

Neonatal antigen-presenting cells are functionally more quiescent in children born under traditional compared with modern environmental conditions

Joanne G. Lisciandro, BSc/Hon,^{a,b} Susan L. Prescott, MD, PhD,^b Marie G. Nadal-Sims, BSc/Hon,^a Catherine J. Devitt, BSc/Hon,^a Peter C. Richmond, MBBS, MRCP, FRACP,^b William Pomat, MSc,^c Peter M. Siba, PhD,^c Patrick G. Holt, DSc, FAA,^a Deborah H. Strickland, PhD,^{a*} and Anita H. J. van den Biggelaar, PhD^{a*†} *Perth, Australia, and Goroka, Papua New Guinea*

Background: One explanation for the high burden of allergic and autoimmune diseases in industrialized countries is inappropriate immune development under modern environmental conditions. There is increasing evidence that the process of immune deviation already begins *in utero*, but the underlying immunologic mechanisms are not clear.

Objective: We sought to identify differences in the function of neonatal antigen-presenting cells (APCs) in children born in settings that are more traditional versus those of modern societies.

Methods: Cord blood mononuclear cells were collected from newborns from Papua New Guinea (PNG; traditional) and Australia (modern) and compared for differences in APCs and T-cell phenotype and function.

Results: Australian cord naive T cells (CD4⁺CD25⁻CD127⁺ cells) showed an enhanced and more rapid proliferative response in an autologous, APC-dependent culture system, a result of differences in neonatal APCs rather than T-cell function. This included an increased capacity to process antigen and to upregulate activation markers after stimulation. In contrast, resting PNG APCs exhibited higher baseline levels of activation and inhibitory markers and were less responsive or nonresponsive to stimulation *in vitro*.

Conclusions: This study supports the hypothesis that prenatal environments can influence the developing immune system *in utero*. Children born under modern environmental conditions exhibit increased APC reactivity at birth compared with children born under traditional environmental conditions. The functionally more quiescent nature of PNG neonatal APCs might protect against the development of harmful inflammatory responses in early life. (J Allergy Clin Immunol 2012;■■■■:■■■■-■■■■.)

Key words: Antigen-presenting cell, cord blood, hygiene hypothesis, neonatal, T cell, Papua New Guinea, Australia

Following trends in industrialized countries, allergic and autoimmune diseases are increasing in populations transitioning to more modern lifestyles,¹⁻³ suggesting that the characteristics of such lifestyles, including improved hygiene and reduced microbial burden, contribute to this heightened risk. Indeed, epidemiologic studies have demonstrated inverse relationships between allergy risk and markers of microbial exposure, including living in farming or rural environments, larger family size, pet ownership, and parasitic infection.⁴⁻¹⁰ According to the hygiene hypothesis, microbial exposure in early life educates the developing immune system to react appropriately to stimuli, including harmless allergens, a process in which antigen-presenting cells (APCs) presumably play an important role.¹¹⁻¹³

Although the hygiene hypothesis initially focused on postnatal events, there is increasing interest in the influence of the *in utero* environment on postnatal immune function and hence susceptibility to allergic disease.¹⁴⁻¹⁷ Findings from human studies include reports that children born in traditional settings display altered neonatal innate immune responses compared with those of children born in developed settings resulting in downstream effects on postnatal T-cell development,¹⁸ and contact with farm animals during pregnancy decreased the expression of innate immune receptors at birth and protected against atopic dermatitis during the first 2 years of life.¹⁹ A landmark study by Conrad et al²⁰ has provided the first evidence of a central role of the maternal and fetal innate immune systems. In mice microbial exposure during pregnancy enhanced maternal Toll-like receptor-mediated inflammatory responses, leading to downregulated Toll-like receptor and cytokine expression in placental tissues and reduced risk of allergic disease development in offspring. Although it is yet unknown how disease protection is afforded or which mechanisms are involved, current opinion favors fetal APCs as likely initial targets of microbial effects.^{21,22}

Complementing earlier studies reporting on neonatal innate and regulatory T-cell immune responses in children born under

From ^athe Telethon Institute for Child Health Research, Centre for Child Health Research, and ^bthe School of Paediatrics and Child Health, University of Western Australia, Perth, and ^cthe Papua New Guinea Institute of Medical Research, Goroka.

