following cytokines: interleukin-2 (IL2), interleukin-4 (IL4), Interleukin-5 (IL5), Interleukin-10 (IL10), Tumor Necrosis Factor (TNF) and Interferon-γ (IFNγ) protein levels in a single sample.

The procedure used in this assay is similar to the procedure used for the BD™ CBA Human Inflammation Kit (cf. *1.21*).

1.22 IFNα quantification by ELISA

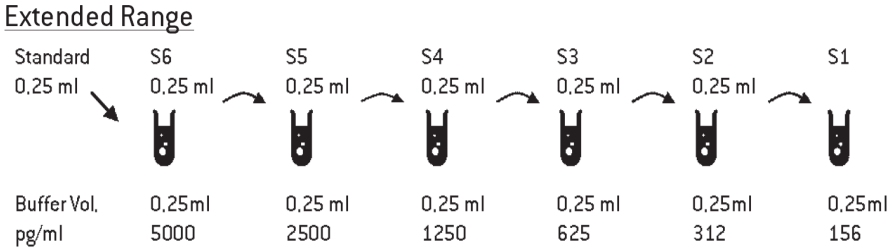
To measure concentrations of IFNα a pre-fabricated ELISA kit was used. This kit quantifies human interferon alpha in supernatants (specific for human IFNα, no cross reactivity with human IFNγ, human IFNβ or human IFNω) using a sandwich immunoassay. The kit is based on an ELISA with anti-secondary antibody conjugated to horseradish peroxidase (HRP). Tetramethyl-benzidine (TMB) is the substrate.

The principle of the test is such that cytokines dissolved in culture supernatant bind to specific antibodies coated to the bottom of the pre-coated ELISA 96 well-plate wells. The plate is then washed carefully. In a second step, a second antibody to which HRP is conjugated binds to the cytokine now immobilized on the bottom of the wells. The plate is washed again to eliminate unbound HRP and thus avoid unspecific reactions. In a third step, the substrate for HRP, TMB is added and incubated in the wells. The horseradish peroxidase catalyses a quantifiable reaction with the tetramethyl-benzidine that is proportional to the amount of cytokine present in the sample and is visible by a color change from blue to yellow in the solution in the wells.

The reaction is stopped with hydrogen peroxide (40%, ‘Stop Solution’). Extent of absorption created by the now converted coloring agent TMB is done by the ELISA reader at a wavelength of 450 nm. A standard curve was established by measuring of double values. Analysis results were evaluated by comparison to the standard curve.

1.22.1 ELISA procedure

The Human IFNα Standard was provided at a concentration of 10.000 pg/ml and diluted in dilution duffer as shown below for the Extended Range (cf. Fig 6). An Extended Range standard curve was also constructed for 0-5000 pg/ml: Six polypropylene tubes (S1-S6) were labeled and filled with Dilution Buffer as indicated. Human IFNα Standard was added to S6 and mixed to establish a standard for 5000 pg/ml. 250 µl was removed from S6 and added to S5 (2500 pg/ml), from this tube 250 µl were added to S4 (1250 pg/ml) and so on via S3 (625 pg/ml) and S2 (312 pg/ml) to S1 (156 pg/ml). The tubes were stored at 4°C until later use.



(Source: PBL Interferon Source, 131 Ethel Road West, Suite 6, Piscataway, NJ 08854, USA)

Fig 6 Dilution for the Extended Range assay

Test samples were thawed and used for duplicate analysis to eliminate variations. Antibody concentrate was diluted with dilution buffer according to lot specific certificate of analysis. Antibody solution was refrigerated until later use.

HRP Conjugate Concentrate was diluted with HRP Conjugate Diluent, according to lot specific certificate of analysis. HRP Conjugate Concentrate was stored at 4°C until later use. In Table 13, the assay procedure is listed as explained below.

1.22.2 Assay procedure

All incubations were performed in a closed chamber at RT using plate sealers to avoid concentration and/or volume change and external influences. In between washing steps, fluid was removed by inverting and blotting on lint-free absorbent paper, tapping dry twice. To wash, wells received 250 µl of diluted wash solution.

First, the number of micro-plate strips required to test the desired number of samples plus the appropriate number of wells needed to run blanks and standards was determined. As explained above, each standard, blank and sample was run in

duplicate. Extra microtiter strips were removed from the frame, sealed in a foil bag and stored at 4°C until later use. Per well, 100 µl of the interferon standards, blanks and samples were added, covered and incubated for 1 h. After incubation, plate contents were discarded and wells were washed once with diluted wash buffer. 100 µl of diluted antibody solution was added to all wells, covered and incubated for 1 h. After incubation, plate contents were discarded and wells were washed three times with diluted wash buffer. 100 µl of diluted HRP solution was added to all wells, covered and incubated for 1 h. During this incubation period, TMB Substrate solution was warmed to RT. After 1 h, plate contents were discarded and wells were washed four times with diluted wash buffer. 100 µl of the TMB Substrate solution was added to each well and incubated for 15 minutes without a plate sealer in the dark. After incubation with TMB 100 µl of Stop solution was added to each well. Using a microplate reader, absorbance was determined at 450 nm within 5 min after addition of Stop solution.

By plotting the optical densities (OD) using a 4-parameter fit for the standard curve, the interferon titer in the samples could be determined. Blank ODs were subtracted from the standards and sample ODs to eliminate background.

Step	Reagent	Volume/ well	Incubation	Wash	Comments
1	Standard and Samples	100 µl	60 min	1X	Include blanks containing Sample Diluent only
2	Diluted Antibody Solution	100 µl	60 min	3X	
3	Diluted HRP Solution	100 µl	60 min	4X	During incubation, **warm TMB to room temp**.
4	TMB Substrate	100 µl	15 min	DO NOT WASH	Incubate in the dark; no plate sealer
5	Stop Solution	100 µl	0 min	DO NOT WASH	Read plate at 450 nm within 5 minutes
6	Read Plate at 450nm				

(Source: PBL InterferonSource, USA)

Table 13 Assay procedure for IFNα-ELISA

1.23 Statistical analysis

Results are displayed as arithmetic mean in a boxplot if n was ≥ 5 and skewness was <1. If number of cases analyzed was <5, median or single values are displayed in a scatter plot. Number of cases refers to numbers of independent experiments conducted.

In most experiments, means of adult and neonatal experiments were compared using the Mann-Whitney-U test for independent samples if skewness was >1, if skewness was <1, a Student-t test was used. To compare paired samples (mean cytokine production by autologous T cells incubated with pre-activated PDC day 0 – day 3 and day 3 – day 5), the paired Student-t test was used, respectively the Mann-Whitney-U test, depending on skewness. P values were calculated as quoted in Results (cf. ??).

Results

In order to study maturation and differentiation of PDC after stimulation with CPG oligonucleotides (CPG ODN) and CD40 ligand (CD40L), the expression of surface molecules and phosphorylation of intracellular markers by PDC, production of cytokines by T cells and T cell division were analyzed. First, PDC were separated from PBMC, used directly or cryopreserved for later use, then stimulated to promote maturation and examined for surface marker expression or intracellular phosphorylation. To evaluate the effect of CPG- and CD40L-stimulated PDC on T cells, T cell division and cytokine production were assessed.

1.24 Effects of freezing

The frequency of PDC in the peripheral blood of human newborns is very low, so that a large sample needs to be taken in order to obtain sufficient PDC numbers for analysis. Cord blood provided the best solution to this problem. However, this also meant that multiple sampling of large quantities was out of question. Thus, in order to obtain T cells for long course experiments, PDC were isolated first then T cells were isolated from the eluted fraction and stored in FCS and DMSO. Although carefully performed, the freezing process in itself, and additionally the thawing conditions represent the major source for damages or ill activation of cells.

1.24.1 Percentage of PDC

In literature, the frequency of PDC among PBMC in neonatal fresh blood is given at around 0.8 %. Our studies concur with these values, showing a frequency of around 0.7 % mean in neonatal fresh blood. In previously frozen samples from neonates, the frequency rose to 1.5 %. A similar phenomenon was noted in samples obtained from adults. Here, the frequency of PDC among PBMC was 1.17 % in fresh blood and 2.68 % in previously frozen samples. Note that total cell counts refer to the number obtained prior to PDC isolation via MACS, not prior to freezing.

	Frozen PBMC samples	Fresh PBMC samples
Adult blood (AB)	2.68 %	1.17 %
Cord blood (CB)	1.5 %	0.7 %

Table 14 Percentage of PDC obtained from total PBMC numbers after isolation via MACS

1.25 Co-stimulatory and differentiation markers

The frequency of PDC among PBMC was not the only difference between fresh and frozen samples. During the establishing phase of the experiments, when comparing frozen and fresh samples, differing expression of co-stimulatory and differentiation markers was noted on unstimulated fresh and frozen cells.

The following graphs each refer to a distinct surface marker. The influence of the different stimulatory agents on surface marker expression is shown separately in the different graphs so that each graph shows adult and neonatal PDC in comparison with their respective columns or boxplots representing distinct stimulatory conditions.

1.25.1 Cryopreserved samples

Frozen PDC were better activated by CPG A and B than fresh PDC. Also, cord blood PDC seemed to tolerate freezing better than adult PDC when it comes to marker expression.

CD40 expression however was lower in cryopreserved neonate than in cryopreserved adult PDC, consistent with results in fresh PDC.

Fig 7 shows the results of measuring the dendritic cell maturation marker CD83 and the co-stimulatory marker CD86 on cryopreserved PDC**.**

CPG B upregulated CD83 and CD86 expression in cryopreserved PDC, with neonate donors showing a slightly higher baseline in some cases and CPG B providing a more effective stimulation than CPG A.

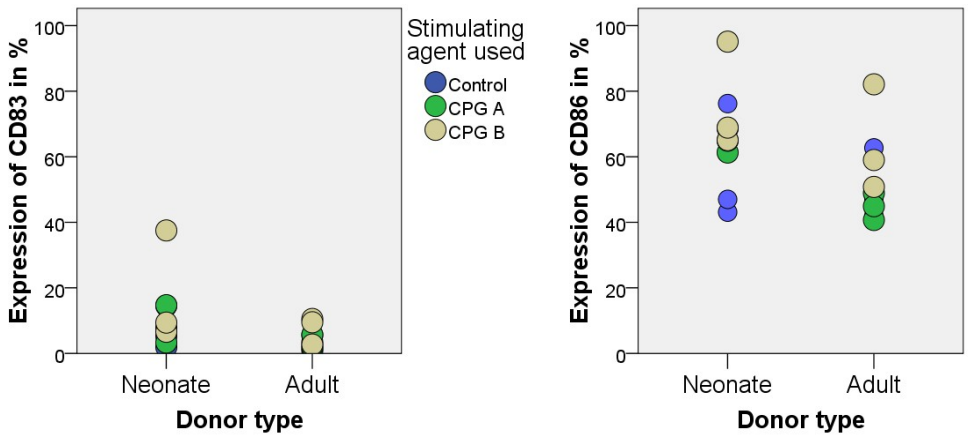


Fig 7 Frozen PDC: Expression of CD83 or CD86
after no stimulation (control), stimulation with CPG A and CPG B (n=3).

This effect was even more pronounced when combined CD83 and CD86 upregulation was measured and more noticeable in cord blood. Upregulation was more pronounced in samples from neonates. When only the percentage of cells expressing high levels of CD86 was monitored, **neonatal PDC seemed to be more sensitive to CPG B stimulation than adult PDC.**

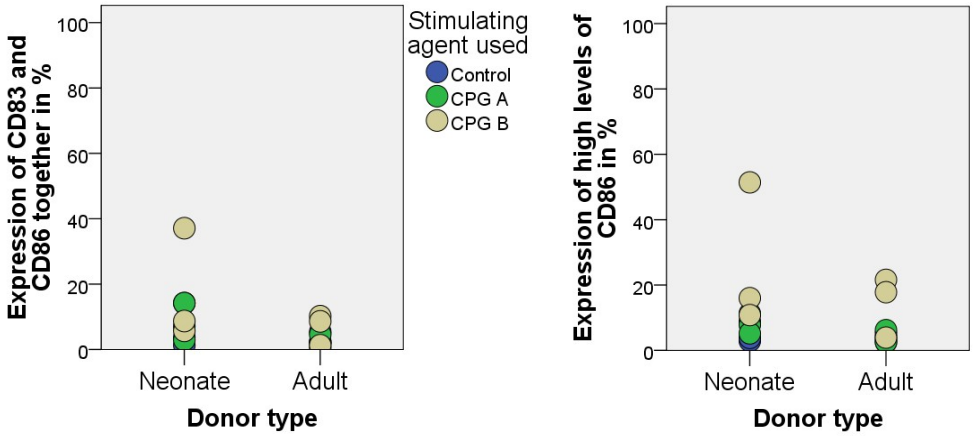


Fig 8 Frozen PDC: Expression of combined CD83 and CD86 and high CD86
expression on all gated PDC after no stimulation (control),stimulation with CPG A and CPG B (n=3).

Two more proteins were measured after stimulation, one of them co-stimulatory protein CD40. Unlike with CD83 and CD86, **similarly increased expression of CD40 could be observed after stimulation both with CPG A and B in neonates** while little to no stimulatory effect was observed in adult PDC. CD80 on the other

hand showed a slight, consistent rise in expression, most likely due to the effects of CPG B.

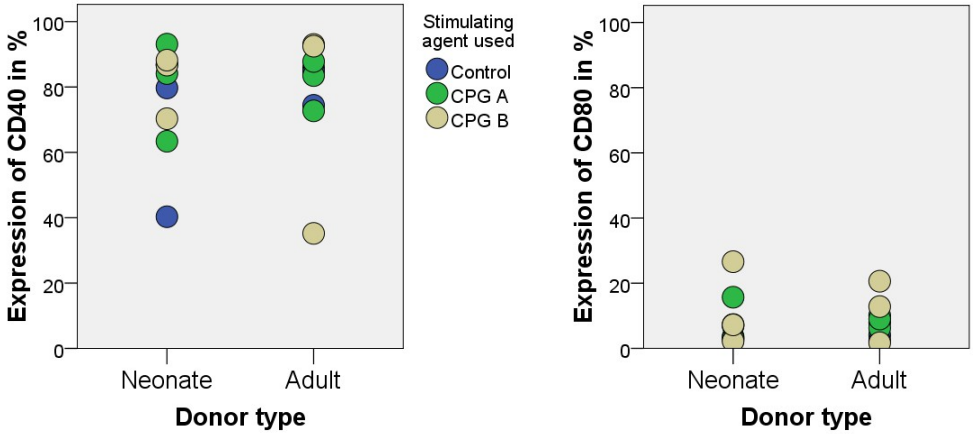


Fig 9 Frozen PDC: Expression of CD40 and CD80
on all gated PDC after no stimulation (control), stimulation with CPG A and CPG B (n=3).

The combined measuring of CD40 and CD80 did not obtain clearer results, as shown in Fig 10.

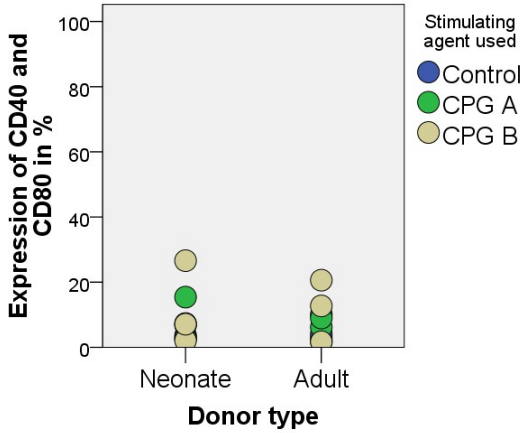


Fig 10 Frozen PDC: Expression of combined CD40 and CD80
after no stimulation (control), stimulation with CPG A and CPG B (n=3).

1.25.2 Fresh samples

The aim of this study was, however, not the functional analysis of cryopreserved PDC but of fresh blood PDC. These, among other antigen-presenting cells, constitute one of main targets in today's research on the initiation of the immune response. Consistent over nearly all measured cell surface parameters, CPG B provided more effective stimulation compared to stimulation with CPG A and control studies.

As in experiments on cryopreserved PDC, **neonatal PDC showed similar upregulation of CD83 compared to adults upon CPG B stimulation**, **while upregulation of CD83 in response to CPG A was slightly lower.** When looking at CD86 expression on fresh PDC, it could be seen that cord blood PDC did not react as strongly to stimulation with CPG B as adult PDC and were also less susceptible to CPG A stimulation, although **CD86 did rise in neonatal PDC after CPG B stimulation.**

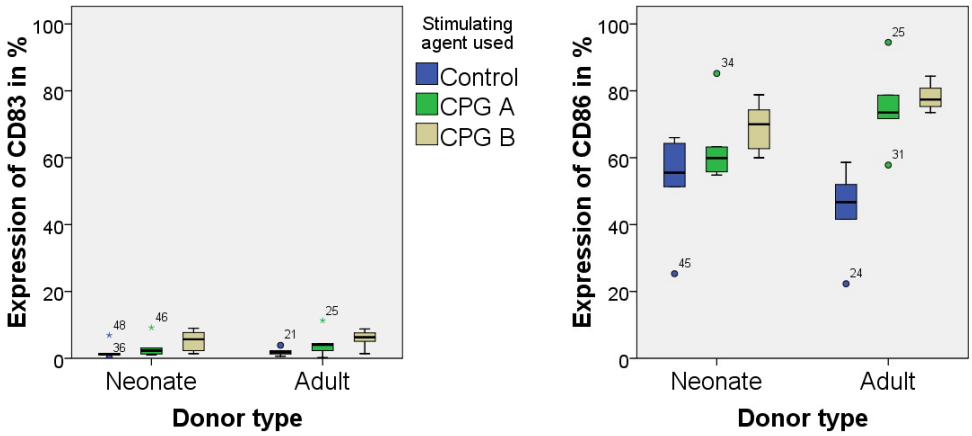


Fig 11 Fresh PDC: Expression of CD83 and CD86
after no stimulation (control), stimulation with CPG A and CPG B (n=5).
Value labels refer to donor to identify outliers.

As shown in single CD83 and CD86 analysis, combined CD83 and CD86 analysis showed that cord blood PDC were less prone to be activated by CPG A, and were only slightly more receptive to CPG B. In contrast to cryopreserved PDC, it seemed that **high CD86 expression upon stimulation was more common in adult PDC than in neonatal PDC** while CPG A even proved to be slightly more stimulatory in neonates than CPG B.

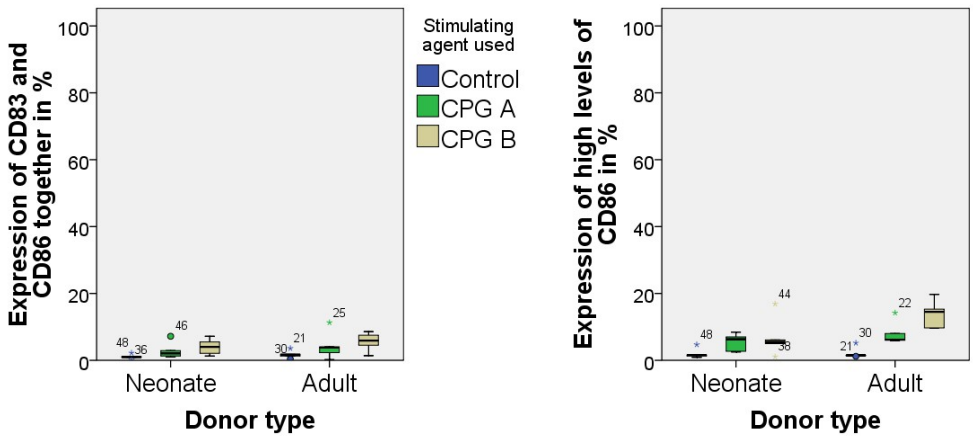


Fig 12 Fresh PDC: Expression of combined CD83 and CD86 and high CD86 after no stimulation (control), stimulation with CPG A and CPG B (n=5). Value labels refer to donor to identify outliers.

When looking at CD40 expression, similar results as in high CD86 expression could be observed: **CD40 expression upon stimulation was higher in adult PDC** than in neonatal PDC while **CPG A even proved a more potent stimulator than CPG B both in adults and neonates. For both CPG A and CPG B stimulation, a difference in CD40 expression could be shown (CPG A: p=0.000; CPG B: p=0.006; t-test).** CD80 showed, as in cryopreserved PDC a slight, consistent rise in expression, most likely due to the effects of CPG B with cord blood PDC showing a higher baseline.

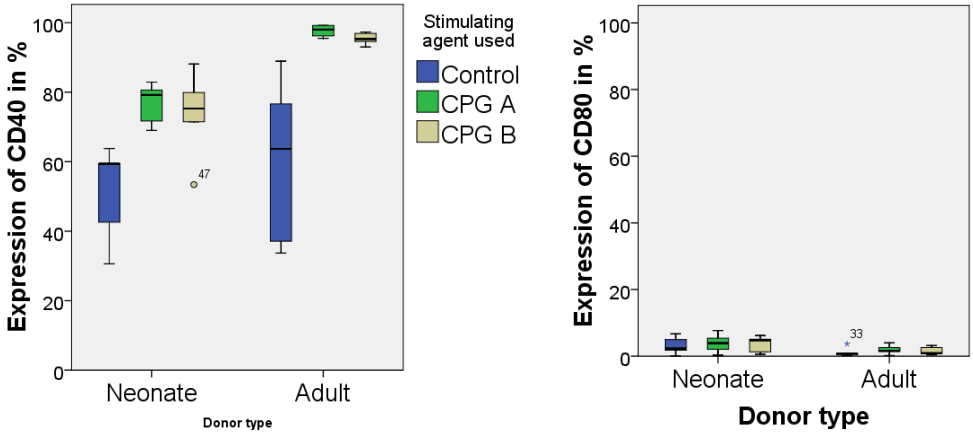


Fig 13 Fresh PDC: Expression of CD40 and CD80 after no stimulation (control), stimulation with CPG A and CPG B (n=5). Value labels refer to donor to identify outliers.

When we combined observation of CD40 and CD80, CPG A became a slightly more potent stimulatory agent in cord blood PDC, due to its effect on the expression of CD40. The effect on combined upregulation was however low because of low baseline expression and low stimulatory capacity of CPG for CD80.

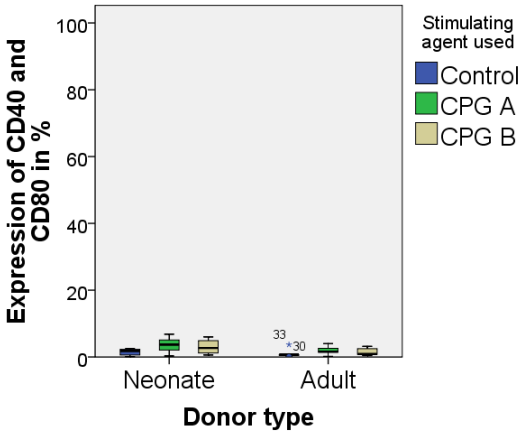


Fig 14 Fresh PDC: Expression of combined CD40 and CD80 after no stimulation (control), stimulation with CPG A and CPG B (n=5). Value labels refer to donor to identify outliers.

1.26 IFNα production

CPG A improved IFNα production in adult PDC much more than in cord blood PDC which furthermore showed a lower production capacity after cryopreservation.

Based on the observation that CPG stimulation could increase PDC co-stimulatory and maturation marker expression, we were interested in the effect of CPG stimulation on PDC cytokine production.
In particular, we were interested in IFNα, the key interferon in immune response to viral infection.

The CPG-induced production of IFNα was particularly stimulated by CPG A, which is contrary to the observation that CPG B was a more powerful stimulator of overall maturation and activation of PDC. A statistic difference in IFNα production by cord blood and adult PDC could be shown for CPG B (cryopreserved PDC: p=0.046; fresh PDC: p=0.047; Mann-Whitney U-Test). Cryopreserved cord blood PDC seemed to produce less IFNα than fresh cord blood PDC upon CPG A stimulation.

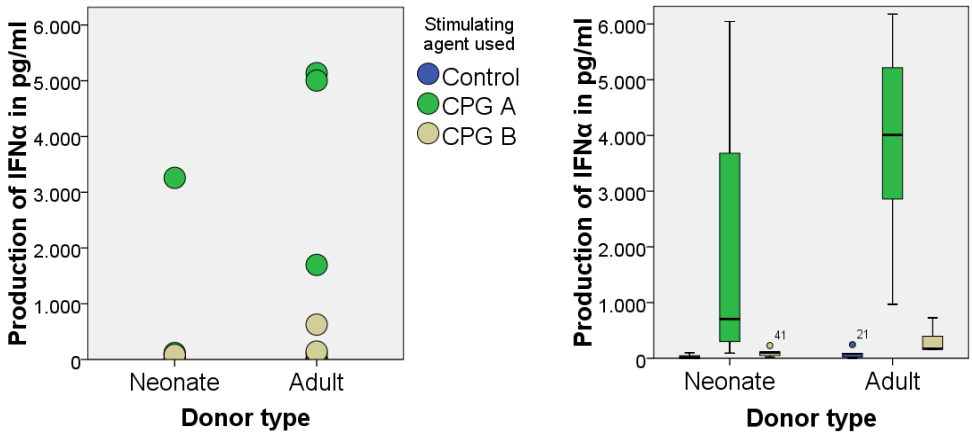


Fig 15 Production of IFNα
in pg/ml by cryopreserved PDC (left graph, n=3) and fresh PDC (right graph, n=5)
and after no stimulation (control), stimulation with CPG A and CPG B.
Value labels refer to donor to identify outliers.
IFNα levels were obtained via conventional ELISA.

1.27 Cytokine production by activated PDC

In flow cytometric experiments and IFNα ELISA, it was proven that CPG A and B did indeed induce maturation and co-stimulatory activation in PDC, as shown by CD expression profiles and IFNα production. Nonetheless, a large part of PDC function is the production of immune system-activating ("inflammatory") cytokines. These were measured by cytometric bead assay.

As in cell-surface marker expression experiments, notable differences between adult and cord blood PDC as well as between cryopreserved and fresh PDC could be detected.

1.27.1 Cryopreserved samples

Interferon and interleukin production was virtually non-existent in cryopreserved neonatal PDC, while adult PDC donors reacted to CPG A and B stimulation with some IL12p70 production, mixed TNF and IL1β production. Only CPG B stimulation of adult PDC led to a noticeable IL12p70, IL6 and IL8 production. In the case of IL10 CPG stimulation down-regulated interleukin production in adult donors. Only one neonate PDC donor produced IL8.

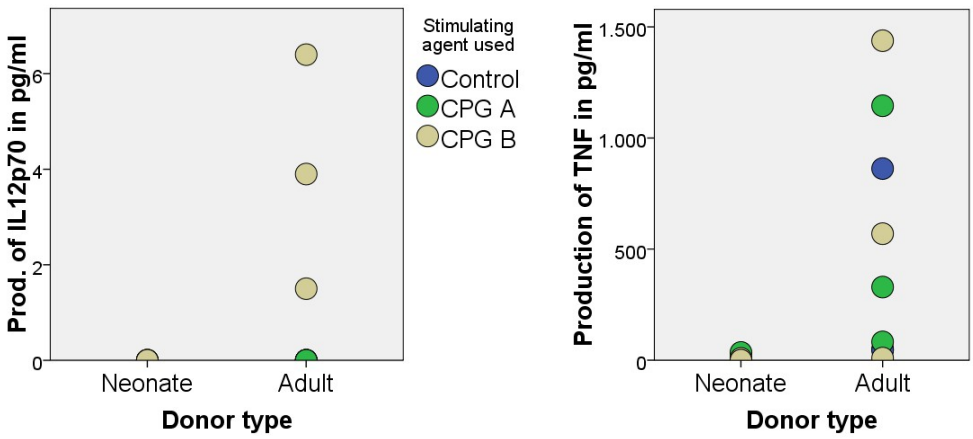


Fig 16 Frozen PDC: Production of IL12p70 and TNF
in pg/ml after no stimulation (control), stimulation with CPG A and CPG B (n=3)

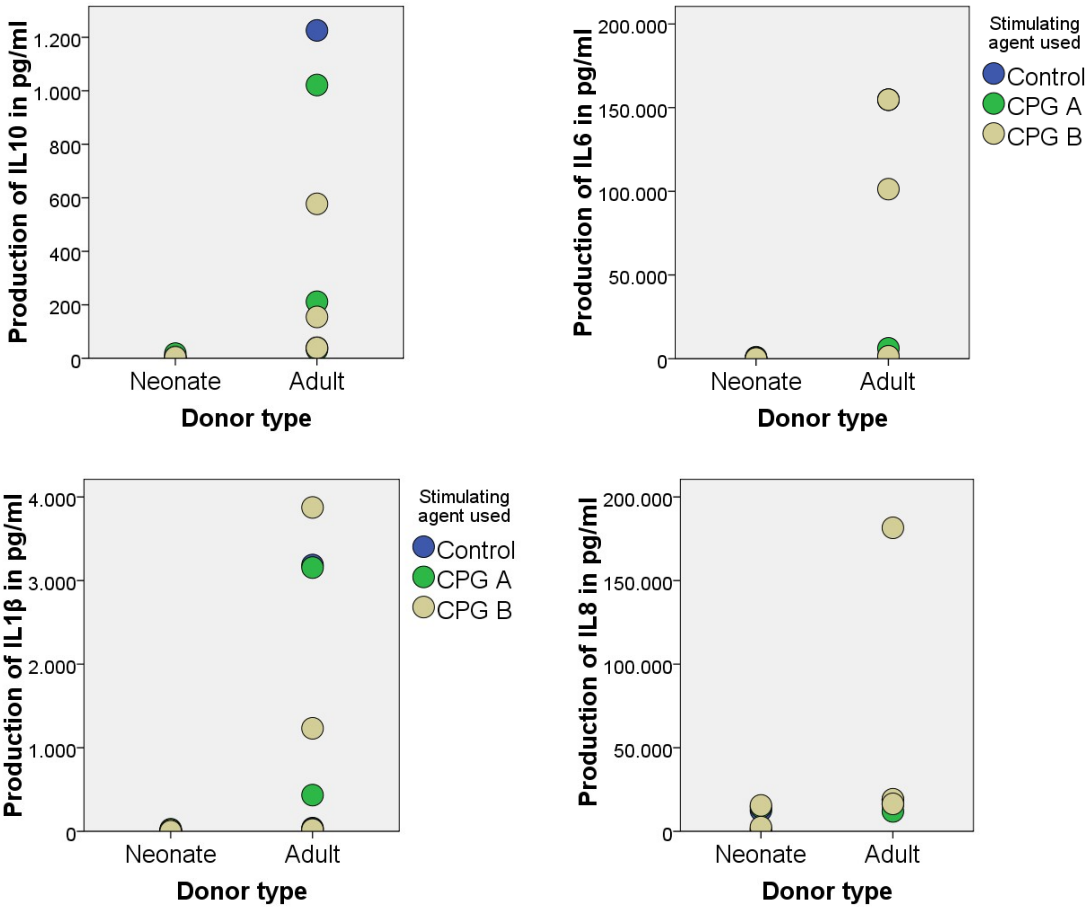


Fig 17 Frozen PDC: Production of IL10, IL6, IL1β and IL8
in pg/ml after no stimulation (control), stimulation with CPG A and CPG B (n=3)

1.27.2 Fresh samples

Fresh PDC were wholly incapable of producing IL12p70 (data not shown), unlike the adult cryopreserved PDC. TNF, on the other hand, was freely produced by adults upon CPG A stimulation, much like IFNα (cf. 1.26). CPG A was the best inducer of **TNF production** in adults (median 171 pg/ml vs. median 41 pg/ml for CPG B and 3 pg/ml for controls), while **in neonates, neither CPG A nor CPG B (median 12 resp. 14 pg/ml) surmounted the control group (median 3 pg/ml) by much.** MWU testing however showed no significant differences.

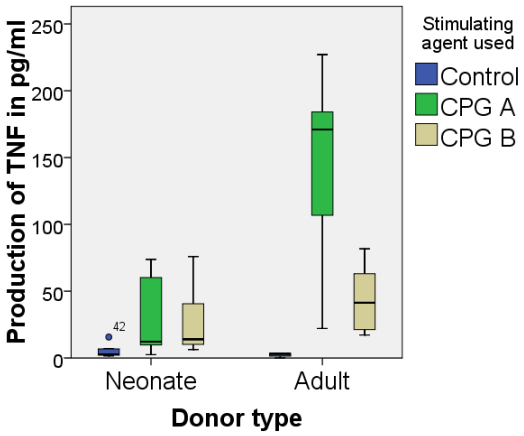


Fig 18 Fresh PDC: Production of TNF in pg/ml after no stimulation (control), stimulation with CPG A and CPG B. Value labels refer to donor to identify outliers. (n=6)

IL10 and IL6 analysis provided a surprise, however: **In neonatal PDC, CPG B triggered an increase of IL10 production** (median 20 pg/ml) while CPG A had little effect here (median 4 pg/ml, median of control group 0 pg/ml). In adult PDC CPG A and B caused similar IL10 responses (CPG A: median 3, maximum 41 pg/ml; CPG B median 3 pg/ml, maximum 126 pg/ml; control group 2 mg/ml). Looking at **IL6 production**, it could be demonstrated that **CPG A and B triggered similar amounts of IL6 both in neonates** (control group median 144 pg/ml, CPG A median 217 pg/ml, CPG B median 224 pg/ml) **and adults** (control group median 81 pg/ml, CPG A median 170 pg/ml, CPG B median 86 pg/ml). MWU testing did not reveal significant differences between adults and neonates.

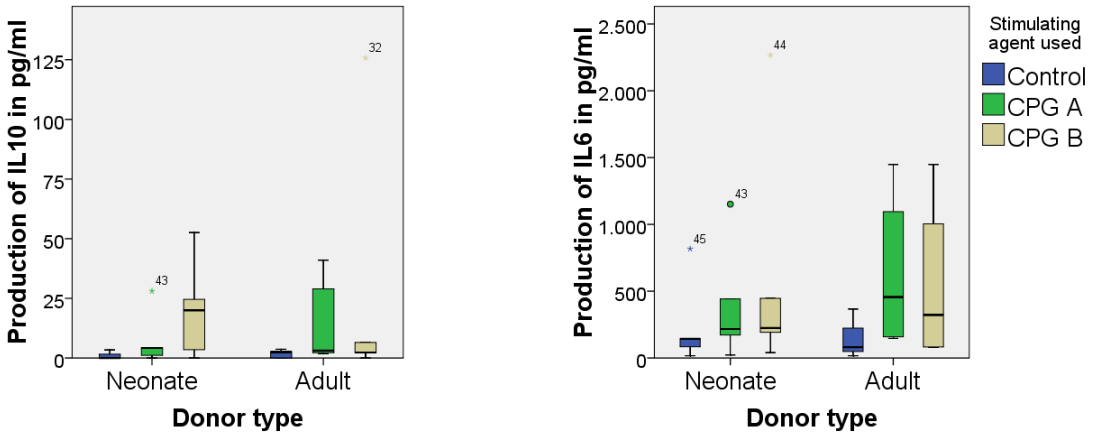


Fig 19 Fresh PDC: Production of IL10 and IL6
in pg/ml after no stimulation (control), stimulation with CPG A and CPG B.
Value labels refer to donor to identify outliers. n=6

IL1β production was not increased by CPG B stimulation in adult PDC (median 11 pg/ml) when compared to CPG A stimulation (median 12 pg/ml, control group 9 pg/ml) while **no marked difference could be noted in neonates** (control group median 5 pg/ml, CPG A median 6 pg/ml, CPG B median 8 pg/ml). **CPG stimulation did not increase IL8 production** (adults: control group median 6225 pg/ml, CPG A median 6453 pg/ml, CPG B median 5951 pg/ml, neonates: control group median 8301 pg/ml, CPG A median 8683 pg/ml, CPG B median 9145 pg/ml). Neonatal PDC showed a higher IL8 baseline than adults. MWU tests showed no significant differences between adults and neonates regarding IL1β and IL8 production.

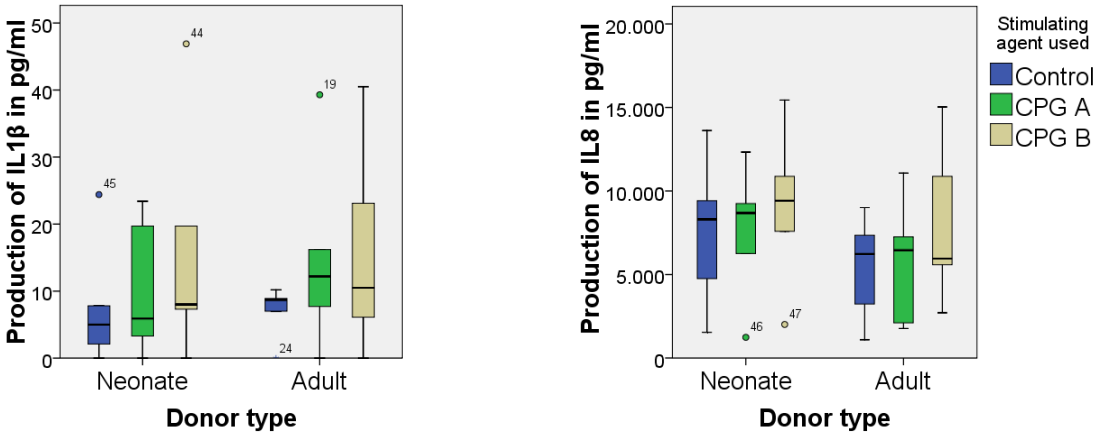


Fig 20 Fresh PDC: Production of IL1β and IL8
in pg/ml after no stimulation (control), stimulation with CPG A and CPG B.
Value labels refer to donor to identify outliers. (n=6)

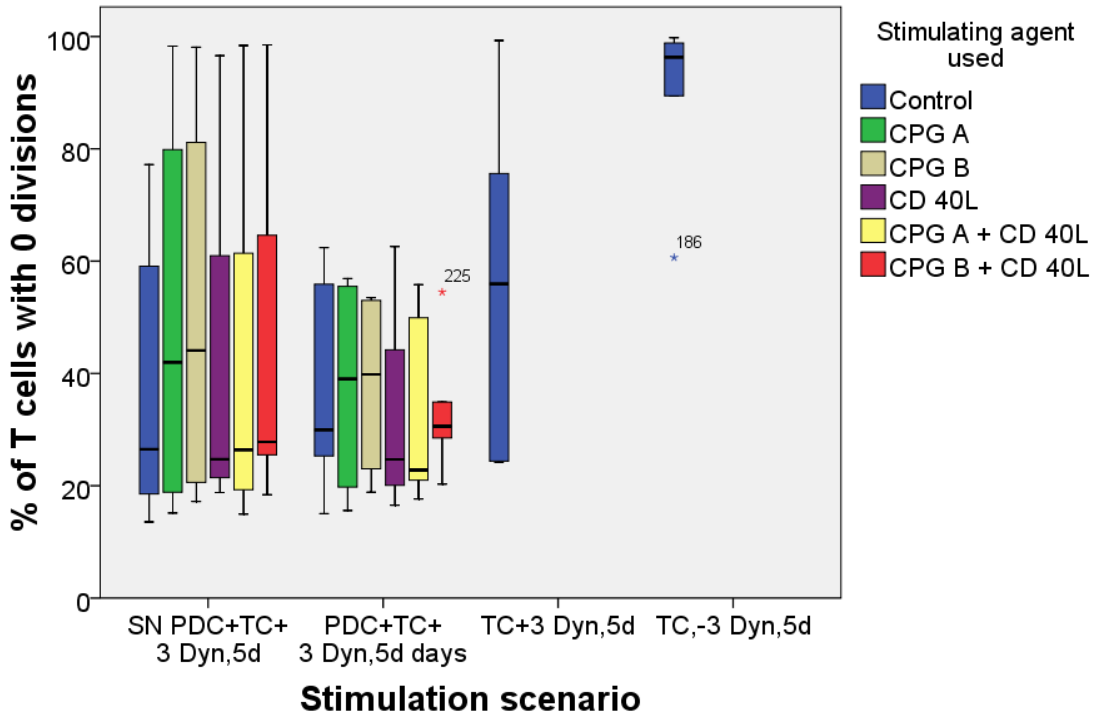
1.28 T cell division stimulated by activated PDC

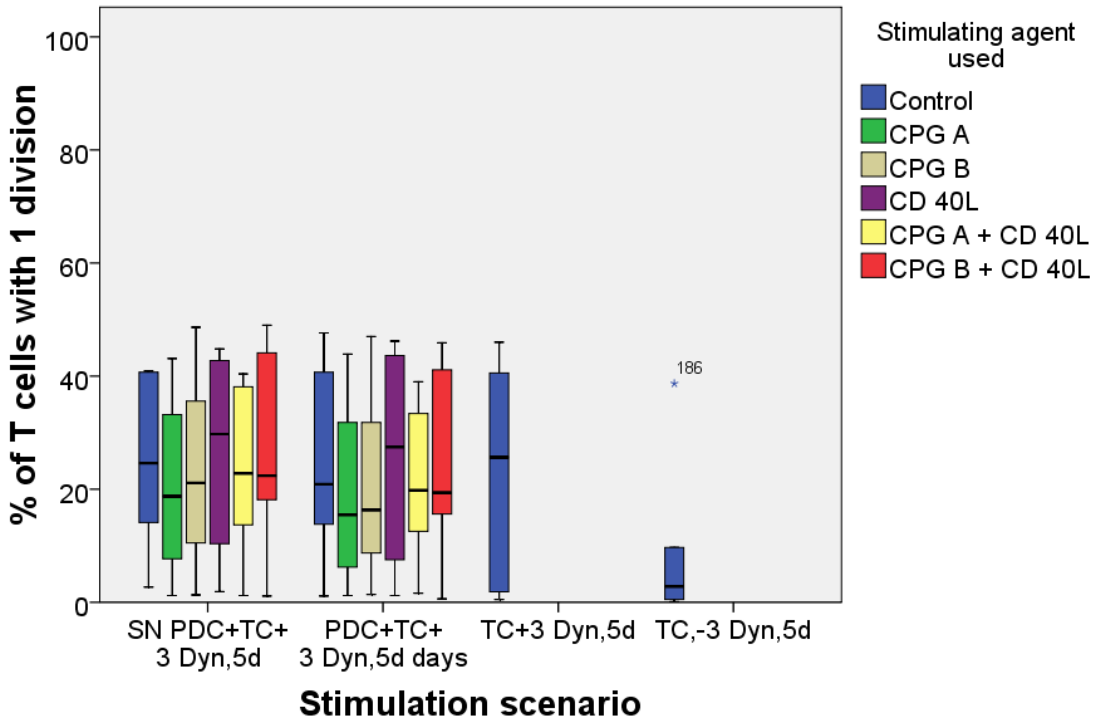
After it had been proven that maturation, co-stimulatory activation and cytokine production in PDC could be induced by CPG A and CPG B; we wanted to test adult and neonatal PDC for their capacity to stimulate T cell proliferation. This attribute of PDC is one of their most important functions and showcases their mediating role between the innate and the adaptive immune system.

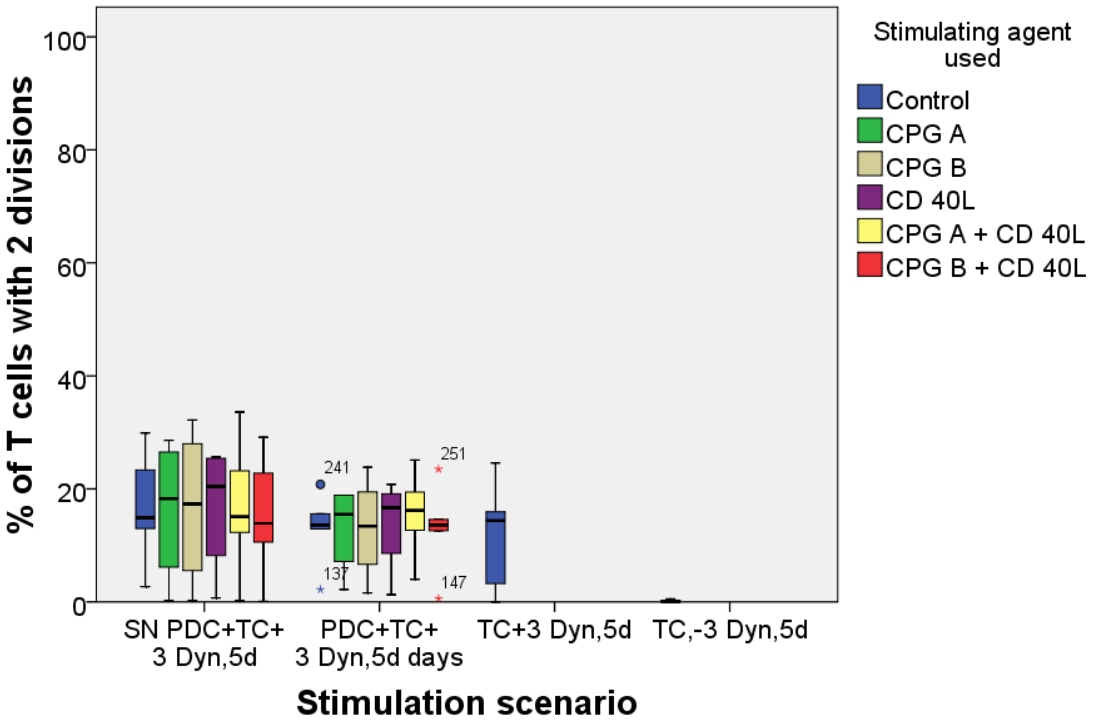
In order to measure T cell activation we chose to focus on T cell division with CFSE dye functioning as a division marker. After CFSE uptake, the dye content is shared between cells upon division in equal measure so that total dye content per cell diminishes with each division. Thus, a certain dye level per cell (comprised in a “gate” in FACS analysis) defines the number of divisions the cell has gone through. We measured first the number of divisions by how many percent of the parent population (the population with one division less than the one gated) appeared in each CFSE dye level gate and second the intensity of fluorescence in each gate.

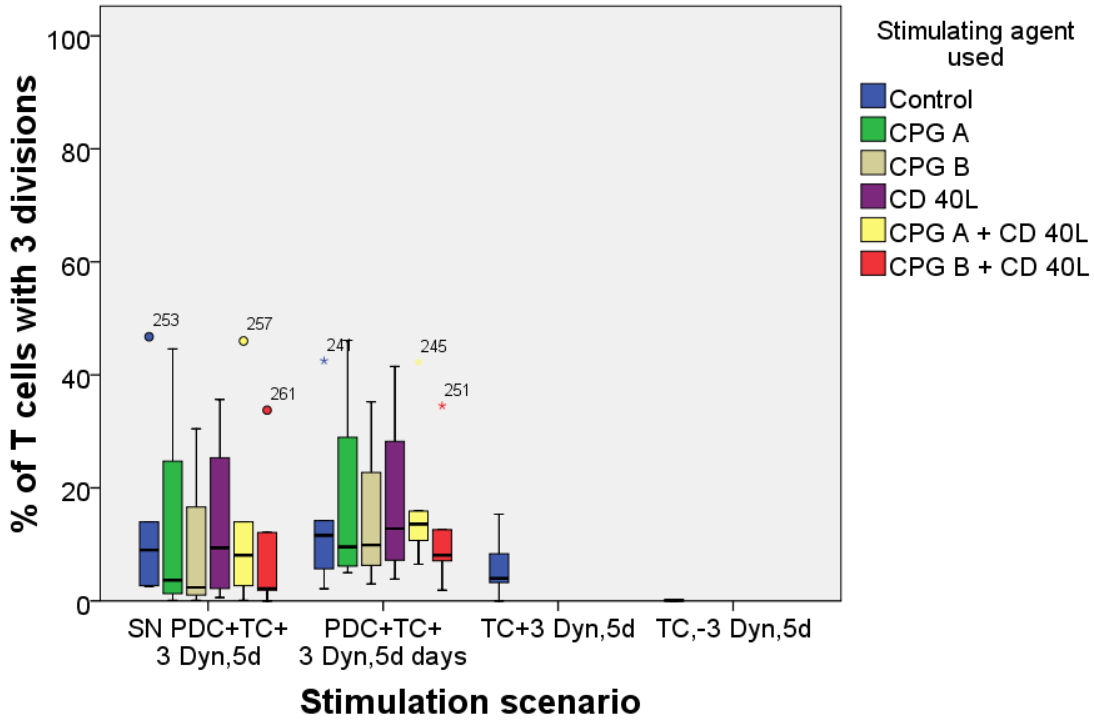
1.28.1 Calibration

To promote T cell activation, CD3/CD28-stimulating beads (Dynabeads) were added in the experiment. A test run with adult cryopreserved T cells stimulated with autologous activated PDC with 3 Dynabeads added to each T cell produced such enormous stimulation that no significant differences in T cell division rate between the different PDC stimuli vs. the control group could be detected, as shown in Fig 21.


% of T cells with 0 divisions
100
80
60
40
20
0
186
225
SN PDC+TC+ 3 Dyn,5d
PDC+TC+ 3 Dyn,5d days
TC+3 Dyn,5d
TC,-3 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


% of T cells with 1 division
100
80
60
40
20
0
186
SN PDC+TC+ 3 Dyn,5d
PDC+TC+ 3 Dyn,5d days
TC+3 Dyn,5d
TC,-3 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


% of T cells with 2 divisions
100
80
60
40
20
0
241
251
137
147
SN PDC+TC+ 3 Dyn,5d
PDC+TC+ 3 Dyn,5d days
TC+3 Dyn,5d
TC,-3 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


% of T cells with 3 divisions
100
80
60
40
20
0
253
257
261
241
245
251
SN PDC+TC+ 3 Dyn,5d
PDC+TC+ 3 Dyn,5d days
TC+3 Dyn,5d
TC,-3 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L

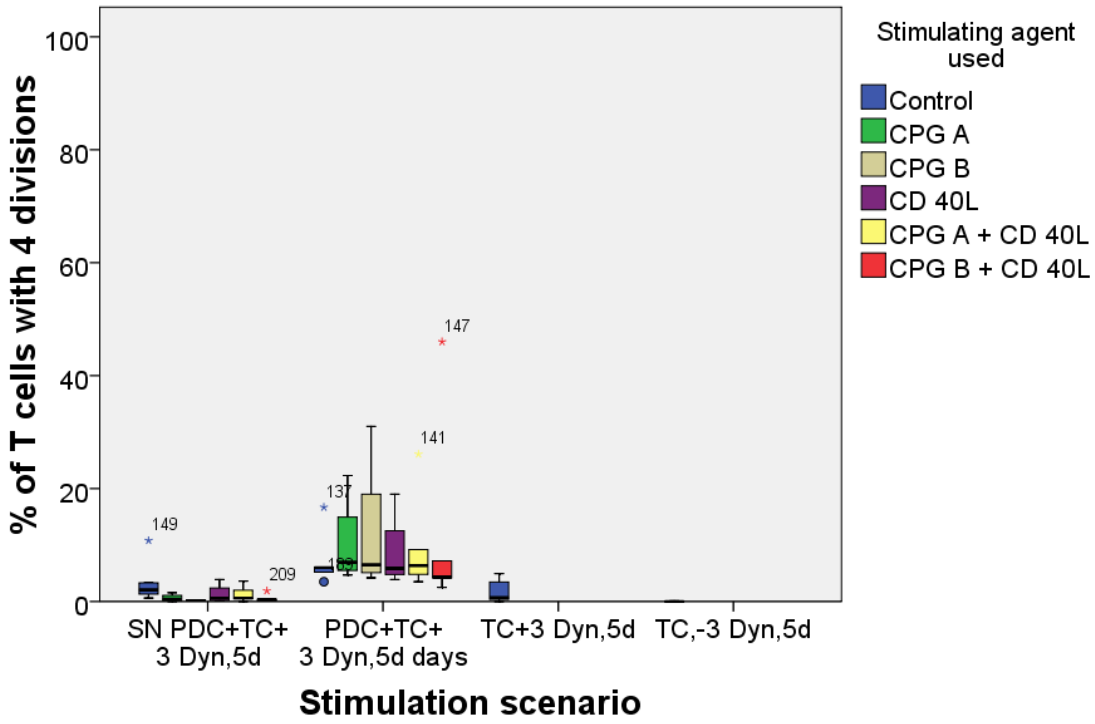


Fig 21 Division of autologous T cells stimulated by PDC - calibration after no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. SN PDC = Supernatant of activated PDC, TC = cells, Dyn = number of Dynabeads/T cell, 5 d = 5 days of co-incubation. Value labels refer to donor to identify outliers. (n=5)

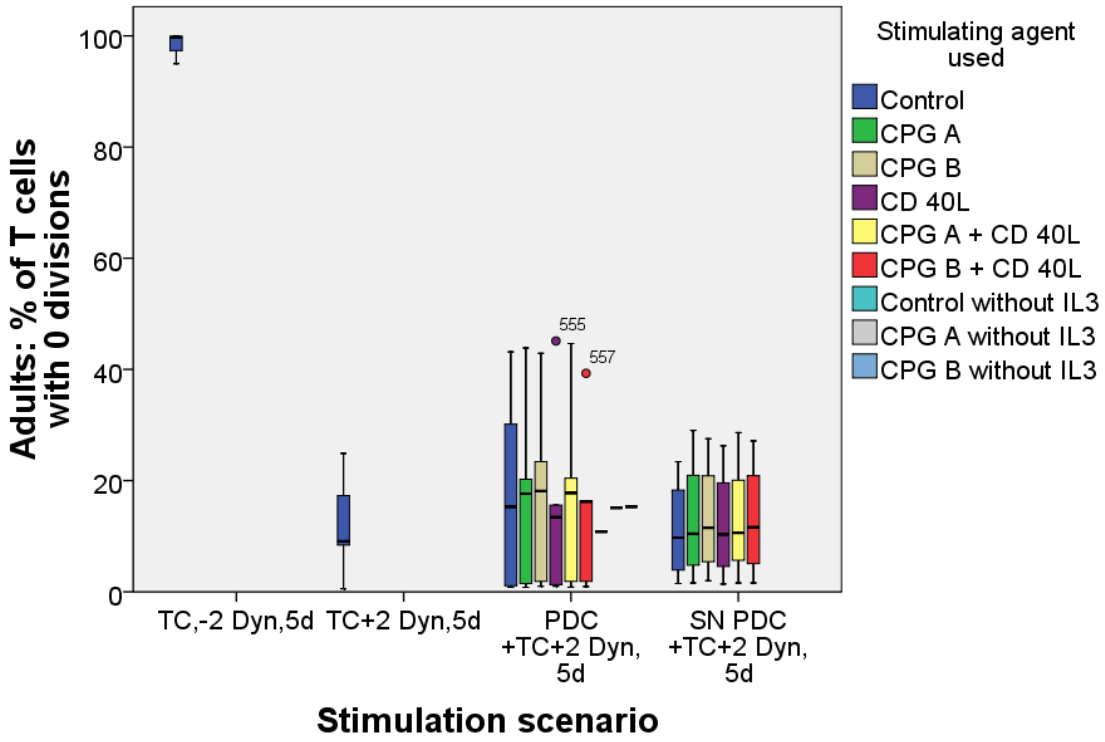
As these graphs show, adding too many Dynabeads per T cell (in our case 3 beads per cell) over-stimulated them. This masked the effect that adding unstimulated or stimulated PDC or their supernatant has on the T cells.