*These authors contributed equally to this work.

†Dr van den Biggelaar is currently affiliated with Crucell, Archimedesweg 4-6, 2333 CN, The Netherlands.

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Corresponding author: Anita H. J. van den Biggelaar, PhD, Crucell, Innovation & Discovery Lab, Archimedesweg 4-6, 2333 CN, The Netherlands. E-mail: Anita.vandenBiggelaar@Crucell.com.

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Abbreviations used

APC: Antigen-presenting cell
 CBMC: Cord blood mononuclear cell
 DC: Dendritic cell
 ILT: Immunoglobulin-like transcript
 mDC: Myeloid dendritic cell
 pDC: Plasmacytoid dendritic cell
 PI: Propidium iodide
 PNG: Papua New Guinea

diverse environmental conditions,^{18,23-25} we investigated the potential effect of a changing lifestyle and environment on *in utero* APC development by comparing the function of cord blood APCs from children born in a modern (Australia) versus a more traditional (Papua New Guinea [PNG]) environment.

METHODS**Study populations**

PNG is a low-income country with a gross national income per capita (PPP international dollars) of \$2,030 compared with \$37,250 for Australia. The majority of the PNG population lives in rural areas (88%), whereas in Australia most people (89%) live in urban centers.^{26,27} The under-5 childhood mortality rate in PNG is considerably higher (69/1,000) than in Australia (5/1,000).^{26,27} According to the United Nations Human Development Index, which takes into account health, education, and living standards, Australia is ranked second worldwide (ie, the Very High Human Development category), whereas PNG is ranked in the Low Human Development category.²⁸

Pregnant women were recruited through private/state antenatal clinics in Perth, the capital city of Western Australia (n = 61), and through Goroka General Hospital, the only tertiary hospital in the relatively remote Eastern Highland Province of PNG (n = 58). Goroka area is highly endemic for respiratory pathogens (particularly *Streptococcus pneumoniae* and *Haemophilus influenzae*) and intestinal helminths and protozoa but not for malaria because of its location at high altitude (1500-1900 m above sea level). Further details on these study populations are described elsewhere.²⁵

Inclusion criteria included uncomplicated pregnancy of at least 37 weeks' gestation, a birth weight of greater than 2000 g, no acute neonatal infection, and no severe congenital abnormality. All PNG mothers were screened for HIV infection during pregnancy and at delivery and excluded from the study if test results were positive. In line with national standard clinical practice, most PNG study mothers (89%) were vaccinated against tetanus in the third trimester of pregnancy. In keeping with the high prevalence of allergy in westernized populations, 47% of the Australian study mothers were atopic, as determined by a positive skin prick test response to common allergens. Although the atopic status of PNG study mothers was unknown, another study in a similar population in PNG found no evidence of atopy.¹

The number of samples used for each experiment is reported in the Results section. Healthy laboratory volunteers (n = 15; mean age $\pm$ SD, 32.6 $\pm$ 7.5 years) were recruited to collect venous blood and isolate adult APCs and naive CD4⁺ T cells, as will be further described.

Written informed consent was obtained from pregnant women (and in PNG also from their partners) and volunteers. Ethical approval was obtained from the PNG Institute of Medical Research Institutional Review Board, the PNG Medical Research Advisory Council, and the Ethics Committee of the Princess Margaret Hospital for Children, Perth, Western Australia.

Isolation of cord and peripheral blood mononuclear cells

Cord (25-50 mL) and venous (laboratory volunteers, 10-25 mL) blood samples were collected in RPMI (Invitrogen-Life Technologies, Melbourne, Australia) containing preservative-free heparin (20 U/mL). Cord blood

mononuclear cells (CBMCs) and PBMCs were isolated by means of Ficoll-Hypaque (Alexis-Shield, Oslo, Norway) gradient centrifugation and cryopreserved in 50% heat-inactivated FCS (JRH Biosciences, Lenexa, Kan) and 7.5% dimethyl sulfoxide. Identical protocols and consumables were used in PNG and Australia. Until thawing for experiments, cells were continuously kept in the liquid nitrogen vapor phase, including during transport by means of a dry shipper from Goroka to Perth. Cell viability was similar between PNG and Australian CBMCs on thawing.