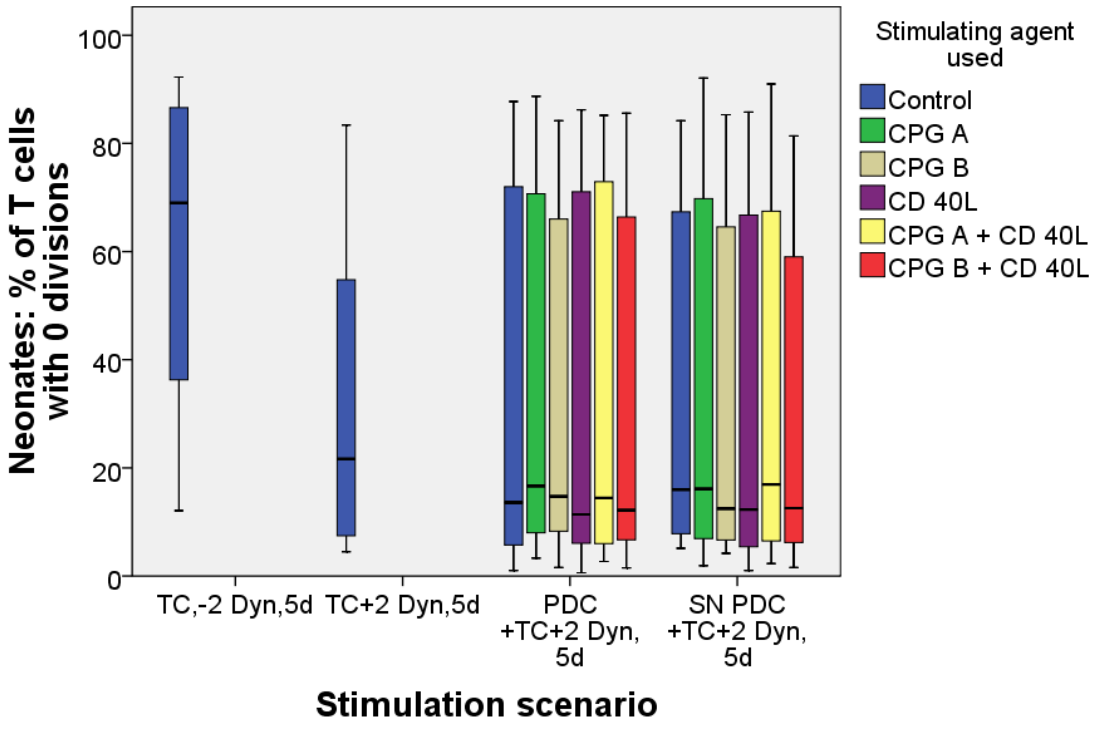
MFI analysis of these overstimulated T cells did not yield different results and is therefore not shown.

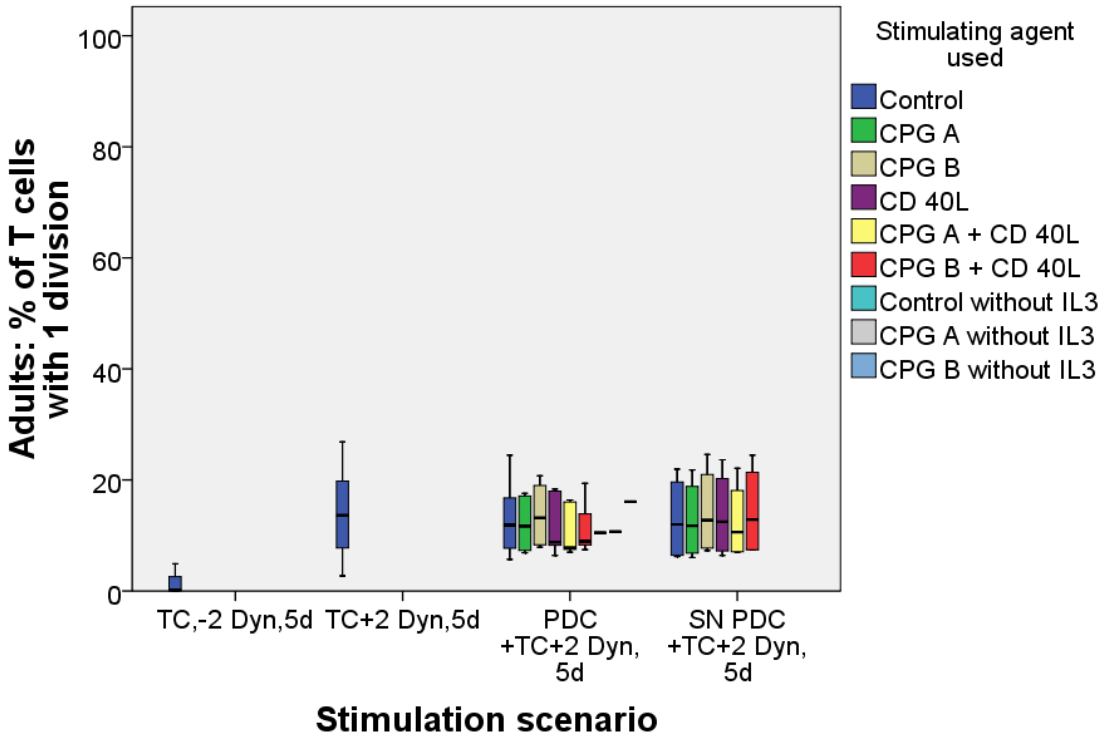
1.28.2 T cell division study

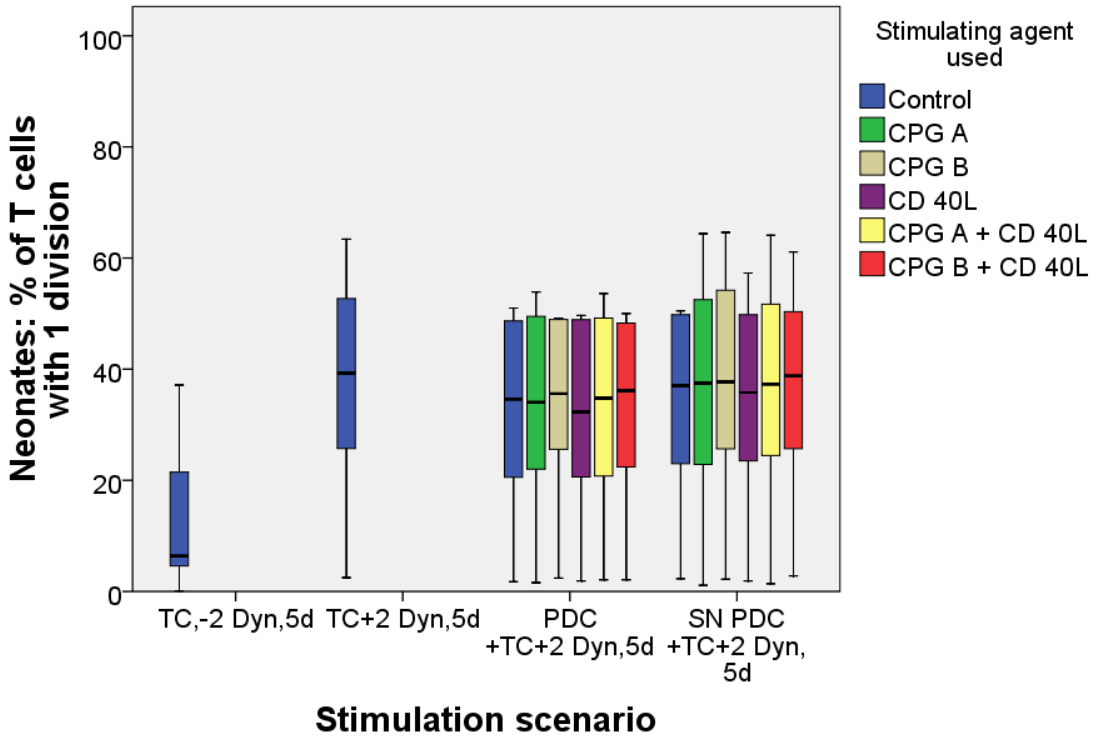
With the unspecific T cell division results from the test run with adult T cells at hand, we decided to reduce the numbers of beads to 2 Dynabeads per T cell so as not to overshadow the effect activated PDC have on T cell division. We also wanted to analyze if direct contact between PDC and T cells was needed for T cell division or if the cytokine micro-environment created by activated PDC was sufficient for T cell division.

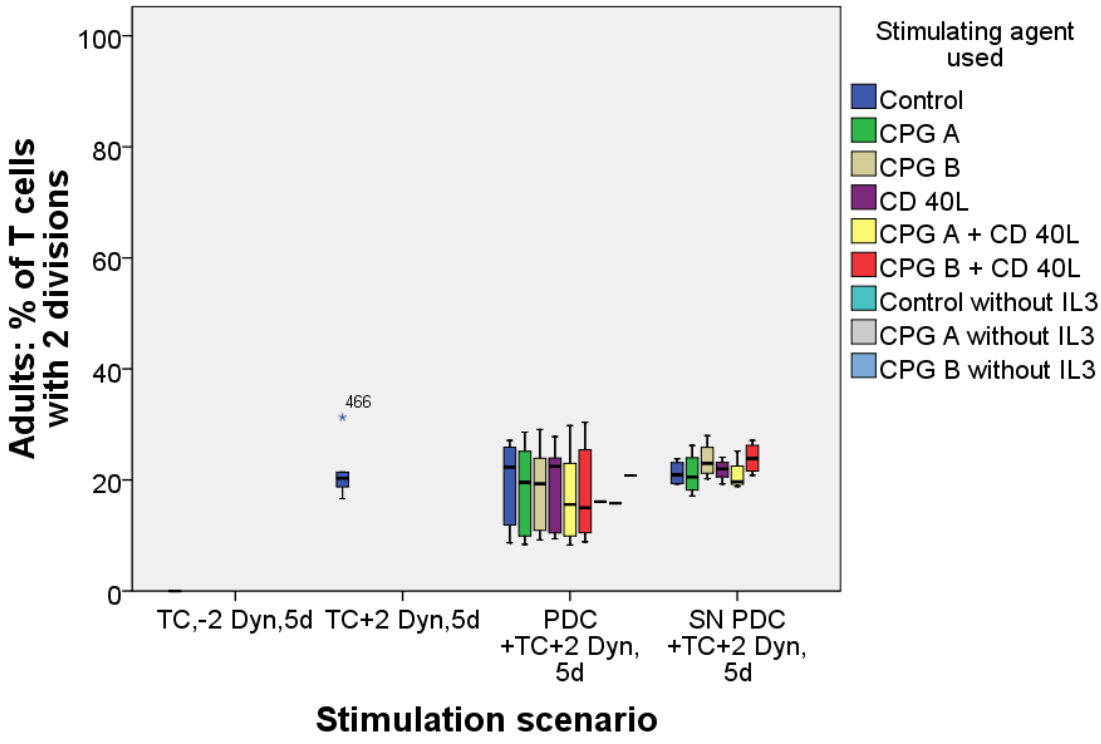
In order to achieve this end, either supernatant of unstimulated controls or PDC activated by CPG A, CPG B, CD40L and combinations of these three stimulants or just the PDC were added to autologous T cells. The results are shown in Fig 22. Due to a pipetting error, 3 adult T cell wells were incubated without the T cell-life prolonging IL3; therefore the results are listed in separate boxplots with the median bar representing the value obtained (bright blue, grey and light blue color codes from left to right).

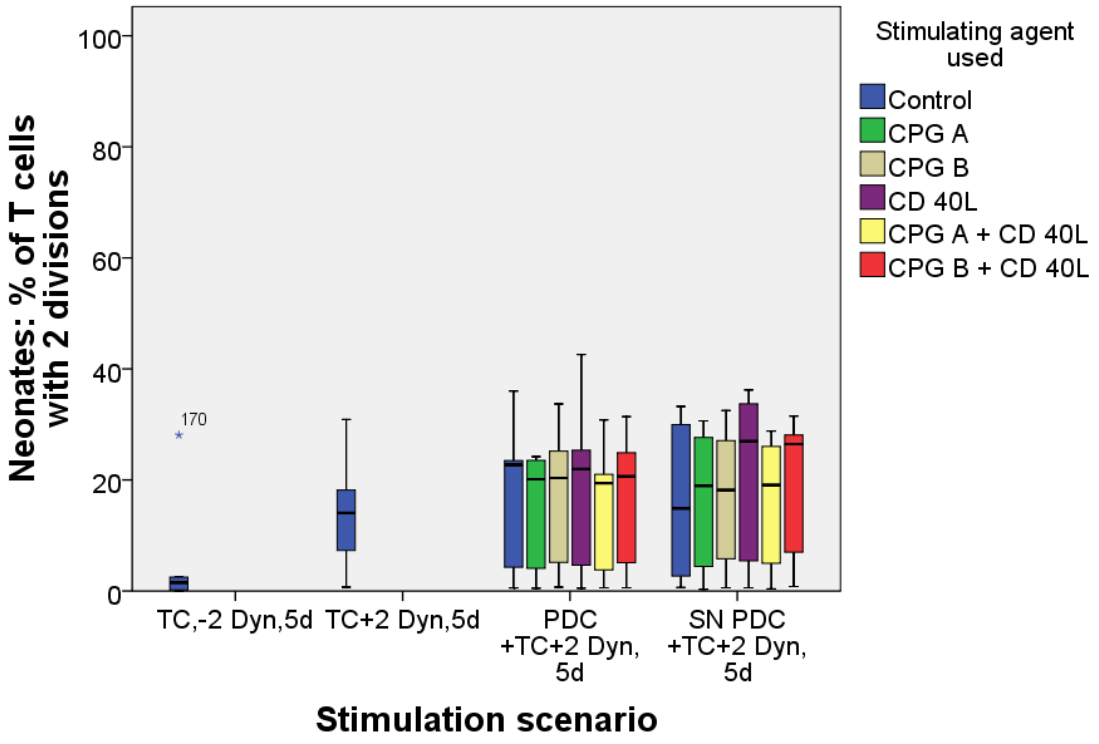


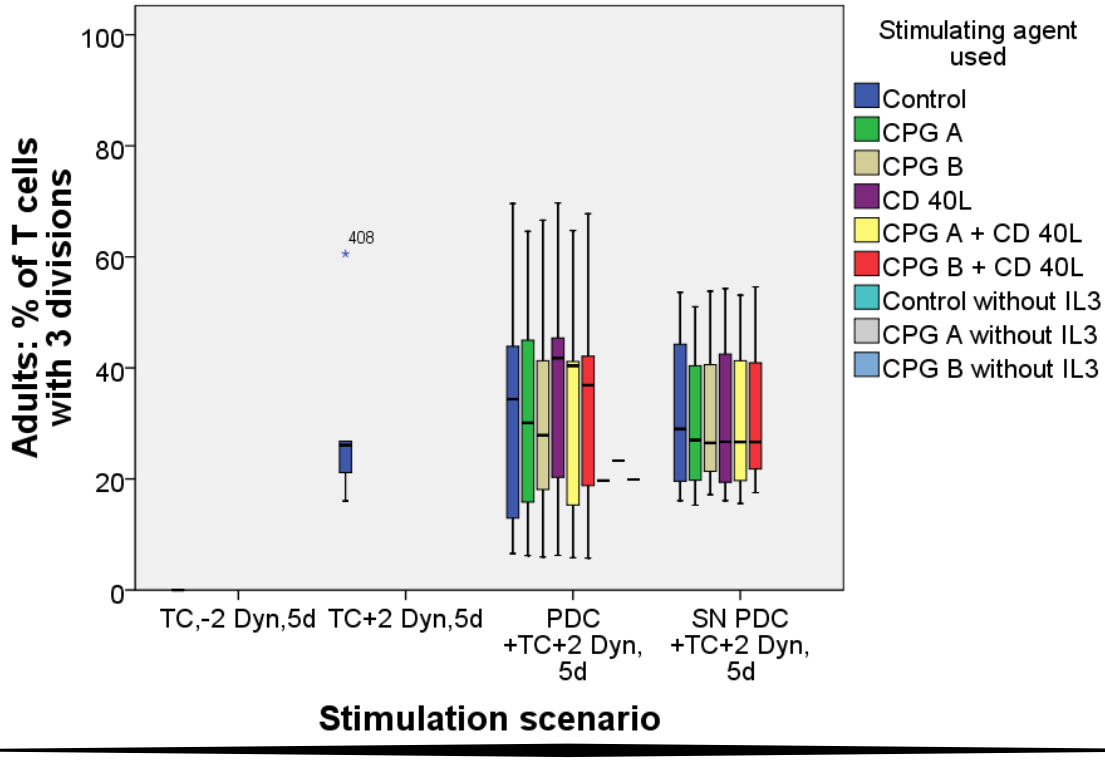

Neonates: % of T cells with 0 divisions
100
80
60
40
20
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L

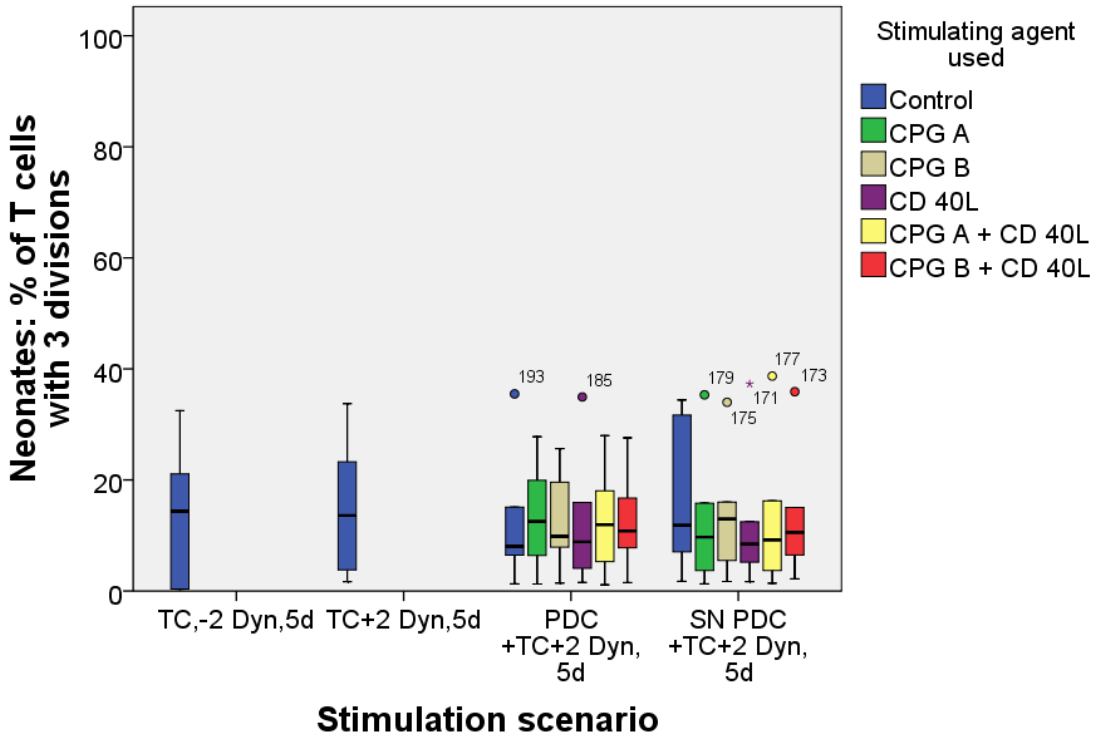

Adults: % of T cells with 1 division
100
80
60
40
20
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3

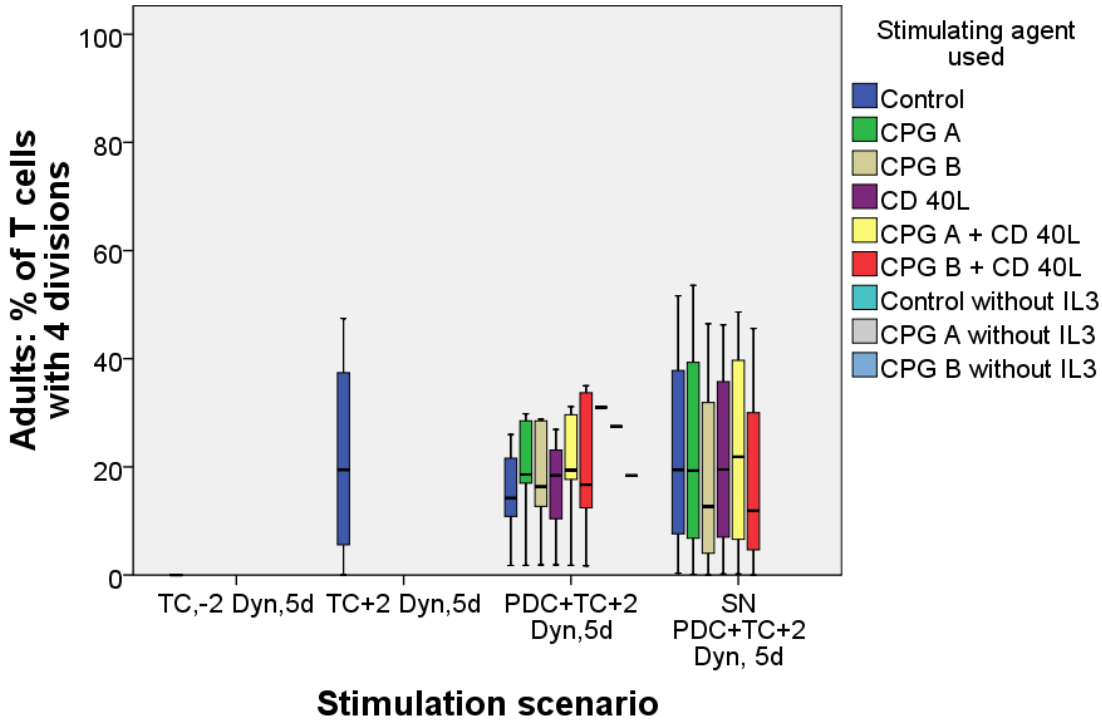

Neonates: % of T cells with 1 division
100
80
60
40
20
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


Adults: % of T cells with 2 divisions
100
80
60
40
20
0
466
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3


Neonates: % of T cells with 2 divisions
100
80
60
40
20
0
170
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


Adults: % of T cells with 3 divisions
100
80
60
40
20
0
408
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3


Neonates: % of T cells with 3 divisions
100
80
60
40
20
0
193
185
179
177
173
171
175
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn, 5d
SN PDC +TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


Adults: % of T cells with 4 divisions
100
80
60
40
20
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC+TC+2 Dyn,5d
SN PDC+TC+2 Dyn, 5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3

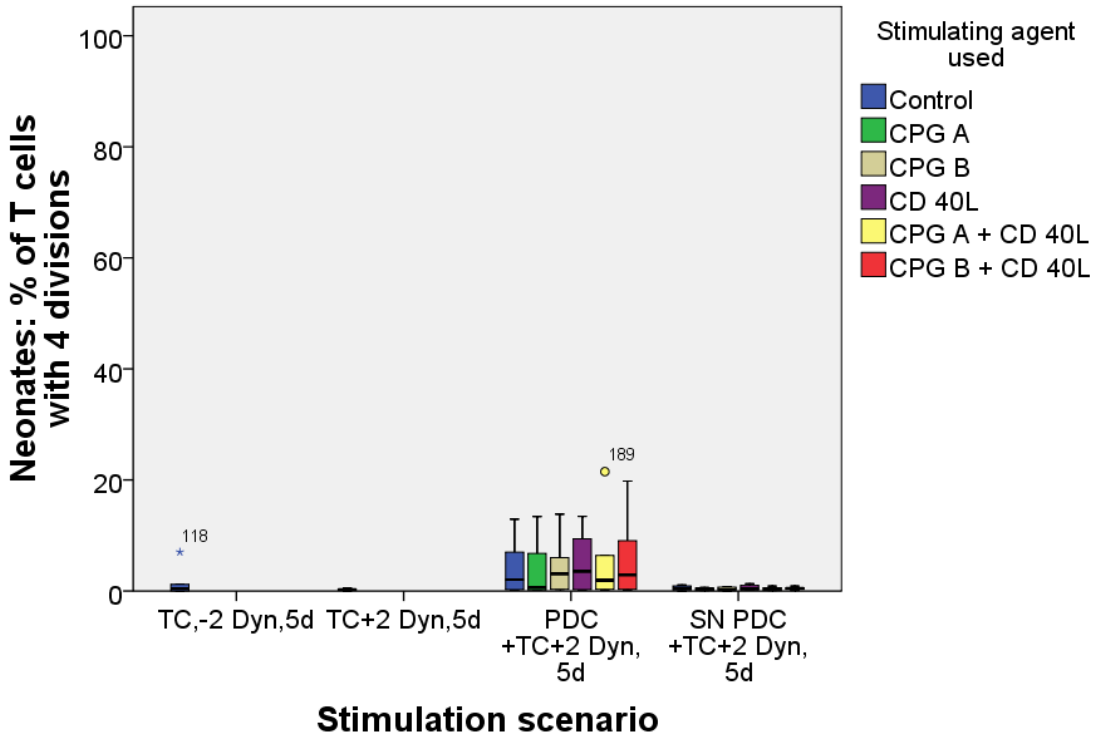


Fig 22 Division of autologous T cells: Percentage of T cells that divided after no stimulation (control), stimulation with Dynabeads (control) and stimulated with PDC after no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. SN PDC = Supernatant of activated PDC, TC = cells, Dyn = number of Dynabeads/ T cell, 5d = 5 days of co-incubation. Value labels refer to donor to identify outliers. (n=6)

Based on the different stimulations scenarios, the graphs above illustrate the following:

1) **Neonates showed a higher spontaneous T cell division rate** (median of 69 % of cells with 0 divisions vs. 6 / 1 / 14 / 0 % with 1 / 2 / 3 / 4 divisions respectively) in the totally unstimulated control group (no Dynabeads added) than adults (median of 100 / 0 / 0 / 0 / 0 %).
2) In the second control group (2 Dynabeads added per cell), **neonatal T cells were less prone to activation** (median of 22 / 39 / 14 / 14 / 0) than adults (median of 9 /13 / 20 / 26 / 19 %).

3) In the third stimulation scenario (direct cell-cell contact), where Dynabeads and pre-stimulated PDC were added to autologous T cells, the following results occurred (all values are medians):

   a. In the control group (unstimulated PDC added to autologous T cells pre-stimulated by Dynabeads), neonates produced a division pattern of 14 / 35 / 23 / 8 / 2 % while adults showed a division pattern of 15 / 12 / 22 / 34 / 14 %.

      Thus, adult autologous pre-stimulated T cells showed a higher division rate after co-incubation with unstimulated PDC than neonates.

   b. In the CPG A group (where PDC stimulated by CPG A were used) neonates showed a division pattern of 17 / 34 / 20 / 13 / 0 % whereas adult T cells had a division pattern of 18 / 12 / 20 / 30 / 19 %.

      Neonate CPG A-stimulated PDC produced a lower responsiveness in autologous T cell division than adult PDC.

   c. In the CPG B group (where PDC stimulated by CPG B were used) neonates showed a T cell division pattern of 15 / 36 / 20 / 10 / 3 % while the adult T cell division pattern was 18 / 13 / 19 / 28 / 16 %.

      Consequently, the T cell division pattern generated by CPG B stimulated PDC was similar to CPG A stimulation in both the adult and the neonate group.

   d. In the CD40L group (PDC stimulated by CD40 ligand), neonatal T cells demonstrated a division pattern of 11 / 32 / 22 / 9 / 4 % while adult T cells exhibited a division pattern of 13 / 9 / 22 / 42 / 18 %.

      In summary, CD40 L stimulation did not overcome the defective T cell stimulation by PDC in neonates.

   e. In the CPG A+CD40L group (PDC stimulated by CPG A+CD40L) neonates showed a division pattern of 14 / 35 / 19 / 12 / 2 % whereas adult T cells had a division pattern of 18 / 8 / 16 / 40 / 19 %.

      In brief, combined CPG A+CD40L stimulation of PDC did not improve T cell division in neonates.

   f. In the CPG B+CD40L group (where PDC stimulated by CPG B+CD40L were used) neonates showed a T cell division pattern of 12 / 36 / 21 /

11 / 3 % while the adult T cell division pattern was 16 / 9 / 15 / 37 / 17 %.

Again, combined stimulation of neonatal PDC, even with CPG B+CD40L did not promote T cell division to adult levels.

g. For adult T cells incubated without IL3 but stimulated with autologous PDC, division rates were as follows: Control group (unstimulated PDC): 11 / 11 / 16 / 20 / 31 %, CPG A group: 15 / 11 / 16 / 23 / 28 % and CPG B group: 15 / 16 / 21 / 20 / 18 %. As these were cells from one single donor, no conclusion should be drawn from this data.

4) In the fourth stimulation scenario (T cell stimulation only by supernatant from PDC), where Dynabeads and supernatant from pre-stimulated PDC were added to autologous T cells, the following results could be demonstrated (all values are medians):

a. In the control group (supernatant from unstimulated PDC added to autologous T cells pre-stimulated by Dynabeads), neonates showed a division pattern of 16 / 37 / 15 / 12 / 0 % while adults produced a division pattern of 10 / 12 / 21 / 29 / 19 %.

Thus, adult autologous pre-stimulated T cells showed a higher division rate even after co-incubation only with supernatant from unstimulated PDC than neonates.

b. In the CPG A group (where supernatant from PDC stimulated by CPG A was used) neonates showed a division pattern of 16 / 37 / 19 / 10 / 0 % whereas adult T cells had a division pattern of 10 / 12 / 21 / 27 / 19 %.

As in direct PDC-T cell contact, CPG A stimulation of neonatal PDC produced a lower responsiveness in autologous T cell division than that of adult PDC.

c. In the CPG B group (where supernatant from PDC stimulated by CPG B was used) neonates showed a T cell division pattern of 12 / 38 / 18 / 13 / 0 % while the adult T cell division pattern was 12 / 13 / 23 / 27 / 13 %.

The T cell division pattern generated by supernatant from CPG B stimulated PDC was similar to CPG A stimulation in both the adult and the neonate group.

d. In the CD40L group (supernatant from PDC stimulated by CD40 ligand), neonatal T cells demonstrated a division pattern of 12 / 36 / 27 / 8 / 0 % while adult T cells exhibited a division pattern of 10 / 12 / 22 / 27 / 20 %.

   Like in the third stimulation scenario, CD40 L stimulation of PDC did not overcome the defective T cell stimulation by supernatant from PDC in neonates.

e. In the CPG A+CD40L group (supernatant from PDC stimulated by CPG A+CD40L) neonatal T cells had a division pattern of 17 / 37 / 19 / 9 / 0 % whereas adult T cells had a division pattern of 11 / 11 / 20 / 27 / 22 %.

   Again, supernatant from PDC stimulated with combined CPG A+CD40L did not improve T cell division in neonates.

f. In the CPG B+CD40L group (where supernatant from PDC stimulated by CPG B+CD40L was used) neonates showed a T cell division pattern of 13 / 39 / 26 / 11 / 0 % while the adult T cell division pattern was 11 / 13 / 24 / 27 /12 %.

   Briefly put, stimulation of neonatal T cells with supernatant from PDC stimulated with combined CPG B+CD40L did not promote T cell division to adult levels.

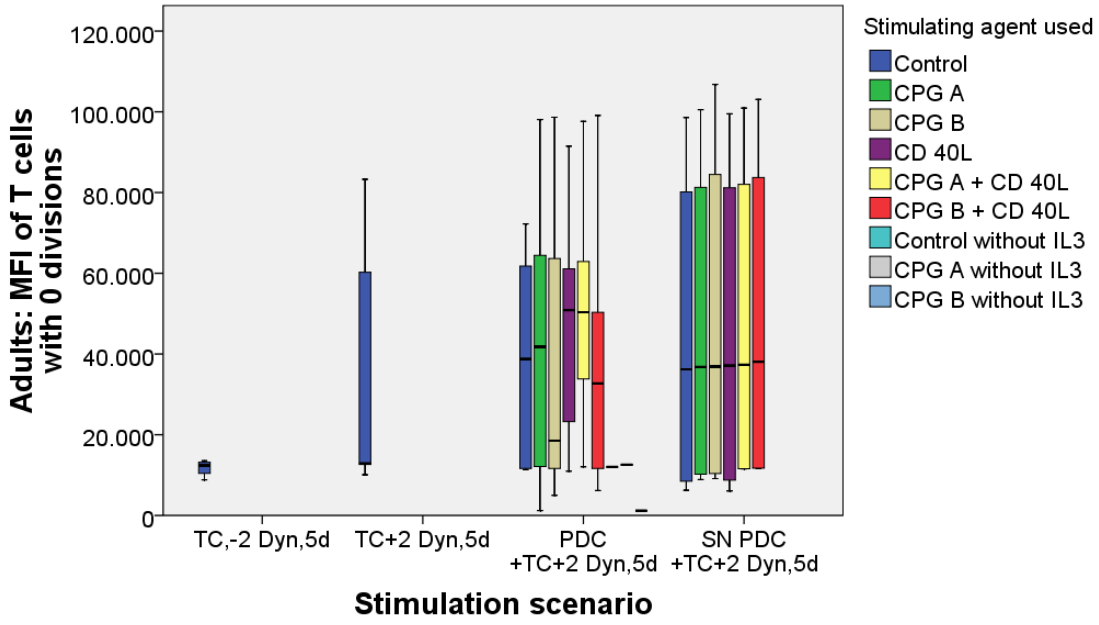
As an overall result of the data presented above, there was no evidence that any of the stimulants tested above could boost T cell division in neonates to a rate similar that that in adults.

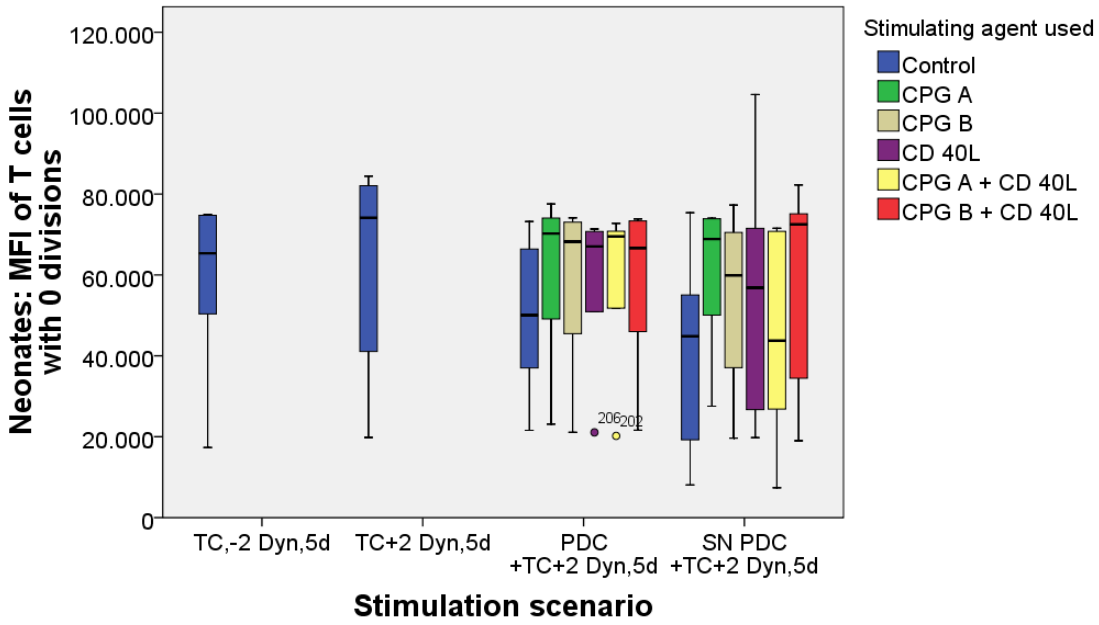
Also, none of the stimulants yielded a consistent and markedly higher T cell division rate than the second control group with 2 Dynabeads per cell.

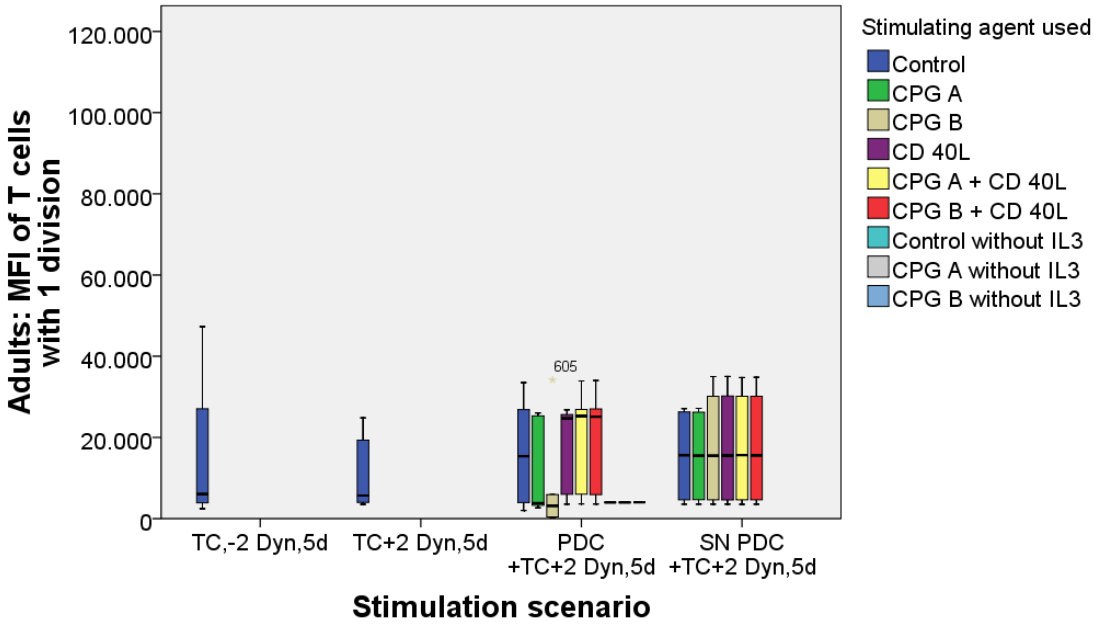
The general division rates between T cells stimulated by direct contact with PDC or by supernatant alone did not display a distinct and consistent difference.

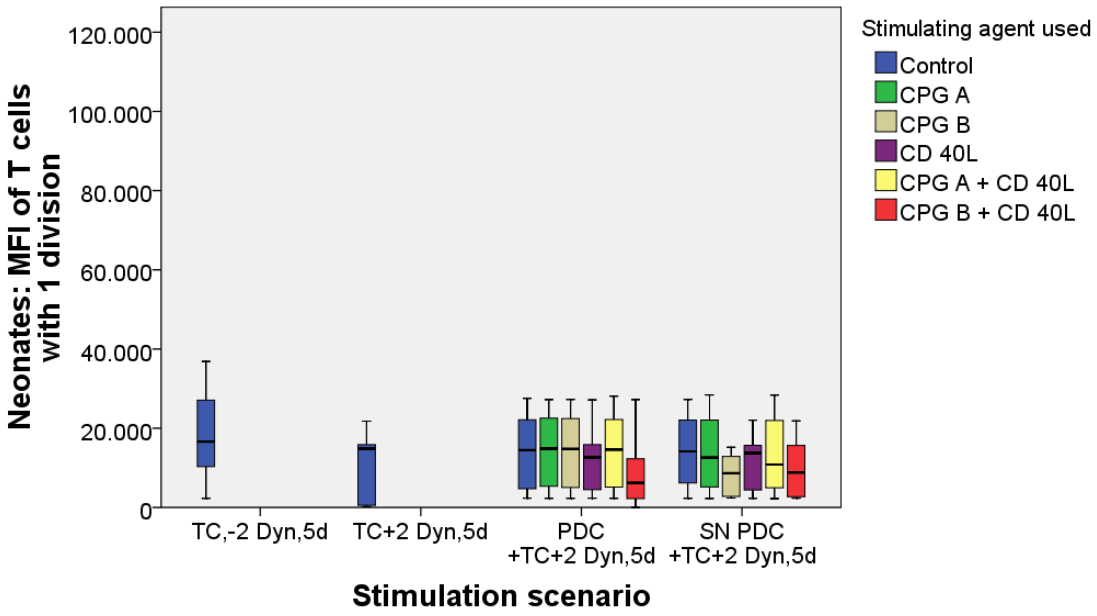
In conclusion, neonatal autologous T cells seem to have a lower capacity to be stimulated to division by activated PDC than adult T cells. CPG stimulation or CD40L priming did not help neonatal PDC overcome this defect.

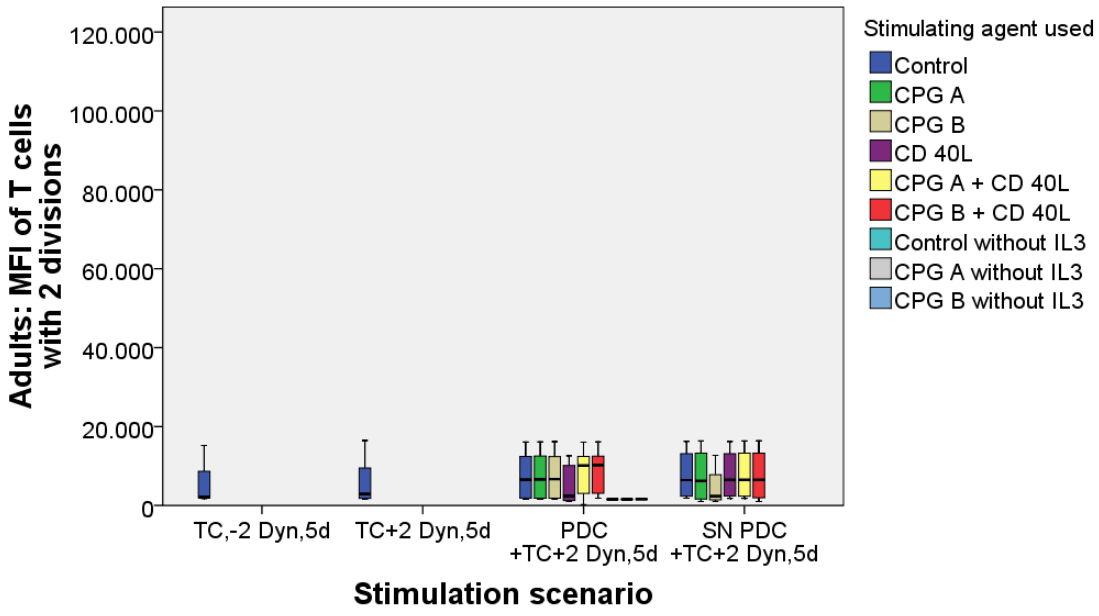
After measuring how many divisions each cell underwent by gating T cell populations we looked at the mean intensity of fluorescence in the divided cells. The results are shown in Fig 23.

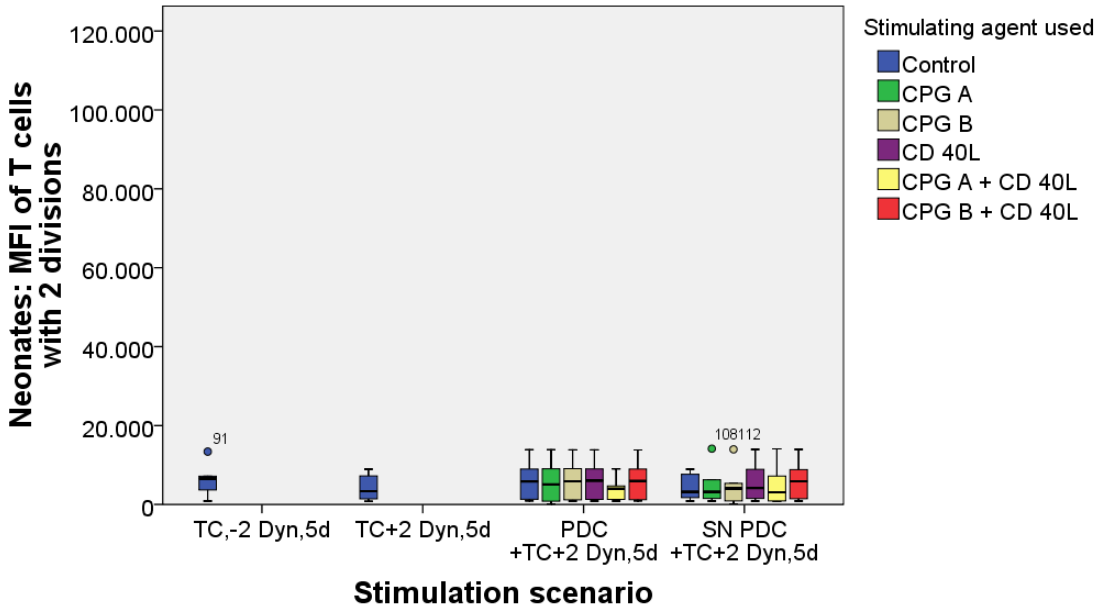


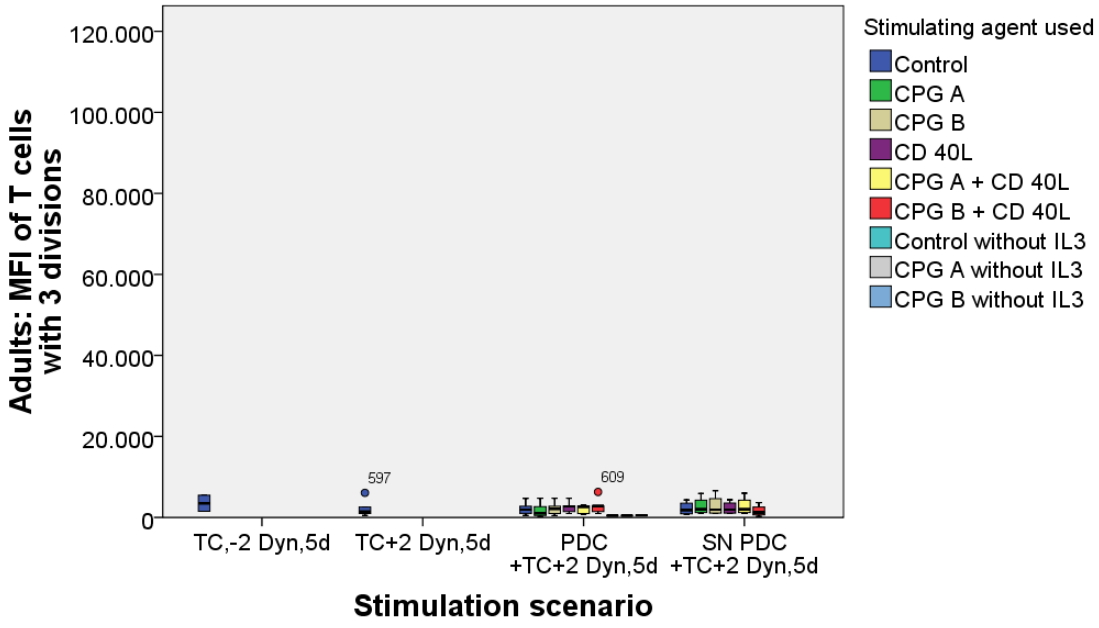


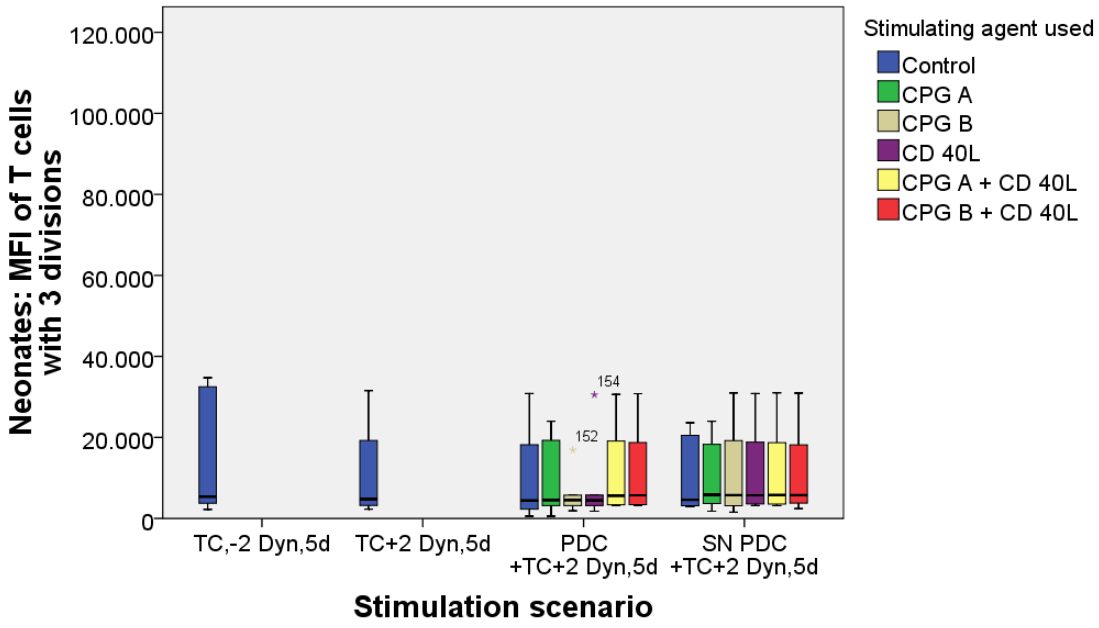

Adults: MFI of T cells with 1 division
120.000
100.000
80.000
60.000
40.000
20.000
0
605
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3


Neonates: MFI of T cells with 1 division
120.000
100.000
80.000
60.000
40.000
20.000
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


Adults: MFI of T cells with 2 divisions
120.000
100.000
80.000
60.000
40.000
20.000
0
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3


Neonates: MFI of T cells with 2 divisions
120.000
100.000
80.000
60.000
40.000
20.000
0
91
108112
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L


Adults: MFI of T cells with 3 divisions
120.000
100.000
80.000
60.000
40.000
20.000
0
597
609
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L
Control without IL3
CPG A without IL3
CPG B without IL3


Neonates: MFI of T cells with 3 divisions
120.000
100.000
80.000
60.000
40.000
20.000
0
154
152
TC,-2 Dyn,5d
TC+2 Dyn,5d
PDC +TC+2 Dyn,5d
SN PDC +TC+2 Dyn,5d
Stimulation scenario
Stimulating agent used
Control
CPG A
CPG B
CD 40L
CPG A + CD 40L
CPG B + CD 40L

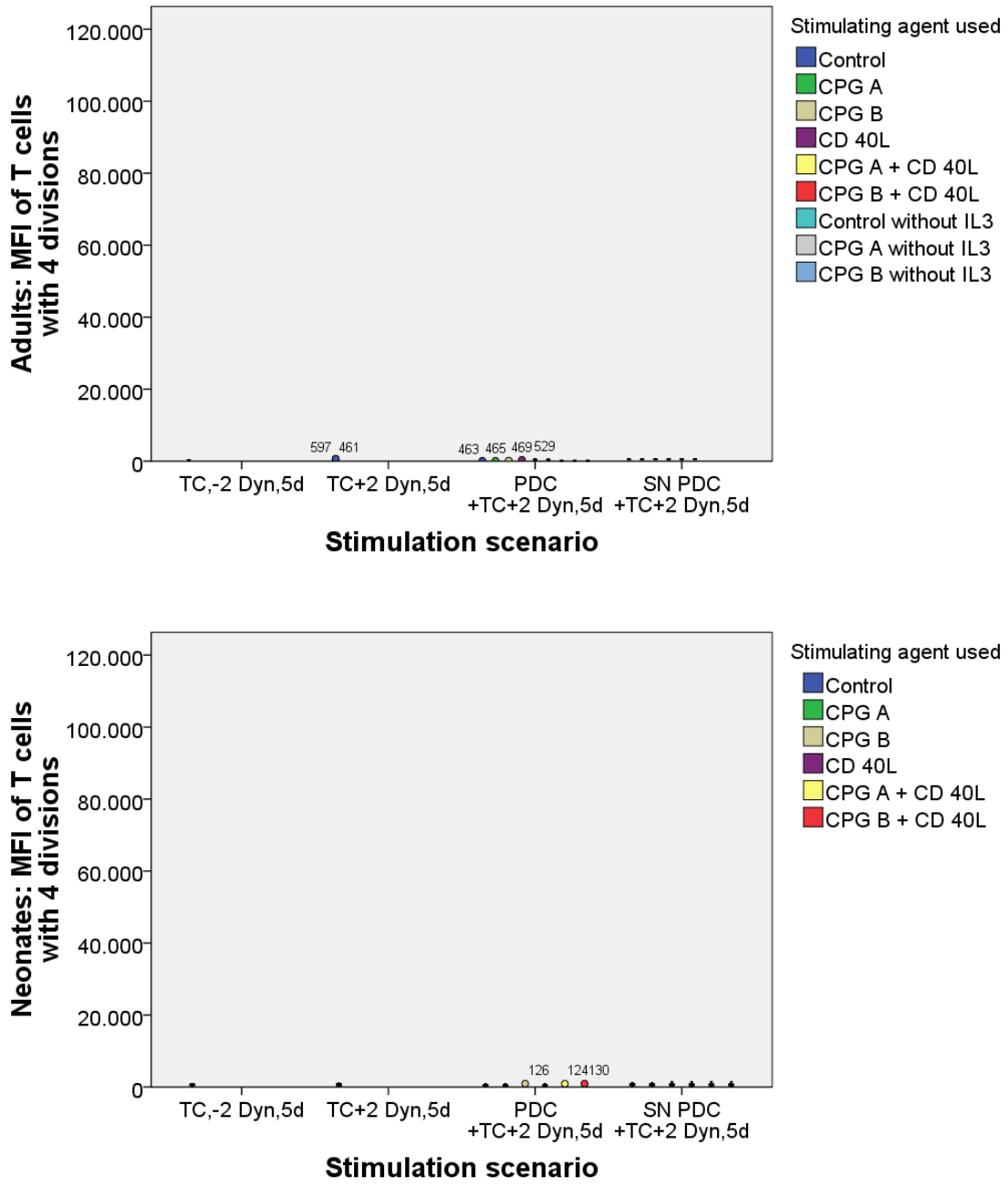


Fig 23 Division of autologous T cells: Mean fluorescence intensity of T cells that divided after no stimulation (control), stimulation with Dynabeads (control) and stimulated by PDC (after no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. SN PDC = Supernatant of activated PDC, TC = cells, Dyn = number of Dynabeads/T cell, 5d = 5 days of co-incubation. Value labels refer to donor to identify outliers. (n=6)

Based on the different stimulations scenarios, MFI was as follows:

1) **Neonatal T cells showed a higher MFI than adult T cells in all division steps** (median 65367 / 16626 / 6555 / 5392 / 691 for cells with 0 / 1 / 2 / 3 / 4 divisions respectively) in the totally unstimulated control group (no Dynabeads added) than adults (median of 12408 / 6074 / 2116 / 3497 / 322).
2) In the second control group (2 Dynabeads added per cell), **neonatal T cells also showed a higher MFI in all division steps** (median of 74122 / 14839 / 3369 / 4770 / 640) than adults (median of 12870 / 5682 / 2919 / 1461 / 444).

3) In the third stimulation scenario (direct cell-cell contact), where Dynabeads and pre-stimulated PDC were added to autologous T cells, the following results occurred (all values are medians):
    a. In the control group (unstimulated PDC added to autologous T cells pre-stimulated by Dynabeads), neonates produced MFIs of 50077 / 14453 / 5856 / 4421 / 465 while adults showed a MFI pattern of 38756 / 15382 / 6546 / 1934 / 420.

       Thus, neonate autologous pre-stimulated T cells showed a lower MFI in division steps 1+2 after co-incubation with unstimulated PDC than adults.
    b. In the CPG A group (where PDC stimulated by CPG A were used) neonates showed a MFI pattern of 70244 / 14840 / 5076 / 4534 / 460 whereas adult T cells had a MFI pattern of 41771 / 3820 / 6617 / 1055 / 415.

       Neonate CPG A-stimulated PDC thus produced a higher MFI in autologous T cells than adult PDC with the exception of division step 2.
    c. In the CPG B group (where PDC stimulated by CPG B were used) neonates showed a T cell MFI pattern of 68228 / 14791 / 5885 / 4548 / 426 while the adult T cell MFI pattern was 18517 / 3150 / 3150 / 6673 / 2114 / 392.

Consequently, the MFI pattern generated by CPG B stimulated PDC was again higher in neonate than in adult T cells, this time with the exception of division step 3.

d. In the CD40L group (PDC stimulated by CD40 ligand), neonatal T cells demonstrated a MFI pattern of 67039 / 12635 / 6036 / 4451 / 443 while adult T cells exhibited a MFI pattern of 50848 / 24708 / 2391 / 2739 / 420.

   CD40L stimulation in adults produced a higher MFI in division step 1 but no other division step.

e. In the CPG A+CD40L group (PDC stimulated by CPG A+CD40L) neonates showed a MFI pattern of 69531 / 14610 / 4006 / 5633 / 443 whereas adult T cells had a division pattern of 50370 / 25279 / 10132 / 2651 / 430.

   In brief, combined CPG A+CD40L stimulation of PDC boosted T cell MFI in adults in division steps 1+2.

f. In the CPG B+CD40L group (where PDC stimulated by CPG B+CD40L were used) neonates showed a T cell MFI pattern of 66637 / 6232 / 5928 / 5709 / 384 while the adult T cell MFI pattern was 32703 / 25114 / 10209 / 2826 / 410.

   Again, combined stimulation of neonatal PDC, even with CPG B+CD40L showed a lower T cell MFI than adult levels in division steps 1+2+4.

g. For adult T cells incubated without IL3 but stimulated with autologous PDC, MFI patterns were as follows: Control group (unstimulated PDC): 12013 / 4013 / 1571 / 476 / 106. CPG A group: 12557 / 4005 / 1558 / 436 / 110 and CPG B group: 1183 / 4031 / 1581 / 519 / 89. As these were cells from one single donor, no conclusion should be drawn from this data.