In vitro unfractionated CBMC cultures

CBMCs (1×10^6 cells/mL) were cultured in RPMI/10% non-heat-inactivated FCS in the presence or absence of 1 ng/mL LPS (Alexis Biochemicals, San Diego, Calif) with or without 10 ng/mL IFN- γ (BD Pharmingen, San Jose, Calif). For intracellular cytokine staining, Brefeldin A (5 μ g/mL; Sigma-Aldrich, Munich, Germany) was added for the last 18 hours of culture. Cells were harvested after 24 hours of culture for flow cytometric analysis.

APC-dependent proliferation assays

CBMCs/PBMCs were labeled with anti-CD3 Dynabeads (Invitrogen Dynal AS, Oslo, Norway) to separate T cells from APCs. The CD3⁺ APC fraction was gamma irradiated at 3000 mCg. Naive CD4⁺CD127⁺CD25⁺ T cells were sorted from the CD3⁺ fraction by using the FACSaria instrument and FACSDiva software (BD Biosciences) and labeled with 11 μ M carboxyfluorescein succinimidyl ester (Molecular Probes, Invitrogen, Eugene, Ore). Gamma-irradiated CD3⁺ cells (5×10^4 per well APCs) and CD4⁺ T cells (2.5×10^4 per well) were cultured in RPMI/5% heat-inactivated human AB serum with or without 1 μ g/mL PHA (Remel Europe Ltd, Kent, United Kingdom) for 68 or 88 hours. In autologous assays T cells and APCs from the same donor were cocultured, whereas in assays addressing the role of neonatal APCs or T cells, adult T cells were cultured with cord blood APCs or cord blood T cells were cultured with adult APCs, respectively.

Antigen processing assay

CBMCs were cultured in RPMI plus 5% FCS (2×10^6 cells/mL) in 24-well flat-bottom plates with 1 μ g/mL DQ-Ovalbumin (DQOVA; Molecular Probes, Invitrogen) at 37°C or 4°C (control) for 1 hour to determine antigen processing and uptake.

Flow cytometry

To determine T-cell and APC proportions, CBMCs were resuspended in fluorescence-activated cell-sorting buffer (1×10^6 cells per tube); incubated on ice with antibodies for CD3, CD4, CD14, CD19, CD56, CD45RO, lineage markers, HLA-DR, CD11c, or CD123 (BD Biosciences); washed; and fixed overnight with 1% formaldehyde (Univar, Seven Hills, Australia) in PBS.

Carboxyfluorescein succinimidyl ester-labeled cells were costained with antibodies for CD25, CD127 and CD4, and propidium iodide (PI) to measure proliferation. Cells were stained with antibodies for CD14, CD56, CD19 and CD3, or HLA-DR, mixed lineage markers, CD123, and CD11c to determine antigen processing.

CBMCs were stained with antibodies for (1) CD14, CD19, HLA-DR, CD40, CD80, and CD86; (2) CD14, CD19, HLA-DR, and immunoglobulin-like transcripts (ILTs) 3 and 4; (3) HLA-DR, mixed lineage markers, CD11c, CD123, and CD40; and (4) HLA-DR, mixed lineage markers, CD11c, CD123, CD80, and CD86 to phenotype APC subsets at baseline.

CBMCs were stained with antibodies for (1) CD14, CD19, HLA-DR, CD40, CD80, and CD86; (2) HLA-DR, mixed lineage markers, CD11c, CD123, CD40, and CD80; and (3) CD14, HLA-DR, and CD19 plus intracellular IL-6, IL-10, and IFN- γ to phenotype APCs in cultures.

Data were acquired by using 4-color FACSCalibur or 10-color LSRII (BD Biosciences) instruments, and results were analyzed with FlowJo software (TreeStar, Ashland, Ore). Dead cells were excluded from analysis by using a forward- versus side-scatter gate or selection of PI⁺ cells.