4) In the fourth stimulation scenario (T cell stimulation only by supernatant from PDC), where Dynabeads and supernatant from pre-stimulated PDC were added to autologous T cells, the following results could be demonstrated (all values are medians):

a. In the control group (supernatant from unstimulated PDC added to autologous T cells pre-stimulated by Dynabeads), neonates showed a MFI pattern of 44843 / 14158 / 3191 / 4603 / 878 while adults produced a MFI pattern of 36219 / 15600 / 6444 / 1834 / 519.

   Thus, neonate autologous pre-stimulated T cells showed a higher MFI than adults after co-incubation only with supernatant from unstimulated PDC than neonates. Only in division step 1+2 did adult T cells show a higher MFI.

b. In the CPG A group (where supernatant from PDC stimulated by CPG A was used) neonates demonstrated a MFI pattern of 66872 / 12595 / 3195 / 5844 / 635 whereas adult T cells had a MFI pattern of 36779 / 15497 / 6213 / 2021 / 525.

   Consequently, CPG A stimulation of neonatal PDC produced a higher MFI in autologous T cell division than that of adult PDC. Only in division step 1+2 did adult T cells show a higher MFI.

c. In the CPG B group (where supernatant from PDC stimulated by CPG B was used) neonates showed a T cell MFI pattern of 59878 / 8663 / 4081 / 5749 / 642 while the adult T cell exhibited a MFI pattern of 36890 / 15519 / 2364 / 1910 / 563.

   Again, the T cell MFI pattern generated by supernatant from CPG B stimulated PDC was similar to the pattern produced by CPG A stimulation in both the adult and the neonate group. Here, a higher adult MFI could only be shown in division step 2.

d. In the CD40L group (supernatant from PDC stimulated by CD40 ligand), neonatal T cells demonstrated a MFI pattern of 56850 / 13758 / 4176 / 5721 / 641 while adult T cells exhibited a MFI pattern of 37131 / 15537 / 6491 / 1920 / 522.

   In the CD40L group, neonatal T cell MFI after stimulation with CD40L stimulated PDC was lower than that of adult T cell in nearly all division steps. Thus, MFI in adult T cells was only higher in step 1+2.

e. In the CPG A+CD40L group (supernatant from PDC stimulated by CPG A+CD40L) neonatal T cells had a MFI pattern of 43758 / 10844 / 3097 /

5775 / 603 whereas adult T cells had a MFI pattern of 37340 / 15637 / 6514 / 2015 / 518.

The same pattern as above presented itself here: MFI in adult T cells was only higher in division step 1+2.

f. In the CPG B+CD40L group (where supernatant from PDC stimulated by CPG B+CD40L was used) neonates showed a T cell MFI pattern of 72489 / 8807 / 5875 / 5769 / 632 while the adult T cell MFI pattern was 38080 / 15563 / 6514 / 1234 / 558.

Stimulation of neonatal T cells with supernatant from PDC stimulated with combined CPG B+CD40L showed the same pattern of lower MFI in division step 1+2.

An overview of the data presented above is given in .

Stimulation scenario	PDC + T cells	Supernatant + T cells
Control 1 (only T cells)	none	
Control 2 (T cells + Dynabeads)	none	
Control PDC (unstimulated)	1+2	1+2
CPG A	2	1+2
CPG B	3	2
CD40L	1	1+2
CPG A+CD40L	1+2	1+2
CPG B+CD40L	1+2+4	1+2

Table 15 T cell MFI in adults higher than in neonates
T cell division steps in which adult T cells showed a higher MFI
than neonatal PDC, sorted by stimulation scenario.

As an overall result of the data presented above on MFI, the conclusion could be drawn that neonatal T cells showed higher MFIs in most of the higher division steps, in particular steps 3+4 (except in the case of CPG B stimulation and direct PDC to T cell contact).

Thus, the study of MFI did not yield any results supporting a superior capacity of neonatal or adult PDC for T cell division after use of the stimulants studied. Cell-to-cell contact generated similar levels of T cell MFI as supernatant, i.e. cytokine stimulation alone. We therefore propose that PDC create a paracrine cytokine milieu in cell culture that allows T cells to divide.

1.28.3 T cell profile differentiation – calibration

After analyzing the direct effect PDC have on T cells after stimulation, we wanted to see which T helper cell type would be produced via PDC stimulation so we focused on cytokine profiles.

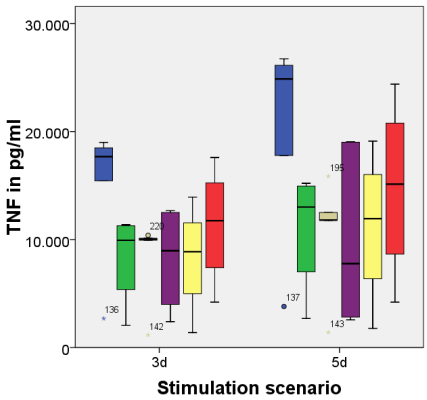
To differentiate between T helper cell types, we focused on the following cytokine profiles:

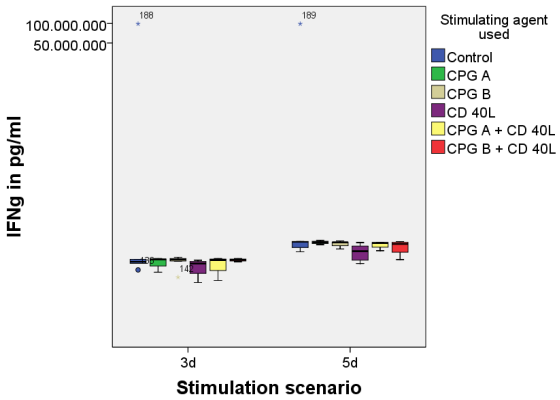
Th1	Th2
IFNγ	IL4
	IL5
TNFβ	IL10
IL2	

Table 16 T helper cell cytokine profiles

In order to analyze cytokine profiles, we compared production of interferon-γ and tumor necrosis factor-β by PDC-activated T cells to production of interleukin-4, interleukin-5 and interleukin-10 on day 3 and day 5 after co-incubation.

First, we looked at the cytokine profiles produced by the adult T cells in our calibration phase, where T cells were supposedly too over stimulated with 3 Dynabeads per cell to make out any kind of effect from the different PDC stimulants.





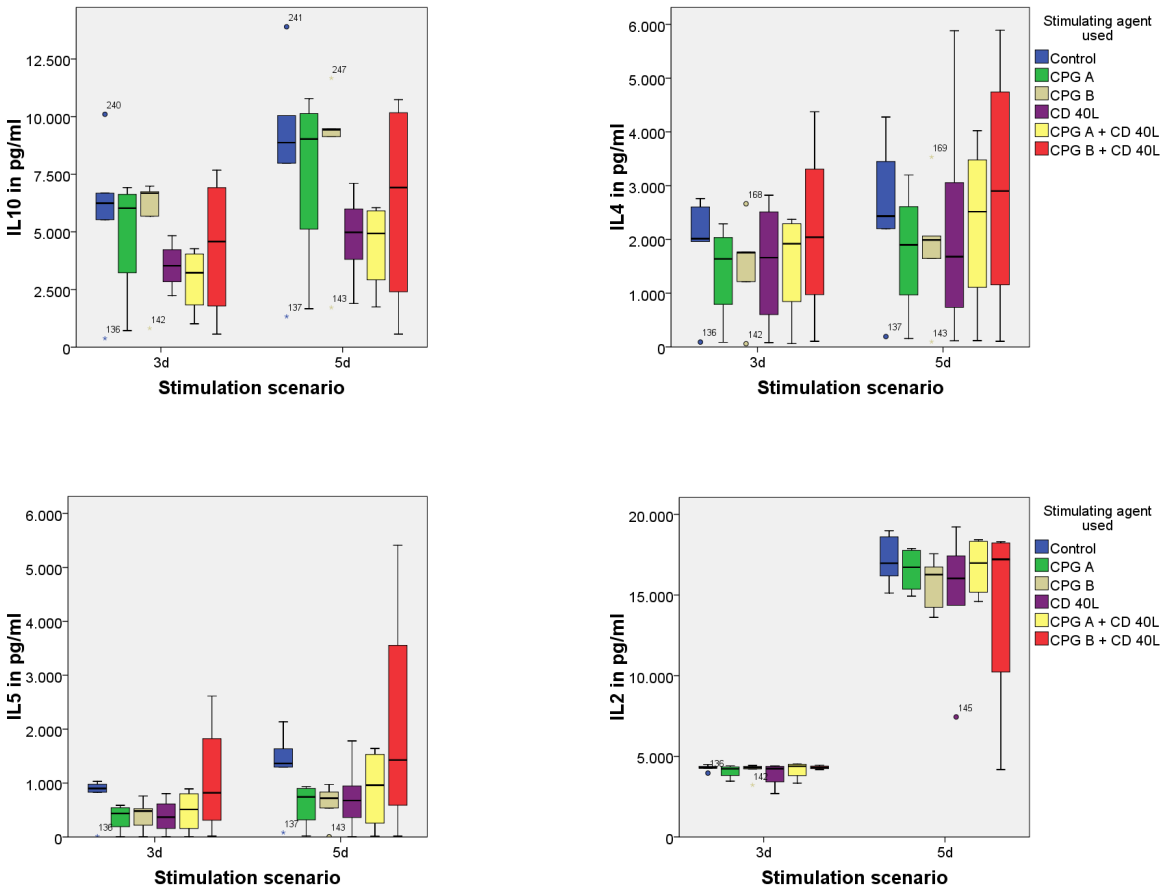


Fig 24 Cytokine production by T cells - calibration

Mean cytokine production by T cells after incubation with 3 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations after 3 and 5 days of co-incubation (3d / 5d). n=5.

Value labels refer to donor to identify outliers. Note logarithmic scale on IFNγ graph.

The graphs clearly show that no PDC stimulant, alone or in combination was more powerful that the control group PDC in activation of T cells.

In the **TNF** scenario, stimulation of T cells with PDC primed with CPG A or CPG B or CD40L and combinations rather suppressed TNF secretion, compared to control, with CPG A+CD40L-stimulation showing a slightly higher cytokine production than the others, though not on a significant level.

Looking at **IFNγ** production, one adult donor showed a very high interferon-γ production by control PDC stimulated T cells, compared to that of otherwise stimulated PDC-stimulated T cells, with an amount of 98 µg IFNγ/ml – so high that logarithmic scale was needed to demonstrate the cytokine amount produced. Compared to around 35 ng/ml produced by other adult controls, this was a sizeable quantity.

IL10 production showed the same pattern as TNF production, with PDC stimulants rather suppressing cytokine production compared to controls with only CPG A and CPG B alone (without CD40L) showing a slight tendency to uphold the same cytokine levels as controls. The same pattern could be noted in **IL4** production – all PDC stimulants rather suppressed IL4 production, with the combinations showing a slightly higher cytokine production on day 5. **IL5** production presented no surprise, either – PDC stimulants suppressed IL5 production, with only the CPG B+CD40L-combination getting nearer to the IL5 amount produced by the control group.
The highest differences in cytokine levels between day 3 and day 5 of co-incubation were detected in **IL2** production. Nonetheless, no PDC stimulatant proved more powerful than control PDCs in promoting cytokine production in T cells. In MWU testing, no differences could be noted.

When looking at the maximum cytokine levels achieved, it is obvious that T cell stimulation with 3 Dynabeads/cell sent the T cells into overdrive, thus producing so many cytokines in all groups that no differences to the control group could be noted. We therefore concluded, as in the T cell division experiments, that we should reduce the Dynabeads to 2 Dynabeads/cell.

1.28.4 T cell profile differentiation – testing

After we had adjusted our experimental setup to 2 Dynabeads/cell, we re-did the cytometric bead array, again measuring IFNγ and TNFβ as well as IL4, IL5 and IL10, this time including cord-blood PDCs.

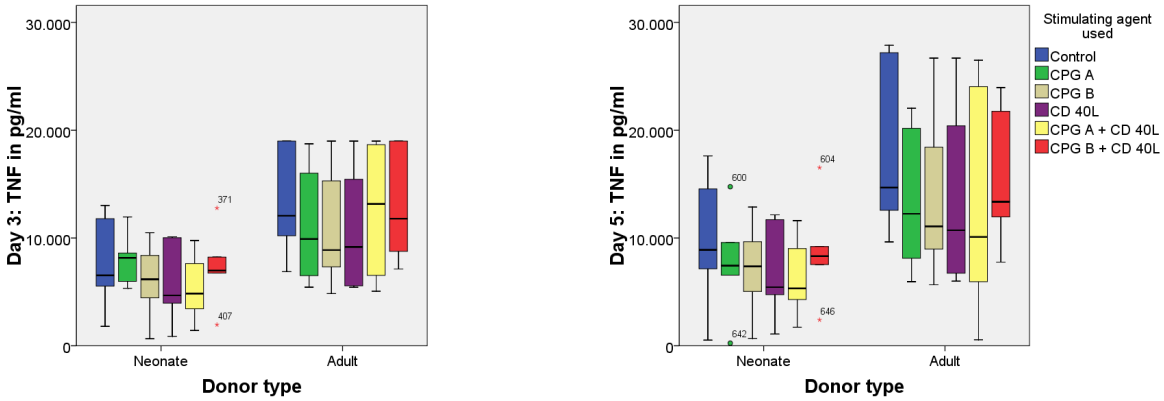


Fig 25 TNF production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

Neonatal T cells showed, in total, a lesser TNF production than adult cells.

On day 3, they showed a median TNF content in their supernatant between 4670 pg/ml (CD40L) and 8153 pg/ml (CPG A), increasing to maximal medians between 8897 pg/ml (control) and 5328 pg/ml (CPG A+CD40L) on day 5. Meanwhile, adult T cells showed medians between 8880 pg/ml (CPG B) and 13163 pg/ml (CPG A+CD40L) in their supernatant on day 3, increasing to maximum medians between 14680 pg/ml (control) and 10097 pg/ml (CPG A+CD40L) on day 5 in their supernatant. The boxplots also show that no PDC stimulant proved to be more effective than controls in inducing TNF production. MWU tests did not reveal any differences between controls and PDC stimulants.

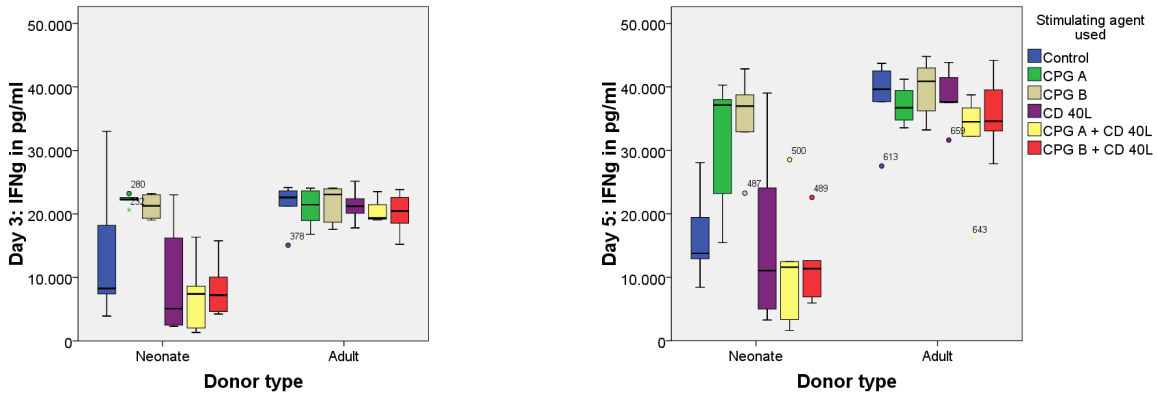


Fig 26 IFNγ production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

Neonatal T cells could be stimulated by CPG A and CPG B primed PDC to produce the same amount of IFNγ as adult T cells. They showed a lower base rate (control group) of IFNγ production. CD40L and combined CPG A (respective CPG B) +CD40L PDC stimulation did not enhance IFNγ production. Even combination of CD40L with CPG A and CPG B did not have a positive effect on IFNγ production in neonates.

Adult T cells did not seem to react to stimulation by primed PDC with a higher IFNγ production. On day 3, **neonatal T cells** showed a median IFNγ supernatant content between 22289 pg/ml (CPG A) and 4670 pg/ml (CD40L), with CPG A and CPG B being the most potent stimulants.

Neonatal IFNγ production increased to maximal medians between 37139 pg/ml (CPG A) and 11060 pg/ml (CD40L) on day 5 (day 5: CPG A vs. control: p = 0.016, CPG B vs. control: p= 0.006). Meanwhile, **adult T cells** showed medians between 22593 pg/ml (control) and 19345 pg/ml (CPG A + CD40L) on day 3, increasing to maximum medians between 40.880 pg/ml (control) and 36.707 pg/ml (CPG A + CD40L) on day 5.

The boxplots clearly show that CPG A and B proved to be an effective stimulant in inducing IFNγ production in neonates, stable over day 3 and 5, actually boosting neonatal IFNγ production to adult levels.

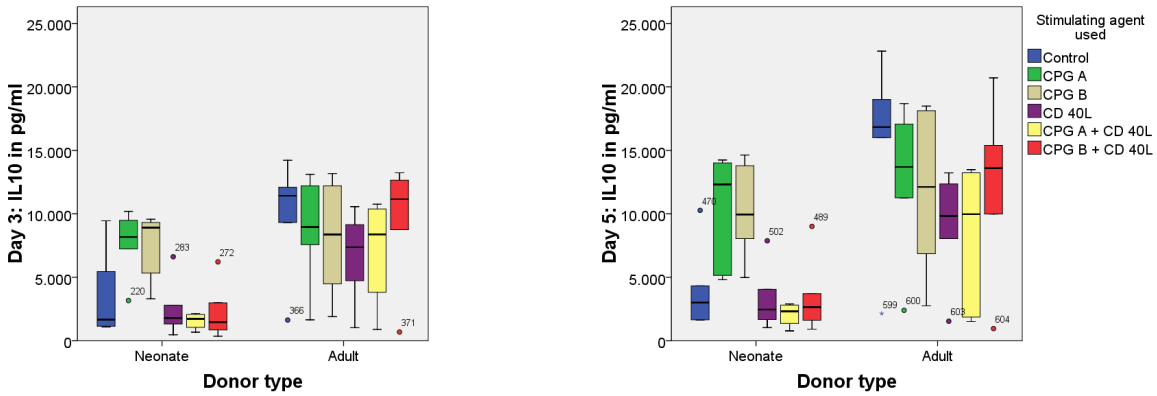


Fig 27 IL10 production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

Similar results could be observed when looking at IL10 production. Neonatal T cells can be stimulated by CPG A (day 3: CPG A vs. control: p = 0.045) and CPG B stimulated PDC to produce similar amounts of IL10 as adult T cells. Neonatal T cells showed a lower base rate of IL10 production. CD40L and combined CPG A (respective CPG B) +CD40L PDC stimulation did not enhance IL10 production in adult or neonatal T cells, rather lowered it. On day 3, neonatal T cells showed a median IL10 production between 8907 pg/ml (CPG B) and 1468 pg/ml (CPG B + CD40L), with CPG A and CPG B being the most potent stimulants.

This increased to maximal medians between 12316 pg/ml (CPG A) (day 5: CPG A vs. control: p = 0.010, CPG B vs. control: p = 0.025) and 2320 pg/ml (CPG A+CD40L) on day 5. Combination with or stimulation of PDC with CD40L alone seemed to suppress the effects that CPG A and CPG B had on the PDCs. Meanwhile, adult T cells showed medians between 11419 pg/ml (control) and 7373 pg/ml (CD40L) on day 3, increasing to maximum medians between 16834 pg/ml (control) and 9834 pg/ml (CD40L) on day 5.

The boxplots show that CPG A and B proved to be an effective PDC stimulant inducing IL10 production in neonatal T cells, stable over day 3 and 5, although not to the same levels as that of adult T cells.

On day 5, adult T cells produced a significantly lesser amount of IL10 upon stimulation by CD40L (day 5: CD40L vs. control: p = 0.045) and combination of CPG A with CD40 L (day 5: CPG A+CD40L vs. control: p = 0.028). **It seems that CD40L-**

stimulation of PDCs not only does not stimulate IL10 production in adult T cells but rather suppresses it.

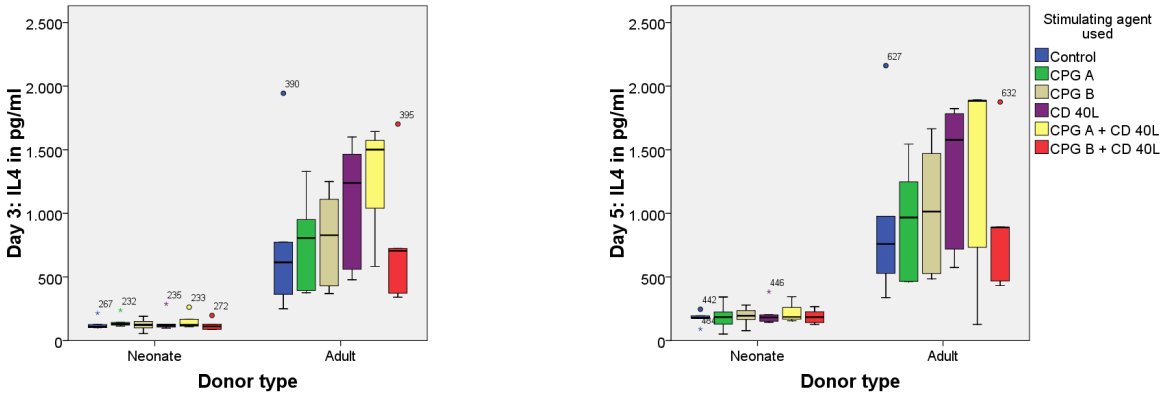


Fig 28 IL4 production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

Results of **IL4** analysis showed unexpectedly clear results: **Neonatal T cell IL4 production did not show any reaction to any kind of stimulation** but stayed at a base rate of median 100-130 pg/ml on day 3 and 178-195 pg/ml on day 5. Adult T cell IL4 production varied between medians of 614 pg/ml (control) and 1500 pg/ml (CPG A+CD40L) on day 3 and medians of 759 pg/ml (control) and 1884 pg/ml (CPG A+CD40L) on day 5. **The highest improvements in IL4 production by adult T cells were achieved by CD40L stimulation and combined CPG A+CD40L stimulation, stable over day 3 and 5**: On day 3, 1.238 pg/ml (CD40L) and 1.500 pg/ml (CPG A + CD40L) and on day 5, 1577 pg/ml (CD40L) and 1884 pg/ml (CPG A+CD40L). Combination of CPG B+CD40L seemed to suppress the effects of stimulation with CD40L alone in adults. This effect could not be reproduced by MWU tests.

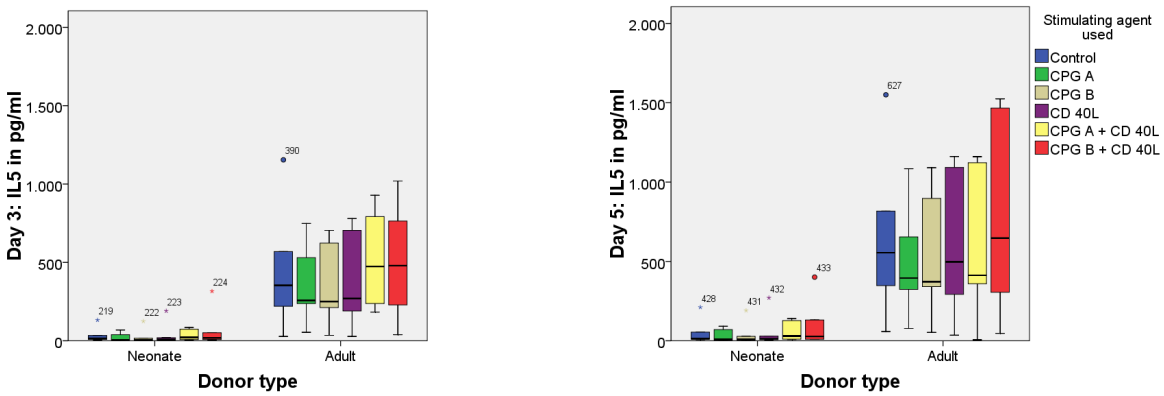


Fig 29 IL5 production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

IL5 production analysis showed similar results to IL4 analysis: **Neonatal T cell IL5 production did not show any reaction to any kind of stimulation** but stayed at a base rate of 5 (control) – 21 pg/ml (CPG A + CD40L) on day 3 and 9 (CPG B) – 30 pg/ml (CPG A + CD40L) on day 5.

Adults had a higher IL5 base rate: Adult T cell IL5 production varied between medians of 250 pg/ml (CPG B) and 480 pg/ml (CPG B + CD40L) on day 3 and medians of 372 pg/ml (CPG B) and 646 pg/ml (again CPG B + CD40L) on day 5. **The biggest improvements in adult T cells were achieved by combining CPG with CD40L stimulation:** On day 3, 474 pg/ml (CPG A+CD40L) and 480 pg/ml (CPG B+CD40L) and on day 5, 646 pg/ml (CPG B+CD40L). MWU testing did not show any differences between the stimulants vs. the control group.

IL2 production did not present any surprises: On day 3, neonatal T cell had produced IL2 at a level between 4246 pg/ml (CPG B) and 4371 pg/ml (CPG A) while adult T cells secreted IL2 on a range between 4179 pg/ml (CD40L) and 4372 (CPG A+CD40L). On day 5, neonatal T cells showed an IL2 production at a level between 15980 pg/ml (CPG B+CD40L) and 17741 pg/ml (control) while adult T cells had produced IL2 on a range between 14855 pg/ml (CPG A) and 16807 pg/ml (CPG A+CD40L). MWU tests did not reveal a difference between PDC-stimulants and the control group.

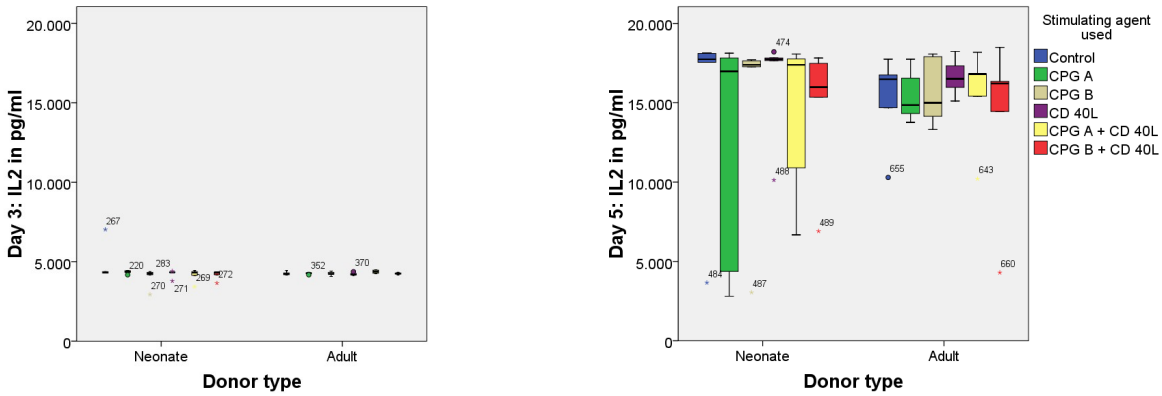


Fig 30 IL2 production by T cells

Mean cytokine production by T cells after incubation with 2 Dynabeads/cell and with PDC that received: no stimulation (control), stimulation with CPG A, CPG B, CD40L and combinations. n=6. Value labels refer to donor to identify outliers.

With regard to Th1 vs. Th2 profile development, TNF secretion in both adult and neonatal T cells seemed to be unchanged by PDC stimulation, though in total, neonatal T cells had a lower base rate TNF production. It could be shown IFNγ and IL10 production neonatal T cells can be induced by stimulation with PDC primed with CPG A and CPG B to reach the cytokine levels attained by adults. This could not be reproduced in adult T cells. CD40L rather suppressed this development (even in combination with CPGs). However, CD40L seemed to improve IL4 production in adult T cells, especially in combination with CPG A, though not with CPG B. This could not be reproduced in neonates. Adult T cells secreted IL5 at a higher base rate than neonatal T cells, though in both populations the secretion rate could not be changed much by PDC stimulation, maybe a little improved by combination of CD40L with CPG B. IL2 production remained at the comparative levels between adults and neonates, with no differences induced by PDC-stimulants.

CD40L stimulation of adult PDC (especially in combination with CPG A) elicited a Th2 response.

A clear decision towards a Th1 or Th2 profile induced by CPG A or CPG B stimulation of neonatal PDC could not be concluded from the cytokine profile analysis. Therefore a general T cell activation seems likely.

1.29 Intracellular phosphorylation

The last step of our experiments was the analysis of intracellular phosphorylation. We used phosphorylation as an intracellular activation marker in order to demonstrate the level of change in intracellular enzyme conformation triggered by stimulation with CPG A and CPG B. As positive controls, we used PMA and IFNγ.

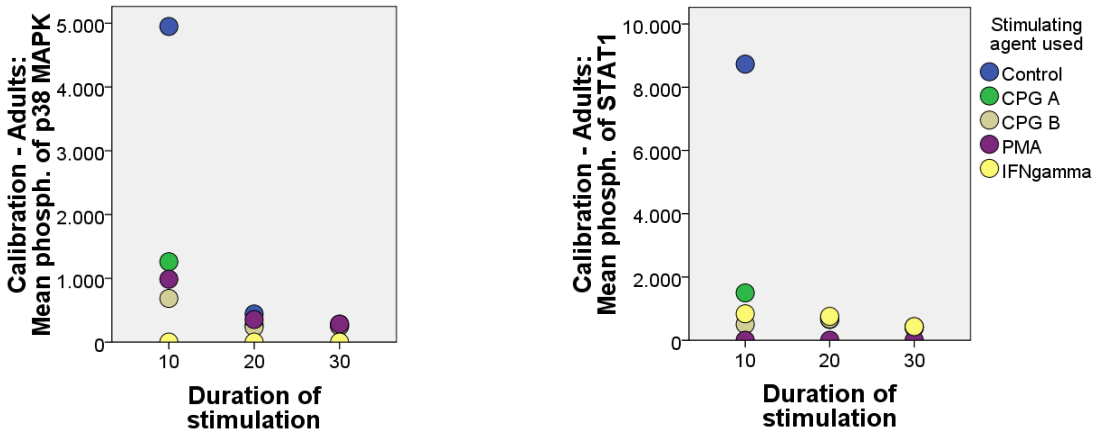
1.29.1 Intracellular phosphorylation – calibration

The following data illustrate the effects of PDC stimulation on intracellular phosphorylation in a collective of cryopreserved PDC from our two well-known donor groups (neonates and adults).

In all following paragraphs, phosphorylation intensity refers to the "mean fluorescence intensity (MFI)" which was measured by FACS.

The values in this paragraph refer to "uncorrected values", meaning that intensities and percentages shown are the median of each group and not differences to the results of positive control stimulations with PMA (positive control for phosphorylation of p38 MAPK) and IFNγ (positive control for phosphorylation of STAT1).

During calibration, we tested which duration (10, 20, 30 min) of PDC stimulation with CPG A, CPG B, PMA and IFNγ would yield the highest amount of intracellular phosphorylation. The PDC used stemmed from frozen adult and neonatal PBMC samples. The results are shown in Fig 31.



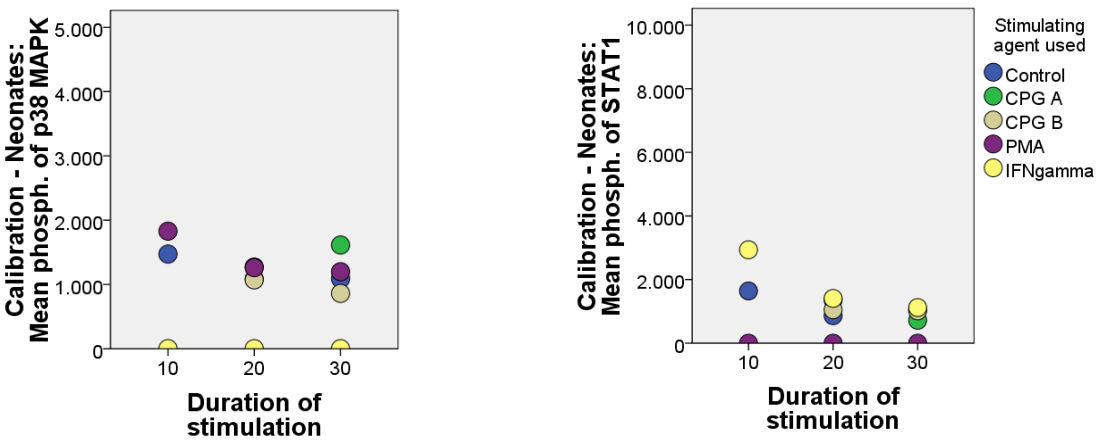


Fig 31 Frozen PDC: Intracellular phosphorylation in terms of MFI of p38 MAPK or STAT1 after no stimulation (control), stimulation with CPG A, CPG B, PMA and IFNγ after 10, 20 and 30 min stimulation. n=3.

Since MFI results for STAT1 and p38 MAPK were best at 10 min, we also looked at the percentage of phosphorylated PDC, as seen in Fig 32.

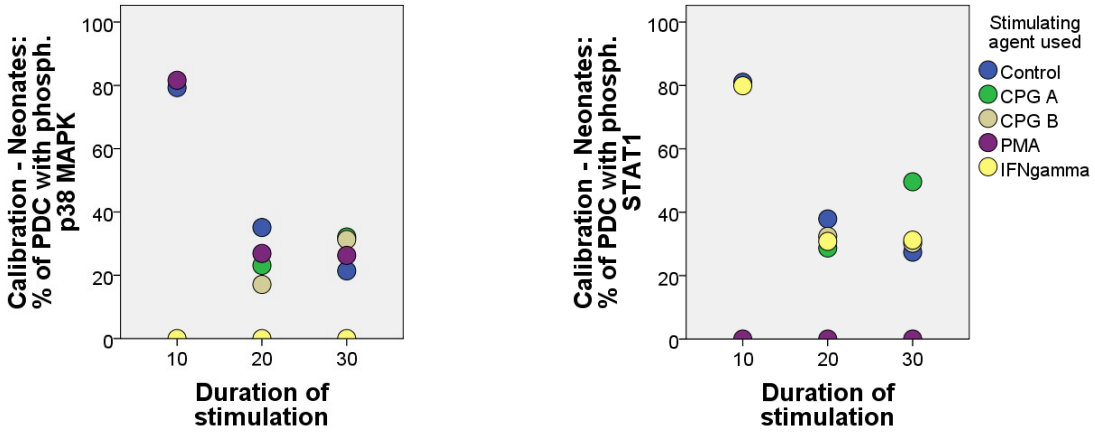
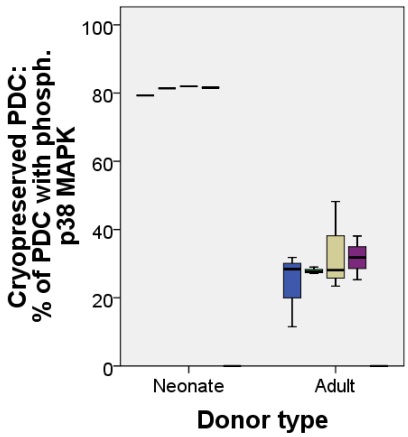


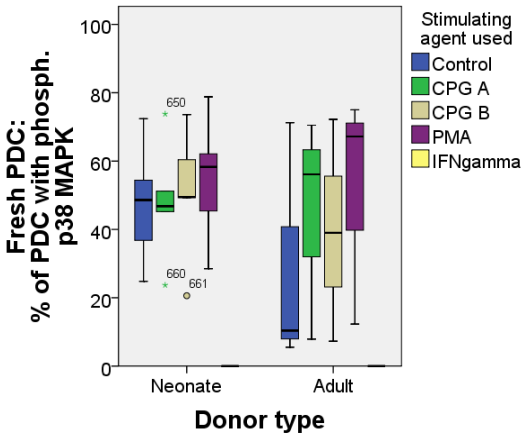
Fig 32 Frozen PDC: Intracellular phosphorylation in terms of percentage of phosphorylated PDC containing p38 MAPK or STAT1
after no stimulation (control),
stimulation with CPG A, CPG B, PMA and IFNγ. n=3.

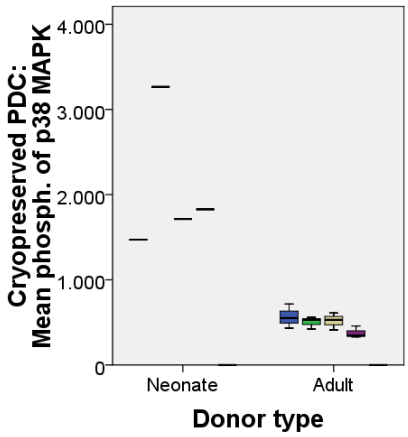
The boxplots show that the best results for percentages of intracellular phosphorylation of p38 MAPK and STAT1 and phosphorylation intensity of these kinases was achieved by stimulation over 10 minutes. Stimulation over longer periods did not seem to be superior therefore we chose to continue the stimulation experiments with 10 minute intervals.

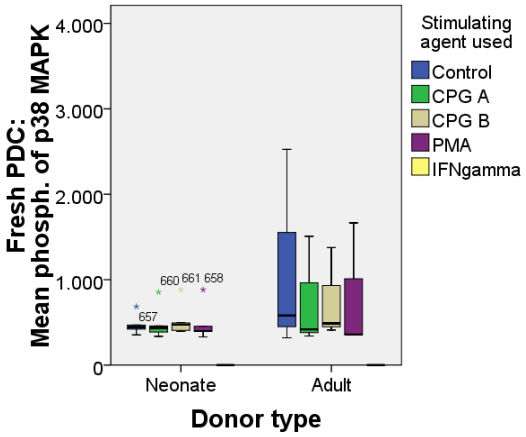
1.29.2 Intracellular phosphorylation in fresh vs. frozen PDC

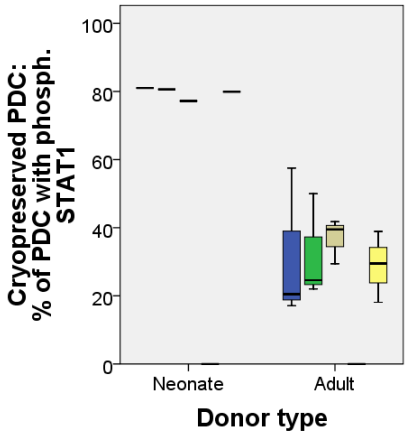
We were unsure if freezing did not hinder proper phosphorylation upon CPG A, CPG B, PMA or IFNγ stimulation (as impairments of cell function had shown in other experiments), therefore we also compared frozen to fresh neonatal PDC, frozen to fresh adult PDC and, last but not least, fresh neonatal PDC to fresh adult PDC.

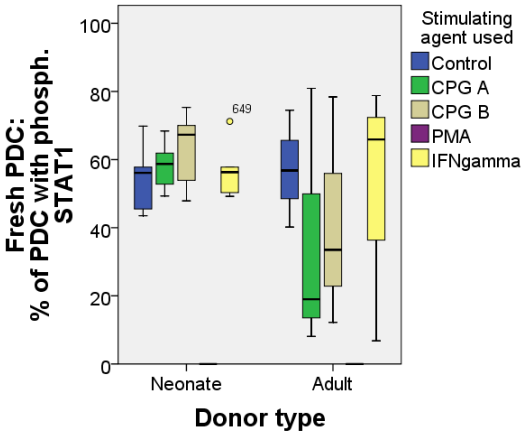












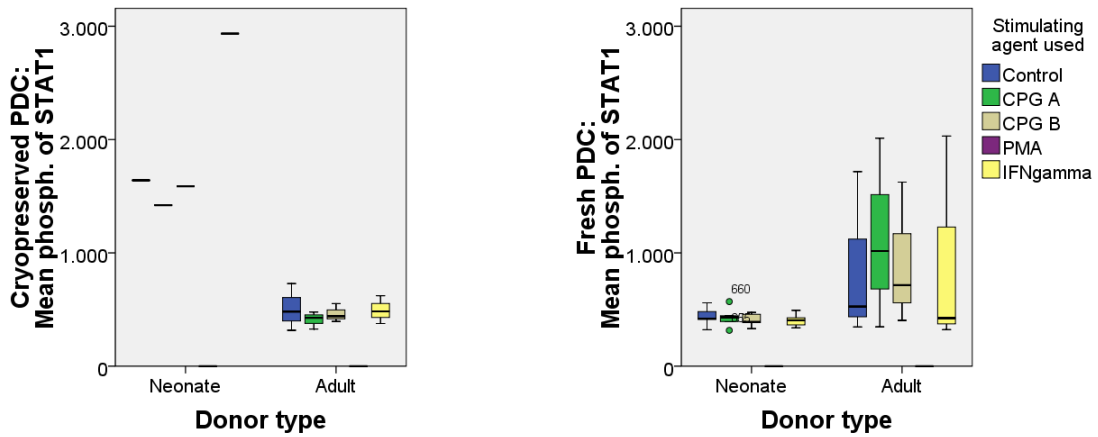


Fig 33 Frozen and fresh PDC: Intracellular phosphorylation in PDC in terms of MFI of p38 MAPK or STAT1 or percentage of PDC containing phosphorylated p38 MAPK or STAT1 after no stimulation (control), stimulation with CPG A, CPG B, PMA and IFNγ. In frozen PDC, n=1 for neonates and 3 for adults, while in fresh PDC, n=5 for neonates and 3 for adults. Value labels refer to donor to identify outliers.

Consistent with prior experiments, **frozen neonatal PDC (the one donor tested) exhibited stronger phosphorylation** (both in intensity and percentages) than adult PDC.

Looking at median **p38 MAPK, phosphorylation in fresh PDC could not be improved** by CPG A or CPG B stimulation in neonates or adults compared to PMA stimulation.
However, looking at the percentages of PDC showing MAPK phosphorylation, CPG A showed to be a potent stimulating agent (close to PMA) in adults (median 56.1% vs. 67.2 %) but not in neonatal PDC. These exhibited a higher percentage of phosphorylated cells in the control group (median 48. %) than adults with only PMA (58.3 %) coming close to the percentages measured in adults after CPG A and CPG B stimulation.

Fresh neonatal PDC did not gain in **STAT1 phosphorylation** intensity after stimulation compared to the control group, while adult PDC reacted best to CPG A (median MFI 1016) and a little to CPG B (median MFI 715) compared to control (median MFI 526).

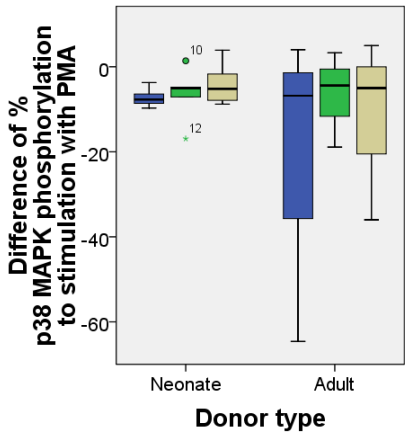
The **percentage of STAT1 phosphorylated PDC** increased in neonates the most after CPG B stimulation (67.3 % vs. control 56.1 %). In adults, percentages actually decreased following CPG A stimulation (19 %) more than after CPG B stimulation (33.5 %), while IFNγ (65.9 %), the positive control remained a little higher than control (56.8 %). This development mirrored phosphorylation intensity, indicating that a number of PDC gained in phosphorylation while others remained nearer the level of the controls.

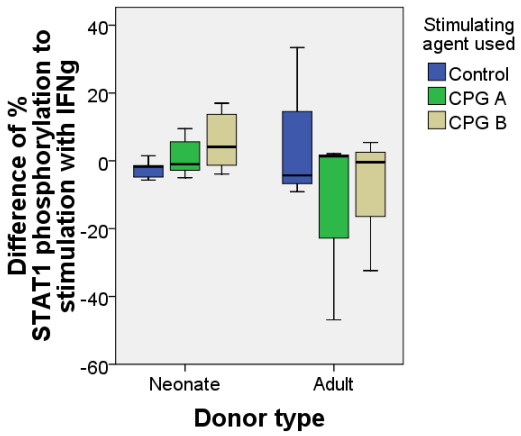
Differences between CPG A and CPG B did not show in MWU tests for any of the four categories of our phosphorylation experiments.

1.29.3 Effects of stimulating agents vs. positive controls

In order to analyze if CPG A and CPG B had a real stimulatory effect superior to the positive controls PMA and IFNγ it was necessary to deduct the values reached by CPG A and CPG B stimulation from the levels attained via PMA and IFNγ stimulation. The positive controls were PMA for p38 MAPK and IFNγ for STAT1. Only the results for fresh blood PDC are shown below.

As the aim of this set of experiments was to compare the effect of CPG A and CPG B stimulation on intracellular phosphorylation to positive controls, the results shown below are for the difference of the results of CPG A to PMA, respective IFNγ stimulation and the difference of CPG B to PMA, respective IFNγ stimulation.





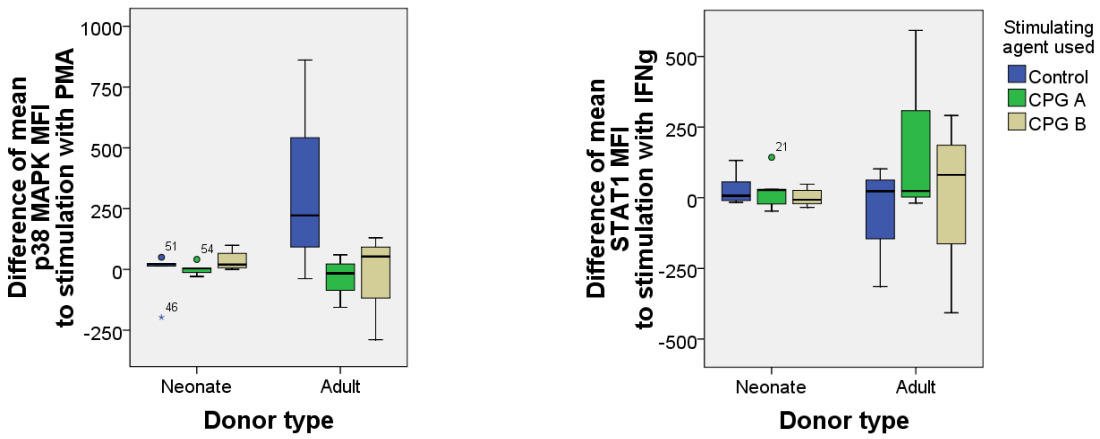


Fig 34 Fresh PDC: Differences in percentages of intracellular phosphorylation of p38 MAPK and STAT1 in PDC and MFI

of PDC after stimulation with CPG A or CPG B as compared to positive controls.

n=5 for neonates and 3 for adults. Value labels refer to donor to identify outliers.

The **median difference in percentage of PDC showing p38 MAPK phosphorylation** in neonate donors was -8 % in the control group and -5 % in both the CPG A and CPG B stimulations.

In adult donors, medians ranged between -7 % in the control group, -4 % in the CPG A and -5 % in the CPG B group.

In conclusion, CPG A or B stimulation did not improve percentage of PDC showing p38 MAPK phosphorylation in neonates more than PMA.

Median differences in phosphorylated p38 MAPK MFI ranged between 22 in the control group, 4 for the CPG A and 20 in the CPG B group in neonates. Adults showed a pattern 222 for the control group, -16 in the CPG A and 53 in the CPG B group.

In brief, CPG A and B did not increase phosphorylated p38 MAPK MFI in neonates more than PMA.

The **median difference in percentage of PDC showing STAT1 phosphorylation** in neonates was -2 % in the control group, -1 % for the CPG A and -4 % in the CPG B group, respectively.

In adults, medians were distributed between -4 % in the control group, 1 % for CPG A stimulation and 0 % in the CPG B group.

CPG stimulation did thus not enhance percentage of PDC showing STAT1 phosphorylation in neonates more than IFNγ.

Looking at **phosphorylated STAT1 MFI**, neonatal PDC displayed a median MFI of 7 in the control group, 27 in the CPG group and -7 in the CPG B group. Adult PDC, meanwhile, showed a MFI of 23 in the control group, 24 in the CPG A group and 81 in the CPG B group.
Differences in MFI of phosphorylated STAT1 did not provide new insights.

P38 MAPK phosphorylation was not boosted by CPG A or B in neonatal fresh blood PDC although they showed a baseline higher than that of adult fresh blood PDC. Stimulation of adult fresh blood PDC with CPG A and B improved p38 MAPK phosphorylation, up to the level of that in neonatal fresh blood PDC.

In conclusion, CPG B improved STAT1 phosphorylation in neonatal fresh blood PDC to levels higher than that of adult PDC while lowering MFI a little. However, IFNγ used as the positive control did not boost STAT1 phosphorylation more than in unstimulated control PDC.

Meanwhile, CPG A and B stimulation of adult PDC rather lowered the percentage of STAT1 phosphorylation while boosting MFI which leads to the assumption that it actually suppressed STAT1 phosphorylation.

Cryopreservation of neonatal PDC seemed to boost p38 MAPK and STAT1 phosphorylation to a level superior to adult PDC.

Discussion

1.30 Challenges to newborn immunity – focus on PDC, CPG and CD40L

The neonatal immune system is severely challenged by viral pathogens. Plasmacytoid dendritic cells with their unsurpassed capacity to produce IFNα play a pre-eminent role in the fight against these pathogens. Due to this capacity they are important for innate immunity (by activating T cells and NK cells) but also for adaptive immunity (by inducing a Th1 type response and promoting the production of antibodies).

This study aimed to examine some of the inherent defects of human neonatal PDC by addressing sensitivity of the TLR9 receptor via stimulation with its ligand CPG and co-stimulation of PDC with CD40L. The PDC is the only dendritic cell to express prominent amounts of TLR9 and TLR7 (45). The endowment of the PDC with these receptors might be the reason they serve as an important interface between innate and adaptive immunity – once those receptors have been stimulated (46).

CPG ODNs are ligands for TLR9. In spite of the ability of CPG ODNs to mimic bacterial and viral DNA there are some important differences between these two groups:

a) The frequency of CPG motifs in CPG ODN is higher.
b) The chemical backbone of CPG ODNs is more resistant to nuclease-induced degradation.
c) The molecular mass of CPG ODNs is much lesser.

The higher amount of type I interferons induced by CPG A compared to CPG B suggests a solely quantitative difference in stimulatory capacity between the two classes. However, CPG actually induce qualitatively different responses in PDC. Krug et al. (35) and Kerkmann et al. (47) demonstrated that CPG B is superior to CPG A in inducing activation markers typically associated with dendritic cells. CPG B caused a higher expression of co-stimulatory molecules and production of pro-inflammatory mediators. Even the mechanism of type I interferon induction differs between the two classes: Only CPG uses a positive feedback mechanism to amplify production of interferon.

CD40 is expressed by B cells, macrophages, dendritic cells and basal epithelial cells. It binds CD40L, a TNF-family transmembrane cytokine which is expressed by dendritic cells and T cells. CD40 is a receptor for a co-stimulatory signal on B cells, promoting growth, differentiation and isotype switching of B cells as well as cytokine production by macrophages and dendritic cells. On dendritic cells, CD40 binds to CD40L expressed by T cells, initiating transmission of activating signals to the T cell and inducing expression of CD80 and CD86 by the dendritic cell. It also enables them to sustain production of the pro-inflammatory cytokine IL12, strengthening the IL12-induced production of IFNγ by T cells. Activation of PDC through TLR9 induces expression of CD40L. Surely, such an important molecule should be tested further for its therapeutic potential.

1.31 Experimental setup

A lot of research has been done in the murine system including vaccinating newborn mice with protein-based vaccines and CPG used as an adjuvant. From our point of view, it was now time to appreciate the documented differences between the murine system as compared to the human situation.

Due to ethical and practicability concerns, we used neonatal PDC from cord blood and adult peripheral blood PDC. We started by characterizing the *in vitro* response of PDC to TLR9 ligand CPG and CD40L in terms of surface marker expression and cytokine profiles before moving on to T cell activation by PDC and their influence on T cell-specific cytokine profiles.

In order to show the direct effect of CPG ODNs on PDC without the help of other cell types or cytokines, this study aimed to selectively analyze PDC response in pure PDC cell cultures instead of using whole blood assays.

For quite some time, studies on the use of CPG ODN have shown a heightened expression of co-stimulatory molecules (CD80, CD86) in some cells. These effects could be not only be demonstrated in cell-cell-interaction but also in supernatant-cell-interaction (13). We therefore decided to include the study of the role of the humoral phase of PDC cultures by adding supernatant of these cultures to T cell cultures.
Effects mediated by supernatants are due to cytokines, chemokines and defensins. Quite a few studies showed that their levels in newborns are influenced by mode of

delivery (48, 49), however according to a recent study by Nguyen et al. (50) this did not seem to make a difference. For practicability reasons, we chose to exclude this possible influence in our model system by only using cord blood from newborns born by caesarean section.

Purity of PDC obtained through MACS was at a median of 93 %, a value concurrent with literature values (51). PDC cultures were given IL3 to promote survival according to a protocol established by Bauer et al. (31).

1.32 PDC maturation

De Wit et al. (42) reported a dose-dependent effect of CPG on PDC. In accordance with these data we tested the lowest possible dose known to be effective in inducing IFNα production for PDC-only experiments (surface markers, cytokine production by PDC, intracellular phosphorylation). However, the same study showed that neonatal PDC show little response to CPG stimulation after short stimulation times. We opted therefore for the lowest-possible but still higher dosage (42)) known to be effective in short stimulation time for experiments on T cell priming by PDC.

Besides CPG, we decided to study CD40L that has been known to modulate PDC function (11). Using CPG as well as CD40L gave us the opportunity to investigate PDC function in an *in vitro* setting while using stimulants modeled on natural bacterial and viral DNA that have not been described as oncogenic so far in contrast to stimulants like PMA (52). Basic PDC maturation was done according to a protocol established by Grouard et al. in 1997 (7) for PDC isolated from tonsils. Using this protocol it had been shown previously that PDC produce specific cytokines after stimulation with CPG (13, 43).
In order to discover the effects of CPG on PDC on a cellular level, we decided to purify PDC from neonatal cord blood and peripheral blood of adult donors. This was done with the aim of analyzing differences in reaction to CPG without the influence of other cell types or cytokines.

1.33 The influence of cryopreservation

In the preliminary phase of analysis, we looked at PDC yield from different donor and storage types on our model system. As we used cryopreserved cells for some of our experiments, we compared cell yield after cryopreservation between donor types.

PDC represent a small subset of human PBMC: In other studies frequencies were around 0.9 % for adults and 0.74 % for neonates (42), respective 0.11 % in adults and 0.05 % in neonates (53). We could show a frequency similar to the first study of 0.7 % in fresh cord blood, while freezing boosted PDC percentages to 1.5 %. Thus, relative frequencies of PDC in cord blood were lower than in adult blood. The same phenomenon could be observed in adult samples (1.17 % in fresh blood and 2.68 % in cryopreserved) samples. Lower frequency of PDC in cord blood samples was also noted in studies on cord blood transplantation (54) but contradicted by other observations (3, 55) which also propagate widely discrepant PDC/MDC ratios in cord blood.

Cryopreservation has long been associated with decreased immune function, even being used to improve transplant reception. However, the effects of freezing differ between cell types. A reason for better survival of cryopreservation by PDC could not be deduced from our data yet it might be rewarding to explore the effect of cryopreservation on dendritic cells further in order to broaden the use of live-cell vaccines such as dendritic cell vaccines, as well as for flexibility in lab research. Preliminary research in this field has been begun (56).

Cryopreserved PDC differ in their marker expression pattern from fresh PDC (57). Frozen PDC were more easily activated by CPG A and B than fresh PDC. This was mainly visible in the unstimulated control group which underlines that cryopreservation seems to have an effect on intracellular signaling pathways.
As in fresh PDC, CPG B induced higher expression of activation and maturation markers than CPG A in cryopreserved PDC.

In total, neonatal PDC tolerated freezing better than adult PDC in terms of marker expression (although CD40 expression was a little lower in neonatal than in adult cryopreserved PDC, upregulation after CPG B stimulation was comparable). This contrasts with results from a whole-blood and purified PDC study by Gold et al. (58)

comparing adult peripheral blood and cord blood, like we did with isolated PDC. They state that fresh and cryopreserved cells showed similar results although they do not present data in proof. As stated before, we could not exclude the possibility that cryopreservation influences cell reactions. We therefore used only comparable cell groups (fresh vs. fresh, cryopreserved vs. cryopreserved) in the present study.