Cytokine protein detection

Cytokine levels were measured in supernatants by using an in-house multiplex assay, as previously described.²⁹

Statistical methods

Differences in continuous variables were tested for significance by using the Student *t* test if data were normally distributed or the Mann-Whitney *U* test when data were not normally distributed. Pearson χ^2 and Fisher exact tests (when cells contained numbers ≤ 5) were performed to test for differences between groups in categorical variables. Wilcoxon signed-rank tests were used to test for differences in related samples. Differences were considered statistically significant when *P* values were less than .05. All analyses were performed with SPSS 15.0 software (SPSS, Chicago, Ill).

RESULTS

Population characteristics

Obstetric and newborn characteristics are described in Table E1 in this article's Online Repository at www.jacionline.org. Australian mothers were significantly older and more likely to have delivered by means of cesarean section and to have used antibiotics during pregnancy. Australian newborns weighed significantly more at birth and had larger head circumferences compared with PNG newborns.

Proportions of APCs and CD4⁺ T cells

Significantly lower proportions of monocytes (CD14⁺), B cells (CD19⁺), and plasmacytoid dendritic cells (pDCs; HLA-DR⁺ lineage⁻ CD123⁺, see Fig E1 in this article's Online Repository at www.jacionline.org) were present in CBMCs of PNG versus Australian newborns. No differences in myeloid dendritic cell (mDC; HLA-DR⁺ lineage⁻ CD11c⁺, see Fig E1) or natural killer cell (CD56⁺) numbers (Table I) were detected.

The frequency of CD4⁺ (CD3-coexpressing) T cells was higher in CBMCs of PNG compared with Australian newborns (Table I). We found no difference in the CD4⁺ T-cell mRNA expression of T cell–receptor excision circles, which is characteristic of recent thymic emigrants (data not shown). The maturation marker CD45RO tended to be expressed on relatively more Australian than PNG neonatal CD4⁺ T cells; however, this difference was not significant (mean $\pm$ SEM, 13.0% $\pm$ 2.1% and 8.8% $\pm$ 1.0%, respectively; *P* = .085).

Cord APC phenotype

Markers of activation (HLA-DR and CD86) and inhibition (ILT3 and ILT4) were expressed significantly more frequently by monocytes of PNG compared with Australian newborns (Fig 1, A). Monocytes expressing inhibitory markers also expressed the highest levels of HLA-DR (see Fig E2 in this article's Online Repository at www.jacionline.org). Although there was a trend for higher CD80 expression on PNG versus Australian monocytes (*P* = .078), expression was generally low or absent on most APC types. Similar to PNG monocytes, the expression of HLA-DR (geometric mean fluorescence intensity) was higher on DC subsets from PNG compared with Australian newborns (Fig 1, D). A higher frequency of mDCs from PNG compared with Australian newborns (but not B cells) expressed CD86 (Fig 1, B and C). There were no significant differences in the proportion of

TABLE I. Frequency of APCs and T cells in PNG and Australian CBMCs

	PNG CBMCs	Australian CBMCs	<i>P</i> value
Monocytes	13.4 (5.3)	18.9 (7.2)	.027
B cells	13.0 (5.2)	21.0 (8.5)	.010
NK cells	10.0 (3.7)	9.6 (6.7)	.424
pDCs	0.3 (0.1)	0.5 (0.3)	.010
mDCs	0.5 (0.3)	0.4 (0.2)	.527
CD4 ⁺ T cells	36.9 (9.7)	30.9 (9.0)	.020
CD8 ⁺ T cells	11.4 (3.4)	10.3 (4.5)	.185

Data present the proportions (means and SDs) of different APC and T-cell subsets in PNG and Australian CBMCs: *n* = 11 (PNG) and 12 (Australian) for natural killer cell proportions; *n* = 12 (PNG) and 14 (Australian) for monocyte and B-cell proportions; *n* = 19 (Australian) and 20 (PNG) for pDC and mDC proportions; *n* = 25 per group for CD4⁺ T-cell proportions; and *n* = 20 per group for CD8⁺ T-cell proportions. NK, Natural killer.

monocytes or dendritic cells (DCs) expressing CD40; however, more PNG than Australian B cells expressed CD40 (Fig 1, B).

The relative ratio of CD80- to CD86-expressing APCs at baseline was significantly higher on B cells from PNG compared with Australian neonates; similar trends were observed for monocytes (*P* = .109) but not cord DCs (Fig 1, E).