1.34 Effects of CPG A and CPG B on surface markers and IFNα production

Comparison of fresh cord blood and adult peripheral blood samples provided evidence for a superior capacity of CPG B to stimulate superior maturation and co-stimulatory marker expression than CPG A in fresh blood PDC. We determined that neonatal PDC can be stimulated by CPG B to express CD83, CD86, $CD86^{high}$, combined CD83/CD86 and CD40 on a level with adult PDC. CD80 expression was not much affected by CPG stimulation; therefore we got similar results for combined CD40/CD80 analysis. Our data validate the observation that maturation can be best induced by CPG B in neonatal PDC, as it can in adult PDC. Our results match those described previously by De Wit et al. who state that cord blood PDC function is impaired (42) and other earlier and later studies (31, 44, 47, 50, 59, 60). In 2008, Danis et al. described a similarly impaired maturation of PDC following TLR7 ligation (61).

Ito et al. have demonstrated that IFNα itself is a potent survival factor of PDC (62). We recognized hat CPG A improved IFNα production in adult PDC much more than in cord blood PDC (up to nanogram range, consistent with prior studies (44)). Cord blood PDC furthermore showed a lower IFNα production capacity after cryopreservation. Combination of CPG with CD40 ligand might increase CPG B-induced IFNα synergistically (47); however we did not study IFNα production under that condition.

In summary, the examination of the effects of CPG A and B on PDC alone proved that the same effects can be induced in neonatal PDC (CPG A induces IFNα expression whereas CPG B promotes survival, activation and maturation of PDC) that have been described for adult PDC (44). This matches the results of De Wit et al.'s whole-blood assay (42) which showed decreased ability of CPG A to stimulate IFNα production. Gold et al. (58) in contrast demonstrated similar levels of IFNα for neonates in adults in whole blood while their analysis of purified PDC revealed a

higher IFNα production by adults, again confirming our results. Different constellations of inhibitory factors in whole blood (such as B cells and IL10 secretion (63)) might be responsible for these seemingly divergent results.
Danis et al. confirmed our results in 2008. Danis' group also proved previous hypotheses stating that lower responsiveness to CPG stimulation is not due to lower TRL9 expression but to impaired nuclear translation of IRF7 (61). This was corroborated by live virus stimulation in the same study and again confirmed by Nguyen et al. two years later in their longitudinal study (50). According to Danis et al., defective IFNα/β production is already apparent at mRNA levels.

The maturation process of PDC in the first year of life has since been described elegantly in the study by Ngyuen et al. in which the authors of the De Wit paper of 2004 have participated (50).
Maturation, however, does not solve the problem that neonates are exposed to infection while "waiting" for acquisition of immunocompetence.

1.35 Cytokine production by PDC stimulated with CPG A and CPG B

In the first phase of our experiments, we had proven that neonatal cord blood PDC can overcome inherent maturation deficits. Next, we wanted to study if neonatal PDC can bring about T helper cell polarization by PDC. In order to do this, we studied the cytokine profiles expressed by PDC.

A good overview of the polarization of TLR-mediated cytokine response of neonatal APC (cf. Table 17) is given by Levy et al. in their 2007 paper on innate immunity of the newborn (4).

Table 1 | **Polarization of TLR-mediated cytokine responses of neonatal cord-blood-derived monocytes and antigen-presenting cells**

Cytokine	Relative expression in newborns	Effect of cAMP on cytokine production	General function	Comment	References
TNF	↓	↓	Pro-inflammatory; activates neutrophils; T_H1-cell response	TNF associated with spontaneous abortion and pre-term labour	71
IFNα	↓	↓	Antiviral; contributes to vaccine responses	Important for MHC class I expression	108,163
IFNγ	↓	↓	Activation of macrophages; induction of IL-12; T_H1-cell response	Newborns have impaired killing of intracellular pathogens	11
IL-12	↓	↓	p40–p35 heterodimer activates cell-mediated immunity; T_H1-cell response	Neonatal defect in p35 promoter nucleosome remodelling	126
IL-1β	↓	↓	Endothelial adhesion; fever; acute-phase response	Newborn febrile response is blunted	164
IL-6	↑	↑	Acute-phase response; inhibits tissue neutrophilia; inhibits T_{Reg} cells and promotes T_H17 cells	May contribute to acute-phase response at birth	12,165
IL-8	↑	↔	Neutrophil chemoattractant	Expression increased during hypoxia; role in parturition	166
IL-10	↑	↑	Anti-inflammatory; inhibits TNF, IL-1 and IFNγ production	Blocking IL-10 can restore IL-1 production	14,167
IL-23	↑	↑	p40–p19 heterodimer promotes T_H17 cells	IL-17 enhances epithelial expression of antimicrobial peptides	13,125

↑, increased; ↓, decreased; ↔, unchanged; cAMP, cyclic AMP; IFN, interferon; IL, interleukin; T_H, T helper; TLR, Toll-like receptor; TNF, tumour-necrosis factor; T_{Reg}, T regulatory.

(Source: Levy 2007 Innate immunity of the newborn)

Table 17 Polarization of TLR-mediated cytokines responses of neonatal cord-blood derived monocytes and antigen-presenting cells

Notable differences between adult and cord blood PDC as well as between cryopreserved and fresh PDC could be detected. This has been described earlier by our study group for the overall group of neonatal DC (64).

In the data on hand, we demonstrated that for cryopreserved samples, neonatal interferon and interleukin production was virtually non-existent. Only one neonate PDC donor produced IL8 after CPG B stimulation which can be seen as adult-like function. Contrary to the results for adult PDC in prior reports (PDC produce TNF in response to IL3 and thus promote their own maturation in an autocrine fashion), neonatal cryopreserved PDC did not show TNF production in response to IL3 priming. The results for adult cryopreserved PDC match those achieved in fresh blood adult PDC. As no research was found that specifically mentions cytokine productions by cryopreserved PDC, more data is needed to compare our results to those of other groups.

The outcome we saw in fresh blood samples was very different from cryopreserved samples:

Adult PDC did not produce IL12p70, as demonstrated in previous studies (31) though this might have been remedied by combination with CD40L (19, 35) or stimulation with other types of CPG (4).

CPG A proved to be the best inducer of TNF production in adult, more so than CPG B or control. This has been reported in a study by Bauer et al. in 2001 (31) but contrasts with results by Kerkmann et al. from 2003 (47) where CPG A and B achieved nearly similar cytokine levels. CPG A and B caused similarly low IL10 responses by adult PDC (median 3-4 pg/ml), around the same levels as controls. Duramad et al. described IL10 levels between 50 and 300 pg/ml, however they used a CPG dose twice that of ours (65). IL1β production in response to CPG B stimulation was not increased. Comparisons from literature could not be made because of lack of comparable material.

CPG stimulation did not increase IL8 production more than unstimulated controls, contrary to the aforementioned work by Bauer et al. (31). Our results differ from those achieved by Kerkmann (47) who showed, as we did, that even unstimulated PDC produce IL8. Furthermore, in their work IL8 levels ranged between 2000 and 8000 pg/ml with a noticeable increase of IL8 upon CPG A less than for CPG B stimulation. Our IL8 levels ranged between 5000 and 10000 pg/ml with no noticeable increase after CPG stimulation, regardless of CPG type.

Neonatal PDC did not produce IL12p70, a mediator of Th1 polarization (which again might have been remediated by CD40L administration though we did not use CD40L in this study). No CPG increased TNF production much more than for the control group. CPG B triggered an increase of IL10 production while CPG A had little effect. CPG A and B triggered similar amounts of IL6 both in neonates (again conform to a study by Prescott et al. (66). For IL1β no marked difference could be noted in neonates between types of CPG stimulation. Neonatal PDC showed a higher IL8 baseline than adults, but CPG stimulation did not increase IL8 production.

In summary of cytokine production by fresh blood PDC, we propose that neonatal PDC are not in fact inherently deficient in production capacity but present a lower baseline cytokine secretion (except for IL8 whose baseline secretion was higher than that of adult fresh blood PDC) which can moreover be stimulated by CPG to adult levels.

These results match those of a previous study by Krug et al., which demonstrated that CPG DNA as a unique microbial stimulus synergizes with CD40 ligand to induce high amounts of IL12 in adult PDC (35), and those of Duramad et al. where CD40L stimulation was needed for IL12 production (65).
Our results match those of the study of immunity in the first year of life by Nguyen for IL6, IL8, Il1β production (50). They studied whole blood samples, although stimulation was done with a CPG type to which PDC react alone. However, comparison should be done with caution because of interaction with other interleukin-producing cell types in whole cord blood. They also state that the results obtained from purified cord blood PDC in another study (61) do not match their whole blood results. Importantly enough though, both CPG A and CPG B were used in the first study and only CPG A in the study on purified PDC which accounts for the differences in cytokine production and thus matches our results well.

Interestingly, the study by Nguyen's group suggests that PDC reach adult-like function later in life than monocytes and MDC. The lack of IL12p70 production may be due to negative autocrine feedback via IFNα as postulated by McRae (67).
Higher cytokine levels after TLR stimulation in neonatal DC have been demonstrated before for IL10 and IL1β (64). The PDC in our hands partly matched these results. This may point to preferred induction of regulatory T cells in neonates (68), a process in which IL10 and IL1β are heavily involved. Inhibition of activation and maturation as described by Duramad et al. (65) was not explicitly studied by us. However, the strong maturation and activation signal sent by CPG A and B did not seem to be inhibited by autocrine IL10 secretion.

1.36 Cell-cell and supernatant-cell effect of CPG-stimulated PDC on T cells

After studying PDC cytokines profiles, we looked at the ability of primed PDC to promote division of autologous T cells. Mouse PDC activated by CPG and influenza virus have been shown to expand naïve $CD8^+$ T cell populations *in vivo* in response to respectively endogenous and exogenous antigens (69).
Cord blood, which we used as a donor of neonatal PDC, has been shown to contain T cells with a normal proliferative answer to primary allogeneic stimulation (70). Frequencies of alloreactive helper and cytotoxic T cells in cord blood are equal to or even beyond those encountered in adult peripheral blood (71).

The protocol used for T cell division was based on the study by Krug et al. (35). In this setting, we used a higher CPG dose (50 µg/ml) to accommodate for the dose-dependency shown in whole blood assay by De Wit et al. (42) in order to achieve maximum effect in T cells. Beside co-incubation of PDC and naive T cells, we also examined if PDC supernatant alone could prime naïve T cells. This concept had been established by our study group for neonatal DC (64).

No data could be found to compare our results to. A recapitulation of our results including a thorough interpretation is all that can be provided here.
We observed that neonates showed a higher spontaneous T cell division rate and that the same donor group is less prone to activation by CD3/CD28 stimulating beads (Dynabeads).
When direct cell-to-cell contact was permitted between PDC and T cells, neonatal PDC showed an overall reduced capability to promote T cell division, regardless of the initial PDC-priming stimulant, compared to their adult counterparts. The same can be said for T cells stimulated by supernatant from primed PDC.

There was no evidence that any of the stimulants tested above could boost T cell division in neonates to a rate similar to that of adults. Also, none of the stimulants yielded a consistent and markedly higher T cell division rate than the second control group with 2 Dynabeads per cell. This may be due to an “overshadowing” by the Dynabead stimulation and would explain the contrast to Duramad’s study from 2003 where his group achieved T cell proliferation after co-incubation with CPG-primed PDC (65). Harris et al. demonstrated in 2008 (72) that use of Dynabeads affects not only T cells but augments CPG-induced phenotypic maturation and IFNα production of PDC. This is seemingly mediated by the high cytokine content of medium conditioned by anti-CD3/antiCD28-activated T cells which they revealed to contain GM-CSF (granulocyte macrophage colony-stimulating factor), IFNα, IFNγ, IL1β, IL2, IL3, IL4, IL6, IL8, IL10, IL12, TNF and CD40L plus a number of other chemokines and soluble factors. It is important to note that the IFNα production and maturation and/or activation-marker levels in our study were measured without influence of Dynabeads or Dynabead-conditioned lymphocytes on the PDC cultured. We can thus exclude any Dynabead-mediated effect on these results. As our experimental setting

directly analyzed cell-cell-interaction between PDC and T cells, it should be repeated without Dynabead stimulation in further studies.

Analysis of interaction between T cells and PDC was supplemented by looking at T cell mean fluorescence intensity (MFI) after co-incubation with primed PDC. From the results gained, the conclusion could be drawn that neonatal T cells showed higher MFI in most of the higher division steps, in particular steps 3+4 (except in the case of CPG B stimulation and direct PDC to T cell contact). Thus, the study of MFI did not yield any results supporting a superior capacity of neonatal or adult PDC for T cell division after use of the stimulants studied.

In conclusion, neonatal autologous T cells seem to have a lower capacity to be stimulated to division by activated PDC than adult T cells. CPG stimulation did not help neonatal PDC overcome this defect.
Cell-to-cell contact generated similar levels of T cell MFI as supernatant, i.e. cytokine stimulation alone. The idea that supernatants of stimulated DC induce cytokine production naïve T cells by means of type I interferons was introduced earlier by our study group (Krumbiegel et al. 2007 (64)). Type I interferons released by PDC can affect T cell function, inducing early activation markers such as CD69, long-term T cell survival, IFNγ production and Th1 differentiation (64).
We therefore propose that PDC create a paracrine cytokine milieu in cell culture that allows T cells to divide.

It is also possible that in order to induce expansion of T cells in vivo, DC are needed with which PDC can cooperative to achieve this aim. Krug et al. presented data indicating that PDC can effectively stimulate pre-activated or memory T cells and thus wield an immune-regulatory function (73). This may account for the fact that while T cell profile differentiation can be elicited by PDC, the T cell populations in our hands did not expand following stimulation with CPG ODN.

Impaired T cell function in human neonates has also been described by Marchant et al. (74) and Velilla et al. (3) and it may be possible that PDC only amplify responses first initiated by MDC (75).

In summary, our results are in keeping with data from Krug et al. (73), underlining that PDC can only expand antigen-experienced but not naïve T cells in both the neonatal and the adult system.

1.37 T cell cytokine profiles after stimulation with primed PDC

After asserting the capability of neonatal PDC to expand autologous T cells, we looked at the T helper cell cytokine profile created by PDC-priming. The human immune system has two separate mechanisms for protection against different types of microbes. In response to intracellular microbes such as bacteria, viruses, fungi, and intracellular parasites, DC are made to produce IL12 and type I interferons. The primed DC then attempt to stimulate $CD4^+$ helper T cells to differentiate into IFNγ producing Th1 cells.

Activated Th1 cells activate macrophages and $CD8^+$ cytotoxic T cells to kill intracellular pathogens. Extracellular parasites such as worms cause Th2 cells to become activated. Th2 cells produce IL4, IL5 and IL13 which bring about IgE production. This activates mast cells and eosinophils to attack the extracellular pathogens.

To differentiate between T helper cell types, we focused on the following cytokine profiles: IFNγ and TNFβ were regarded as Th1 polarizing, while IL4, IL5 and IL10 were viewed as Th2 polarizing. It is now common knowledge that IL2 production cannot be viewed as either Th1 or Th2 polarization but promotes T cell survival in general. The fact that certain CPG ODN can enhance neonatal Th1 cell responses had been proven before (76) so we wanted to ascertain which T helper cell cytokine profile could be elicited by neonatal vs. adult PDC.

Neonatal T cells showed, in total, a lesser TNF production than adult cells. Neonatal PDC stimulated by CPG A and B improved TNF production a little, although TNF production by adult control T cells was markedly higher. CPG A/CPG B/CD40L suppressed TNF production in the long run in both age groups. Neonatal T cells could be stimulated by CPG A and CPG B primed PDC to produce the same amount of IFNγ as adult T cells, thus corresponding with earlier studies (1, 77).

Analysis of IL10 production showed that neonatal T cells can be stimulated by CPG A and CPG B-stimulated PDC to produce similar amounts of IL10 as adult T cells. Neonatal T cells showed a lower base rate of IL10 production. This matches Nguyen's results on the matter (50).
CD40L and combined CPG A (respective CPG B) + CD40L PDC stimulation did not enhance IL10 production in adult or neonatal T cells, rather lowered it, conform to the study by Bauer et al. (31).
It seems that CD40L-stimulation of PDC not only does not stimulate IL10 production in adult T cells but rather suppresses it. This is in contrast to the study by Grouard et al. that used mainly CD40L (7) and a newer review by Gilliet et al. (68) but corresponds to Prescott's study (66) on the matter.
Influencing the production of immunoregulatory cytokines such as IL10, TNF and IFNγ may be important in neonates with a high risk for allergic disease (78).

Neonatal T cell IL4 production did not show a reaction to any kind of stimulation. The greatest improvements in IL4 production by adult T cells were achieved by CD40L stimulation and combined CPG A+CD40L stimulation, stable over day 3 and 5. Combination of CPG B+CD40L seemed to suppress the effects of stimulation with CD40L alone in adults. The improvement of IL4 production by adult T cells primed with CPG A+CD40L or CD40L alone surpassed those described previously (33).

The results for IL5 production matched these results: Neonatal T cell IL5 production did not show any reaction to any kind of stimulation. The highest improvements in adult T cells were achieved by combining CPG B with CD40L. The results for IL4/IL5 fit those of older studies which showed that IFNα causes direct induction of IFNγ in T cells and inhibits IL-4/IL-5 production (79, 80) via activation of the STAT4 pathway (80).

IL2 production did not reveal a difference between PDC stimulants and the control group for both donor groups in keeping with earlier studies (80).

To sum up, TNF secretion in both adult and neonatal T cells seemed to be unchanged by PDC stimulation, though in total, neonatal T cells had a lower base rate TNF production. It could be shown that IFNγ and IL10 production by neonatal T

cells can be induced by stimulation with PDC primed with CPG A and CPG B to reach the cytokine levels attained by adults. This could not be reproduced in adult T cells. CD40L rather suppressed this development (even in combination with CPGs). Nonetheless, IL10 production is often found in strongly Th1-polarized T cell clones (17) so this may also mean that in our hands, neonatal T cells were in fact made to produce a Th1 response.

However, CD40L seemed to improve IL4 production in adult T cells, especially in combination with CPG A, though not with CPG B (matching Bauer's study of the subject (31)). This could not be reproduced in neonates. Adult T cells secreted IL5 at a higher base rate than neonatal T cells, though in both populations the secretion rate could not be changed much by PDC stimulation. IL2 production remained at the comparative levels between adults and neonates, with no differences induced by PDC stimulants.

Earlier work (35) revealed that simultaneous ligand-binding of CD40L on PDC and stimulation with CPG induces production of large amounts of both IFNα and IL12, which are both essential for Th1 cell formation. This was also demonstrated for stimulation with influenza virus and CD40L (34). However, in our setting, CD40L stimulation of adult PDC (especially in combination with CPG A) elicited a Th2 response. A negative feedback loop in the Th2 response of adults has been described for IL4 (33), although cognate interaction with T cells seems to inhibit this as seen in this study. Rissoan et al. described Th2 trending in T cells stimulated by PDC primed with IL3 and CD40L (33).

Our studies have shown on the one hand that adult T cells stimulated by autologous PDC activated by CD40L and combinations of CD40L plus CPG A or B are more prone to a Th2 response.
Neonatal T cells, on the other hand, stimulated by autologous PDC primed with CPG A or B are more prone to high IFNγ production, typical for a Th1 response (though not TNF which is also classically associated with Th1) while also producing higher IL10 levels upon CPG A and B PDC stimulation, congruent with early studies by Demeure et al. (80).

We hypothesize therefore that a clear decision towards a Th1 or Th2 profile induced by CPG A or CPG B stimulation of neonatal PDC cannot be concluded from the cytokine profile analysis in our hands. This may mean that trending towards a Th1 or Th2 profile depends on the type of direct stimulant plus other factors, as has been proposed before (31). If we take IL10 to be seen as a Th1 polarizing cytokine, it may conversely be deduced that CPG A and B stimulation initiated in fact a Th1 polarization. If IL10 is seen as Th2 polarizing, we might yet be looking at a mixed Th1/Th2 response.

Mixed Th1/Th2 primary responses to protein antigens in neonates have been known for decades (40). These could then be forced into a Th1 response by co-administration of CPG. Meanwhile, secondary responses seemed to be “locked into” Th2 response (40, 41).

The divergent results found in current literature might be due to more than one cause: IL3 gradually increases production of IL12 by PDC (19, 35). Thus, the discrepancy between the ability of CD40L-activated PDC to stimulate discrete T helper cell responses and the reciprocal function IFNα and IL12 in T cell priming might be owed to different time lengths of preincubation with IL3 used for maintaining PDC viability (33, 34). Inducing higher levels of presumably inhibitory cytokines also does not necessarily promote an inverse relationship with the production of effector cytokines. In a study by Dunstan et al. on atopic dermatitis in children, a strong positive correlation between IL10 responses and all other cytokines including Th1, Th2 and pro-inflammatory cytokines was seen (78).
Another cause on a cellular level might be that development of a certain T helper cell cytokine profiles may depend on the ratio of stimulator/responder as shown in studies on T cell activation by virus-primed DC (81).

The answer to the paradox of mixed Th1/Th2 responses may even lie in the limitation of the Th1/Th2 model. IL10 in humans suppresses the division and cytokine production of all T cells, but continues to stimulate plasma cells, thereby ensuring that antibody production occurs. As such, IL10 is not believed to truly encourage the Th2 response in humans, but acts to check over-stimulation of helper T cells while still amplifying antibody production.

The function of the ambiguous cytokine profile of neonatal T cells could accordingly be the inhibition of an overly inflammatory response in neonates. Thus, a toxic reaction of the newborn to infection might be avoided while antibodies are still produced, supporting the assumption that CPG A and/or B may be used as an adjuvant to improve the effect of vaccines in newborns.

Nevertheless, one has to be careful when promoting the use of new adjuvants. Olbrich et al. showed in a study on mice infected with Friend virus that para-immunization with CPG can lead to overwhelming illness if performed prior to infection (82) while Tobagus et al. (83) highlighted the possibility that adjuvants could even have paradoxical effects on neonatal immunity compared with adults. They observed in neonatal mice that Th1 or Th2 adjuvants can actually select against the answer they promote in mature individuals. Th1 activated cells might thereby be eliminated by regulatory populations, killing off these clones and favouring survival of Th2 populations.

As seen in our present human *in vitro* study, *in vivo* implications for human vaccine trials are as yet uncertain. Nonetheless, it is clearly oversimplistic to extrapolate results from adult studies to newborns. We suggest that PDC interpret their environmental condition by way of cytokine and chemokine receptors to effect a suitable answer and that the nature of environmental stimuli determines this answer. This underlines the fact that the different aspects of immune system response need to be evaluated further before advocating routine adjuvant use in human newborns. Of course this also means that the inclination of *in vitro* PDC to elicit Th1 or Th2 responses in neonates needs to be studied in front of a background of different activation schemes as Bauer et al. proposed earlier for adults (31).

The question remains: Is the default setting for newborns a Th2 response? Is a Th2 response in neonates needed to keep the immune system in check while immunological memory develops in the overwhelming presence of pathogen without succumbing to septic shock? If so, how can we influence the neonatal immune system so that the scale between Th1 and Th2 response does not tip too heavily towards Th1?

Other immune cells also produce many of these cytokines and, while the original Th1/Th2 is a good working model and provides many insights into the functions of T helper cells, it is more and more proving to be too simple to define its entire role or actions. *In vivo* studies suggest that some helper T cells might not always match a specific Th1/Th2 profile. Many cells even express cytokines of both profiles – like the PDC-primed T cells in our study. That being said, the Th1/Th2 model is important for us to understand the behavior of T helper cells and the cytokines they express during immune response. Further doubt on the basic Th1/Th2 model has been cast by the characterization of another new T helper subtype, T helper 17 cells (Th17) (84). These IL17 producing cells were first described as a pathogenic population, involved in autoimmunity. However, today they are thought to possess distinctive effector and regulatory functions. Gilliet et al. demonstrated in 2002 that PDC are not only immunogenic, but also tolerogenic: PDC-derived DC induced by IL3 with CD40L can stimulate naive $CD8^+$ T cells to evolve into IL10–producing suppressor T cells (85).

Analysis of T cell cytokine production was not repeated for analogous T cell stimulated with PDC supernatant alone. This had been done successfully in our study group for overall neonatal DC (64) and should be considered in future *in vitro* studies.

1.38 Intracellular phosphorylation of PDC under the influence of CPG A and B

The idea that CPG-induced production of IFNα involves p38 MAPK-dependent STAT1 phosphorylation in human PDC has been put forth by Takauji et al. in 2002 (86). Phosphorylation of STAT1 under the influence of CPG A and B has been proven by Kerkmann et al. (47) and Domizio et al. in 2009 for TLR7 (45).

Differences in STAT1 phosphorylation after TLR stimulation between adult and neonatal DC have since been demonstrated by our study group (64). STAT1 was furthermore selected for functional analysis of PDC because of its involvement in IL12p70 production (87) by MDC elicited by type I interferons favoring Th1 responses.

The best results for percentages of intracellular phosphorylation of p38 MAPK and STAT1 and phosphorylation intensity of these kinases were achieved by stimulation

over 10 minutes. Stimulation over longer periods did not seem to be superior therefore we chose to continue the stimulation experiments with 10 minute intervals. This might be due to intracellular dephosphorylation of the kinases studied after longer periods of stimulation.

Due to donor number restrictions, we had to work partly with cryopreserved cells once more. Reminiscent of surface marker expression, IFNα production and cytokine production, we noted big differences between cryopreserved and fresh PDC. Frozen neonatal PDC (one donor tested) exhibited stronger phosphorylation (both in intensity and percentages) than adult PDC.

In fresh PDC, CPG A or CPG B did not improve MFI of p38 MAPK phosphorylation in neonates or adults compared to PMA stimulation. However, looking at the percentages of PDC showing MAPK phosphorylation, CPG A showed to be a potent stimulating agent (close to PMA) in adults (median 56.1 % vs. 67.2 %) but not in neonatal PDC.

Differences in STAT1 phosphorylation after TLR stimulation between adult and neonatal DC had been shown previously by Krumbiegel et al. in our study group in 2007 (64). Fresh neonatal PDC did not gain in MFI of STAT1 phosphorylation after stimulation compared to the control group, while adult PDC reacted best to CPG A. The percentage of STAT1 phosphorylated PDC increased in neonates the most after CPG B stimulation. In adults, percentages actually decreased following CPG A stimulation (more than after CPG B stimulation) while IFNγ (the positive control for STAT1 phosphorylation) remained a little higher than unstimulated control PDC.

In order to analyze if CPG A and CPG B had a real stimulatory effect superior to the positive controls PMA and IFNγ it was necessary to deduct the values reached by CPG A and CPG B stimulation from the levels attained via PMA and IFNγ stimulation. The positive controls were PMA for p38 MAPK and IFNγ for STAT1. The results discussed below are for the difference of the results of CPG A to PMA, respective IFNγ stimulation and the difference of CPG B to PMA, respective IFNγ stimulation.

In conclusion, CPG A or B stimulation did not improve the percentage of PDC showing p38 MAPK phosphorylation in neonates more than PMA. However, both CPG types improved p38 MAPK phosphorylation in adult PDC. CPG A and B did not increase phosphorylation of p38 MAPK in terms of MFI in neonates more than PMA. Decreased p38 MAPK phosphorylation in cord-blood monocytes has previously been described by Levy et al. and others (4).

CPG A stimulation did not enhance percentage of PDC showing STAT1 phosphorylation in neonates much more than IFNγ while CPG B stimulation boosted STAT1 phosphorylation in neonates. Both CPG types suppressed STAT1 phosphorylation in adults. This is contrary to the observation by Kerkmann et al. that STAT1 is equally up-regulated by both CPG A and CPG B (47). Differences in MFI of phosphorylated STAT1 did not provide new insights.

In fresh blood neonatal PDC the phosphorylation of p38 MAPK was not boosted by CPG A or B although they showed a baseline higher than that of adult fresh blood PDC. Stimulation of adult fresh blood PDC with CPG A (more than CPG B) improved p38 MAPK phosphorylation, up to the level of that in neonatal fresh blood PDC. This is surprising as we showed CPG A to induce high levels of IFNα in adult and neonatal PDC. We hypothesize therefore that CPG A-mediated IFNα production in neonatal PDC is at least partly independent of p38 MAPK.

Summing up the results of STAT1 phosphorylation, CPG B improved STAT1 phosphorylation in neonatal fresh blood PDC to levels higher than that of adult PDC while lowering MFI a little. However, IFNγ used as positive control did not boost STAT1 phosphorylation more than in unstimulated control PDC. Meanwhile, CPG A and B stimulation of adult PDC rather lowered the percentage of STAT1 phosphorylation while boosting MFI which leads to the assumption that it actually suppressed STAT1 phosphorylation. CD40L-supported CPG stimulation as studied by Kerkmann et al. (47) was not examined in this context.

Overall, PDC activation, maturation in neonatal PDC (as effected by CPG B) seems to rely more on STAT1 phosphorylation. IFNα production seems to be largely independent of p38 MAPK phosphorylation in neonatal PDC.

1.39 Use of CPG ODN as an adjuvant in neonatal vaccines

The different effect of CPG depending on their structure correlates to their intracellular trafficking: Multimeric CPG A ODN are retained within endosomes while monomeric CPG B ODN are relocated to lysosomal compartments, prompting activation of separate signaling factors (88, 89).The recent work on CPG C ODN, which are able to activate both PDC and B cells (90) has yet to be translated into cord blood studies. Last but not least, optimal stimulation of TLR may not only be a question of which agent to use, but also which combination of TLR stimulations, thus inducing robust Th1 responses (91).

The efficiency of CPG DNA as an adjuvant has been proven by a number of clinical trials in human adults. However there had been little data so far that furnished evidence of a cellular immune response in humans.

This study has shown that CPG DNA may be used to induce a potent humoral immune response in neonatal PDC *in vitro*. A possibility to further study the effects of CPG motifs on the immune system of neonates would be to use human CPG motifs in mice with a humanized immune system (Hu-PBMC-SCID mice) although, of course, the murine and the human immune system still differ to a large extent.

The regulation and dynamic of CPG uptake are being increasingly elucidated and their biological effectiveness in animals has been solidly proven. Clinical trials with human patients are underway.

The effects of cryopreservation also need to be taken into consideration in this context. The ever-broadening field of cord blood transplantation uses standardized operating procedures for freezing and thawing in cord blood banks. These represent major factors in immunogenicity of cord blood transplants (92). The inherent “defects” of cord blood PDC in cytokine production and T cell priming compared to their capacity in adults should be considered when studying increased susceptibility to microbial infections in recipients of cord blood transplants (54, 92).

Last but not least every new component of a medication or vaccine should be considered in terms of autoimmunity. TLR9 has repeatedly been found to play a part in generation of autoimmunity (93). CPG DNA does not cause autoimmunity if it is not bound in immune complexes. Apparently, there are suppressive DNA motifs

which selectively block the immune response via TLR9, which might explain the lack of immune response after stimulation with vertebrate DNA (94). Suppressive DNA sequences might thus represent future therapeutic targets in autoimmune disease (95, 96).

1.40 Limitations of our model system

First of all, all our experiments were performed using low donors numbers – a method common in immunology although it restricts the statistical power of the results. The results need to be analyzed with care: interindividual variability between donors was high so that strict dichotomies should not be presumed. In this respect the character of this study is more of a feasibility study and a lot more research should be undertaken on this matter.

The comprehensive comparison and characterization not only of the results of an experiment but also of the methods used needs to be done before proposing hypotheses on cellular workings.

Use of BDCA-4 allowed us to specifically isolate PDC from cord blood and adult peripheral blood samples from healthy donors as described earlier (19). However, earlier studies (97) already indicated that BDCA-4/Neuropilin-1 was actually involved in the initiation of immune response.

The principle of positive selection method via MACS for example has been shown to indeed have side effects on the cells separated. The dangers of positive selection methods have been mentioned early on (51). Fanning et al. had observed that when PDC were positively selected using anti-CD4 Ab-conjugated microbeads, these positively selected PDC had a reduced capacity to produce IFNα in response to stimulation with herpes simplex virus than would have been predicted by relative-fold enrichment (98). In 2006, a year after initiation of this study when the greatest body of experimental work had been completed, they showed that cross-linking of cell-surface markers such as BDCA-4 and others reduced the frequency of IFNα producing cells. Cross-linking did not inhibit endocytosis (and thus reduced pathogen recognition) or induce apoptosis. This explains why anti-BDCA-4 continued to be used for PDC isolation by many groups for a number of years. BDCA-4 cross-linking also influenced intracellular tyrosine phosphorylation (with intracellular phosphorylation returning to normal within 10 minutes, indicating triggering of a

signaling cascade) and translocation of IRF7 to the nucleus. Kerkmann et al. had previously reported (47) that anti-BDCA-2 but not anti-IFN-receptor antibody or anti-BDCA-4 antibody triggers intracellular calcium flux in PDC. Fanning et al. underlined the possibility that *in vitro* cross-linking of cell surface receptors leads to overstimulation of the cell, which induces a pathway to shut off the cell instead of activating it like a natural ligand might do. BDCA-4 positively selected PDC were still able to react to CPG stimulation in our study and those of other groups (42), however a possible decrease in IFNα levels and thus reduced efficiency in T cell stimulation must be kept in mind.

Negative selection methods have been in use since then, compare for example Danis' study (61). The consequences lead us to conclude that negative selection is the key for purification of PDC if IFNα is to be the vantage point from which to measure PDC activity and BDCA-4 is not solely used as a PDC marker. This finding which is underlined by the present study is in contrast to previous studies which showed that CD304 (BDCA4, Neuropilin-1) antibody binding did not have a substantial effect on IFN type I production in PDC after challenge with e.g. influenza virus. Further studies are needed to investigate this result. The effect of this study has already led to changes in the commercial biotechnology sector. The producer of the microbeads used for the MACS method, Miltenyi Biotec has changed their standard PDC isolation kit to a new method – not enrichment by positive selection, but depletion of non-PDCs (negative selection). Thus, all direct effects on cell functions of the PDC may be avoided so that the further course of the experiment is not impaired.

Another limitation for the study of PDC and many other subpopulations is their rarity. Cord blood provides us with a large number of PBMC. However, the possibility that a maternal influence in cord blood on the characterization of neonatal DC exists (1) sets the stage for neonatal peripheral blood DC as the ideal study population (3). New technologies (such as image streaming included in FACS analysis which enables single-cell analysis) will make it possible in the future to use neonatal peripheral blood in small volumes without causing anemia in the newborn. This will be the base for more longitudinal studies following up neonates immunized at birth.

1.41 Enigmatic PDC

Although discovered long ago, PDC continue to be an enigma – albeit one that is being solved step by step. Recent research demonstrated that PDC can acquire alloantigen in allografts and home to lymph nodes (99) – a process that requires at least partial internalization of cell debris. PDC had been described as poorly endocytic up to this point. The overview of current literature elucidates that PDC not only have the potential to prime T cells to a cytotoxic Th1 or a Th2 response: They even exhibit a tolerogenic function in peripheral blood (68, 99, 100) as well as the thymus (18).

Future research on PDC should include the study of CPG ODN in many varied settings modeling *in vivo* interactions in order not to tip the scale between immunogenicity and tolerogenicity the wrong way. Studying the function of plasmacytoid dendritic cells *in vivo* is not made easier by the plasticity of the dendritic cells (101, 102) and care should be taken not to accept dichotomic results in scientific settings as a given, a view already put forth by Greenspan in 2007 (103).

1.42 Outlook on PDC research

Our study represents one of the earliest studies on cord blood PDC response to CPG stimulation, analyzing responsiveness in terms of maturation and activation marker expression, cytokine production, autologous T cell expansion and intracellular phosphorylation. Some research has been conducted on PDC interaction with B cells but our work constitutes one of the few early studies looking at interaction with naïve T cells and T cell profile delineation.

Later studies, such as the important longitudinal study of Corbett et al. (5) have confirmed our findings in detail and in general. They contradict the notion that the ontogeny of the innate immune response to TLR stimulation linearly progresses from birth to adulthood and instead propose an age-specific response.

Another aspect in reviewing PDC capabilities is polyfunctionality. Kollmann et al. showed in their elegant high-throughput study (55) that neonatal PDC are strikingly less responsive and less polyfunctional than adult PDC. Meanwhile, conventional dendritic cells and mononuclear cells exhibited very similar responses to TLR stimulation. It must be taken into consideration that his group only used CPG A. Our results match theirs for IFNα and TNF production mediated by CPG A (and also

match the difference they found between adult and neonatal CPG A-stimulated production of these cytokines). Interestingly, their direct comparison of cytokine expression between whole blood and cell culture of mononuclear cells also shows that these cytokines are actually inhibited in whole blood samples. Our study did not match theirs on IL1β and IL6 production; however we used CPG A and B for PDC stimulation while they used CPG A and other TLR ligands.

Kollmann and his group concluded on the basis of their measured cytokines profiles that the innate response early in life appears to be directed away from IL12p70 toward IL23 and Th17 dominance. This seems to support the indecisive helper-cell polarization we saw after CPG stimulation, though we could not prove this directly. Single-cell polychromatic flow cytometry as done by Kollmann's group is noteworthy for studies of polyfunctionality, which to our knowledge they were the first to address on a single-cell level in human neonates. However, overall cytokine levels will remain the hallmark in future clinical trials for some time.

Their data shows that the neonatal innate immune response does not simply suffer from quantitative deficiencies compared with adults but rather is characterized by a response pattern that is qualitatively different (55), shifting towards shift an enhanced Th17 type immune response.
Th17 cells induce the production of antimicrobial peptides by epithelium and promote a phagocytic response to extracellular bacterial and fungal pathogens at, but not limited to, the body surfaces. Th1 cells meanwhile are more involved in systemic defense against intracellular pathogens. This may represent a different defense mechanism than current research expected and tried to enforce with trials to effect a Th1 response.
The newborn immune system may thus be inclined to attack invading pathogens before they actually enter the intracellular compartment where an anti-intracellular immune response takes longer to take effect. Our data support this theory. CPG A and B stimulation supports this response type and more research should be done on the effect of C type CPG ODN on neonatal PDC as Poeck et al. have done (104).

In conclusion, neonatal PDC seem to reach maturity within the first years of life (50) and the intra-uterine environment seems to condition dendritic-cell function. Maternal

immunology and history of infection as well as genetics (4) also influence newborn immunology. Consequently, calculating these factors will move us in the same direction as the development of pharmaceuticals based on individual gene and therefore enzyme expression, “easily” studied by gene panels using only small amounts of blood.

1.43 Socio-economic value of neonatal vaccines

The use of personalized vaccines is nonetheless not easily applicable for the setting in which the best use can be made of vaccinating newborns against common pathogens: vaccination programs in resource-poor countries in the developing world.

Birth is a valid point of contact with health-care worldwide. Vaccines given at birth reach a relatively high proportion of the population, thus the potential of certain TLR agonists capable of efficiently activating Th1 polarizing responses from neonatal APC are of substantial interest as novel neonatal vaccine adjuvants (4, 41). New and powerful TLR agonists may embody tools to enhance neonatal defense against microbes (105) and shift innate immunity away from Th2 polarization, thus potentially reducing allergy (106).

Vaccination has been proven to be the single most efficient and cost-effective medical intervention so far. Great steps have been taken in improving the resistance of vaccines against mechanical impact and heat – the great challenges in vaccination logistics. Combining these advances with a highly efficient single-shot vaccine against a broad spectrum of pathogens is the ultimate goal future research on neonatal vaccines should aim for.

Summary in English

Introduction

Neonatal immunity is impaired and/or depressed, favouring a Th2 over a Th1 response and making newborns prone to infection while also inhibiting full immunogenicity in answer to vaccination.

As the main IFNα producing cell type, PDC influence many other cells of the immune systems in the fight against viral and bacterial infection and constitute a key interface between innate and adaptive immunity. CPG ODN induce expression of co-stimulatory cell surface molecules, maturation markers and production of cytokines in PDC. Co-culture with activated PDC can generate an allogeneic Th2 T cell response as well as well as a Th1 response. CPG are therefore considered potential adjuvants to enhance vaccine response. This study aims to aid vaccine development for newborns by establishing the physiological reactions of neonatal PDC to CPG as compared to adult PDC.

We therefore decided to determine how human cord blood PDC react to stimulation with CPG ODN and if these PDC promote division of autologous T cells and induce a bias in T helper cell response towards Th1 or Th2 compared to adult PDC.

Material and methods

Human neonatal cord blood PDC were compared to adult peripheral blood PDC in their response to stimulation with CPG oligonucleotides and CD40L. To judge direct cell response, we analyzed surface activation and maturation markers and cytokine production by PDC. In order to explore PDC interaction with T cells we studied T cell division and cytokine production after co-incubation with primed PDC. Finally we examined changes in phosphorylation in intracellular signaling molecules in PDC stimulated with CPG ODNs.

Results

Neonatal cord blood PDC can be made to up-regulate activation and maturation markers by CPG A and CPG B on similar levels as adult PDC, though their capacity to produce IFNα is reduced.

The competence of neonatal PDC to produce some cytokines is lower than in adult PDC. Their autologous T cells seem to have a lower capacity to be stimulated to

division by activated PDC than adult T cells. CPG stimulation or CD40L priming did not help neonatal PDC overcome this defect.

T cells nudged into cell division by PDC need a paracrine milieu in order to do this.

Examination of Th1/Th2 profiles proved that a clear decision towards a Th1 or Th2 profile induced by CPG A or CPG B stimulation of neonatal PDC could not be concluded from the cytokine profiles. Therefore a general T cell activation is most likely.

On the level of intracellular phosphorylation, only CPG B improved STAT1 phosphorylation in fresh blood neonatal PDC to levels higher than that of adult PDC, consistent with findings of cytokine experiments.

Cryopreservation negatively influences the survival of PDC and their ability to be activated and produce cytokines.

Discussion

Neonatal cord blood PDC can overcome inherent maturation deficits by means of stimulation with CPG ODNs on the level of surface activation and maturation markers. The cytokine profiles expressed by neonatal PDC demonstrate that they are not in fact inherently deficient in cytokine production capacity but in most cases present a lower baseline cytokine secretion which can moreover be stimulated by CPG to adult levels. We observed that neonates showed a higher spontaneous T cell division rate and that they also are less prone to activation. There was no evidence that any of the CPG ODN or CD40L could boost T cell division in neonates to a rate similar to that in adults. Our results underline that PDC can only expand antigen-experienced but not naïve T cells in both the neonatal and the adult system.

A clear decision towards a Th1 or Th2 profile induced by CPG A or CPG B stimulation of neonatal PDC cannot be concluded from the cytokine profile analysis. This may mean that trending towards a Th1 or Th2 profile depends on the type of direct stimulant plus other factors. Mixed Th1/Th2 primary responses could then be forced into a Th1 response by co-administration of CPG.

The function of the ambiguous cytokine profile of neonatal T cells could accordingly be the inhibition of an overly inflammatory response in neonates, thus supporting the assumption that CPG A and/or B may be used as an adjuvant to improve the effect of vaccines in newborns. The answer to the paradox of mixed Th1/Th2 responses may lie in the limitation of the Th1/Th2 model and the influence of neonatal PDC on

another new T helper subtype, T helper 17 cells. PDC seem to interpret their environmental condition by way of cytokine and chemokine receptors to affect a suitable answer. Optimal stimulation of TLRs may not only be a question of which agent to use, but also which combination of TLR stimulants induces the most robust Th1 response.

Differences between adult and neonatal PDC behavior might be attributable to a maturation process. Maturation, however, does not solve the problem that neonates are exposed to infection while “waiting” for acquisition of immunocompetence. Neonatal PDC seem to reach maturity within the first years of life and the intra-uterine environment seems to condition dendritic-cell function. Maternal immunology and history of infection as well as genetics also influence newborn immunology.
Neonatal PDC are strikingly less responsive and less polyfunctional than adult PDC. This is why we looked at the effect of CPG on neonatal PDC: to use CPG ODN as an adjuvant in neonatal vaccines. This study has shown that CPG DNA may be used to induce a potent humoral immune response in neonatal PDC *in vitro.*

Of course, there are limits to what our experiments can tell us. The first is statistics: Low donor numbers mean that the character of this study is more of a feasibility study and a lot more research should be undertaken on this matter on a larger scale. New technologies will make it possible in the future to use neonatal peripheral blood in small volumes without causing anemia in the newborn. The second is the effects of cryopreservation which need to be taken into consideration in this context. The third is the issue of positive cell selection: BDCA-4 positively selected PDC were still able to react to CPG stimulation in our study and those of other groups, however a possible decrease in IFNα levels and thus reduced efficiency in T cell stimulation must be kept in mind. Negative selection methods have become more common in recent time.

Future research on PDC should include the study of CPG ODN in many varied settings modeling *in vivo* interactions in order not to tip the scale between immunogenicity and tolerogenicity the wrong way. Our study represents one of the earliest studies on cord blood PDC response to CPG stimulation, analyzing responsiveness in terms of maturation and activation marker expression, cytokine

production, autologous T cell expansion and intracellular phosphorylation. Our work constitutes one of the few early studies looking at PDC interaction with naïve T cells and T cell profile delineation.

The interesting cytokine profiles exhibited by neonatal PDC suggest that the newborn immune system is inclined to attack invading pathogens before they actually enter the intracellular compartment where an anti-intracellular response takes longer to take effect. CPG A and B stimulation support this response type.

Vaccination has been proven to be the single most efficient and cost-effective medical intervention so far. This is why resource-poor countries in the developing world are the setting in which the best use can be made of vaccinating newborns against common pathogens. Reducing childhood mortality and morbidity is the goal of every vaccination study in pediatric immunology: Improved child health translates into improved health in adults, allowing effective education and increasing economic stability.

Conclusion

Human cord blood PDC react to stimulation with CPG A and CPG B with an increase in cell surface activation and maturation markers and cytokine production, similar to adult PDC. Intracellular phosphorylation in neonatal PDC did not benefit from CPG stimulation, in contrast to adult PDC. Cord blood PDC primed with CPG A, CPG B and CD40L do not promote division of autologous T cells contrary to adult PDC. Priming of neonatal PDC with CPG A or CPG B does not induce a clear bias in T helper cell response towards Th1 or Th2 while adult PDC trend towards a Th2 response.

Zusammenfassung in Deutsch

Einleitung

Das Immunsystem von Neugeborenen ist nur eingeschränkt funktionsfähig. Häufig wird eine Th2-Antwort ausgelöst, die Neugeborene nicht vor Infektionen schützt und eine ausreichende Immunantwort auf eine Impfung verhindert.

Plasmazytoide dendritische Zellen (PDC) produzieren von allen Zelltypen die größten Mengen IFNα. Sie beeinflussen viele andere Zellen des Immunsystems in der Abwehr bakterieller und viraler Infektionen und stellen eine wichtige Schnittstelle zwischen angeborener und erworbener Immunität dar. CPG Oligodeoxynucleotide (CPG ODN) fördern die Expression von kostimulatorischen Molekülen und Reifungsmarkern auf der Zelloberfläche der PDC. Die Ko-Inkubation mit aktivierten PDC kann sowohl eine allogene Th2- wie auch eine Th1-Antwort hervorrufen. CPG ODN werden deshalb als potentielle Adjuvanzien erachtet, die die Impfantwort verbessern sollen. Das Ziel dieser Studie war es, die Entwicklung von Impfstoffen für Neugeborene zu unterstützen, in dem wir die physiologische Reaktion von neonatalen PDC aus Nabelschnurblut mit der Reaktion adulter PDC aus peripherem Blut verglichen.

Wir entschieden uns daher zu untersuchen, wie humane neonatale Nabelschnurblut-PDC im Vergleich mit adulten PDC auf die Stimulation mit CPG ODN reagieren und ob diese PDC die Teilung autologer T-Zellen stimulieren und eine Tendenz der T-Zellen zu einer Th1- oder Th2-Antwort hervorrufen können.

Material und Methoden

Humane neonatale Nabelschnurblut-PDC wurden in ihrer Antwort auf die Stimulation mit CPG ODN und CD40L verglichen. Um den direkten Effekt auf die PDC zu analysieren, untersuchten wir die Expression von Aktivierungs- und Reifungsmarkern auf der Zelloberfläche sowie die Zytokin-Ausschüttung der PDC. Um die Interaktion von PDC mit den T-Zellen zu beurteilen, prüften wir die T-Zell-Teilungsrate und ihre Fähigkeit, nach Ko-Inkubation mit aktivierten PDC, Zytokine zu produzieren. Schließlich erforschten wir die intrazelluläre Phosphorylierung aktivierter PDC, um die Signaltransduktion, die der Aktivierung zugrunde liegt, zu beleuchten.

Ergebnisse

Neonatale PDC aus Nabelschnurblut können Aktivierungs- und Reifungsmarker nach Stimulation mit CPG A und B auf ein adultes Level hochregulieren. Ihre Fähigkeit IFNα und einige andere Zytokine zu produzieren ist jedoch geringer als die adulter PDC. Die autologen T-Zellen neonataler PDC weisen nach Stimulation eine geringere Zellteilungsrate auf als adulte PDC. Ihre Spontanteilungsrate ist jedoch höher. CPG oder CD40L als Stimulanzien konnten die Zellteilungsrate autologer PDC, die mit aktivierten PDC ko-inkubiert waren, nicht verbessern. Jegliche Zellteilungsrate in diesem Kontakt scheint von parakriner Sekretion statt Zell-Zell-Interaktion abhängig zu sein.

Die Zytokin-Profile der oben beschriebenen T-Zellen zeigen keinen klaren Trend zu einer Th1 oder Th2-Profil. Daher erscheint eine generelle T-Zell-Aktivierung wahrscheinlicher.

Auf dem Niveau der intrazellulären Phosphorylierung konnte nur CPG B die Phosphorylierung von STAT1 in neonatalen PDC so verbessern, dass sie der in adulten PDC gleichwertig war. Dieses Ergebnis passt zu den Resultaten der Zytokin-Profile aktivierter PDC.

Die Kryokonservierung beeinflusst die Überlebensrate der PDC negativ, genau wie ihre Fähigkeit aktiviert zu werden und Zytokine zu produzieren.

Diskussion

Neonatale PDC können ihre Reifungsdefizite auf dem Level von Reifungs- und Aktivierungsmarkern auf der Oberfläche durch die Stimulation mit CPG ODN überwinden. Ihre Zytokin-Profile zeigen, dass sie nicht anlagebedingt defizient in der Zytokin-Produktion sind, sondern meist nur eine geringe Basismenge an Zytokinen produzieren und diese durch die Stimulation mit CPG ODN auf ein adultes Level gebracht werden können. Neugeborene zeigten eine geringere spontane T-Zell-Teilungsrate und ihre T-Zellen ließen sich schlechter aktivieren. Leider gelang es auch mit CPG ODN oder CD40L nicht, die Zellteilung zu steigern. Unsere Ergebnisse unterstreichen damit die Erkenntnis, dass PDC nur antigen-erfahrene, aber nicht naive T-Zellen expandieren können.

Eine klare Entscheidung für ein Th1- oder Th2-Zytokinprofil ließ sich aus unseren Ergebnissen nicht ableiten. Das könnte bedeuten, dass die Zuordnung zu einem dieser Profile nicht nur vom dem Stimulans, sondern von weiteren Faktoren abhängt.

Eine derart gemischte Zytokin-Antwort könnte dann mittels CPG-Ko-Stimulation zu einer Th1-Antwort werden.
Die Funktion einer so eindeutigen Zytokin-Antwort könnte in der Verhinderung einer überschießenden Immunantwort bei Neugeborenen liegen, was die Verwendung von CPG als Vakzin-Adjuvans unterstützen würde. Die Antwort auf die paradox gemischte Th1-/Th2-Antwort könnte aber auch in den Grenzen des Th-1/Th2-Modells sowie im Einfluss von PDC auf immunregulatorische Th17-T-Zellen liegen.
PDC scheinen ihre Umgebung über Zytokin- und Chemokin-Rezeptoren wahrzunehmen, um darauf eine passende Antwort zu gestalten. Die optimale TLR-Stimulation könnte also nicht nur von der Frage abhängen, welche TLR-Stimulanzien benutzt, sondern auch wie diese kombiniert werden müssen, um eine gute Th1-Antwort zu erzielen.

Die Unterschiede zwischen adulten und neonatalen PDC könnten von einem Reifungsprozess abhängig sein. Reifung löst aber nicht das Problem, dass Neugeborene potentiellen Infektionen ausgesetzt sind und daher nicht auf die Erlangung voller Immunkompetenz „warten“ können.
Scheinbar reifen neonatale PDC im ersten Lebensjahr aus und schon die intrauterine Umgebung scheint die neonatale Antwort dendritischer Zellen zu konditionieren. Das Immunsystem der Mutter und ihre Krankheitsgeschichte sowie genetische Aspekte spielen eine große Rolle in der Entstehung des neonatalen Immunsystems.
Neonatale PDC sind auffällig anerg und weniger multifunktional als adulte PDC. Daher entschlossen wir uns, die Verwendbarkeit von CPG ODN als Impfadjuvans für die Impfung von Neugeborenen zu untersuchen. Diese Studie konnte zeigen, dass CPG ODN eingesetzt werden kann, um eine potente humorale Immunantwort bei neonatalen PDC *in vitro* zu erzeugen.

Jedoch sind auch unsere Experimente nicht allgemein gültig, sondern unterliegen Einschränkungen. Die erste ist statistischer Natur: Aufgrund der kleinen Spenderzahlen hat diese Studie den Charakter einer Machbarkeitsstudie. Weitere Forschung auf diesem Gebiet sollte mit höheren Spenderzahlen arbeiten. Neue Technologien werden es in Zukunft möglich machen mit kleinen Blutvolumina zu arbeiten, ohne eine iatrogene Anämie hervorzurufen. Die zweite Einschränkung ist die Kryokonservierung, deren Auswirkungen unbedingt Beachtung finden müssen.

Die dritte Einschränkung ist das Problem der positiven Selektion: PDC, die mittels positiver Selektion über BDCA-4 gefiltert wurden, konnten in unserer Studie (und denen anderer Gruppen) noch gut auf die CPG-Stimulation reagieren. Es ist jedoch durchaus möglich, dass ihre Fähigkeit IFNα zu produzieren und so auch T-Zellen zu beeinflussen, deutlich reduziert war. Seitdem dies bekannt ist, werden zunehmend Methoden mit negativer Selektionstechnik angewendet.

Zukünftige Untersuchungen an humanen PDC sollte CPG ODN in vielen verschieden Kontexten untersuchen, die die *in vivo* Bedingungen so gut wie möglich nachstellen, um eine ungünstige Verschiebung zwischen Immunität und Toleranz zu verhindern. Unsere Studie ist eine der frühesten zur Antwort von Nabelschnurblut-PDC auf CPG-Stimulation, die sich mit Marker-Expression, Zytokin-Ausschüttung, autologer T-Zell-Teilung und intrazellulärer Phosphorylierung beschäftigt. Desweiteren stellt unsere Arbeit eine der frühesten Untersuchungen zur Interaktion von neonatalen PDC mit T-Zellen und der Abgrenzung von T-Helferzell-Profilen dar.

Die interessanten Zytokin-Profile, die von neonatalen PDC ausgeschüttet werden, weisen darauf hin, dass das Immunsystem von Neugeborenen Pathogene attackiert, bevor diese überhaupt den intrazellulären Raum erreichen können. Eine Stimulation mit CPG A und B unterstützt diesen Vorgang.

Impfungen sind bewiesenermaßen die effizienteste und kosteneffektivste medizinische Intervention. Darum bringt die Impfung von Neugeborenen gegen ubiquitäre Pathogene den größten Nutzen in Entwicklungsländern mit geringen Ressourcen. Die Reduktion von Mortalität und Morbidität im Kindesalter ist das ultimative Ziel jeder Impfstoffstudie in der pädiatrischen Immunologie. Eine verbesserte Gesundheit im Kindesalter führt zu besserer Gesundheit im Erwachsenenalter und erlaubt somit effektive Bildung und größere ökonomische Stabilität.

Schlussfolgerung

Humane Nabelschnurblut-PDC reagieren auf die Stimulation mit CPG A und CPG B mit einer gesteigerten Expression von Reifungs- und Aktivierungsmarkern auf der Zelloberfläche sowie mit einer vermehrten Zytokinproduktion, analog zu adulten

PDC. Die intrazelluläre Phosphorylierung profitiert im Unterschied zu adulten PDC nicht von der Stimulation mit CPG. Nabelschnurblut-PDC, die mit CPG A, CPG B oder CD40L aktiviert wurden, fördern im Gegensatz zu adulten PDC nicht die Teilungsrate autologer T-Zellen. Während adulte T-Zellen zu einer Th2-Antwort tendieren, führt eine Aktivierung neonataler PDC weder zu einer klaren Verschiebung der T-Helferzell-Antwort Richtung Th1 noch zu Th2.

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