Induction of T-cell proliferation by neonatal APCs

Cord naive CD4⁺ T cells were cultured with autologous APCs (gamma-irradiated CD3⁻ cells) to assess the ability of neonatal APCs to induce CD4⁺ T-cell proliferation. PHA-induced T-cell proliferation was reduced and delayed in PNG versus Australian cultures (Fig 2, A-C, and see Fig E3 in this article's Online Repository at www.jacionline.org). After 68 hours of culturing, significantly fewer PNG compared with Australian neonatal T cells had undergone cell division (mean $\pm$ SEM, 54.8 $\pm$ 4.9% and 81.3% $\pm$ 2.2%, respectively; *P* < .001), whereas significantly more Australian T cells had divided 2 or more times (Fig 2, B and C). Although proliferation of PNG T cells increased by 88 hours in culture, it did not catch up to that of Australian T cells, lagging behind particularly in the number of cells that entered 4 or more divisions (see Fig E3, C).

We next set out to determine the reason for the observed proliferative differences of PNG and Australian neonatal T cells. No difference in PHA-induced T-cell cytokine production (see Fig E4, A, in this article's Online Repository at www.jacionline.org) or cell death was detected: the proportion of live cells (PI⁻) in PNG (mean $\pm$ SEM, 85.3% $\pm$ 1.1%) and Australian (mean $\pm$ SEM, 84.5% $\pm$ 1.1%) cultures was similar (*P* = .664) after 68 hours of stimulation. The proliferative response of PNG and Australian neonatal T cells was studied while culturing in the presence of a "standardized" APC compartment (ie, APCs isolated from adult volunteers) to address the possibility of an intrinsic difference in T-cell function. Of note, no mixed lymphocyte reaction was observed at 68 hours, as evidenced by low background proliferation in nonstimulated (control) cultures. Although a marginally higher proportion of Australian than PNG T cells underwent cell division in response to PHA stimulation (Fig 2, D), proliferation of PNG T cells was greatly enhanced if cultured with standardized APCs instead of autologous APCs (see Fig E3, B), suggesting that PNG neonatal APCs limit autologous T-cell proliferation.

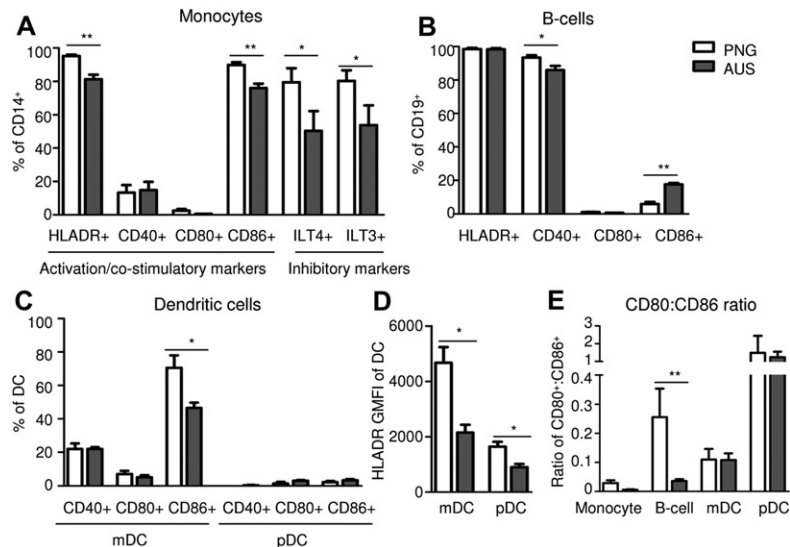


FIG 1. Cord APC phenotype at baseline. **A**, Percentage of CD14⁺ monocytes expressing activation and inhibitory markers. **B** and **C**, Percentages of CD19⁺ B cells (Fig 1, **B**) and mDC- and pDC-expressing activation markers (Fig 1, **C**). **D**, Intensity of HLA-DR expression (geometric mean fluorescence intensity [GMFI]) on DC subsets. **E**, Relative ratio of CD80-expressing to CD86-expressing APCs. White bars, PNG; gray bars, Australian (AUS) (n = 6 per group). Data represent means and SEMs. Significance level is shown as follows: **P* < .05 and ***P* < .01.

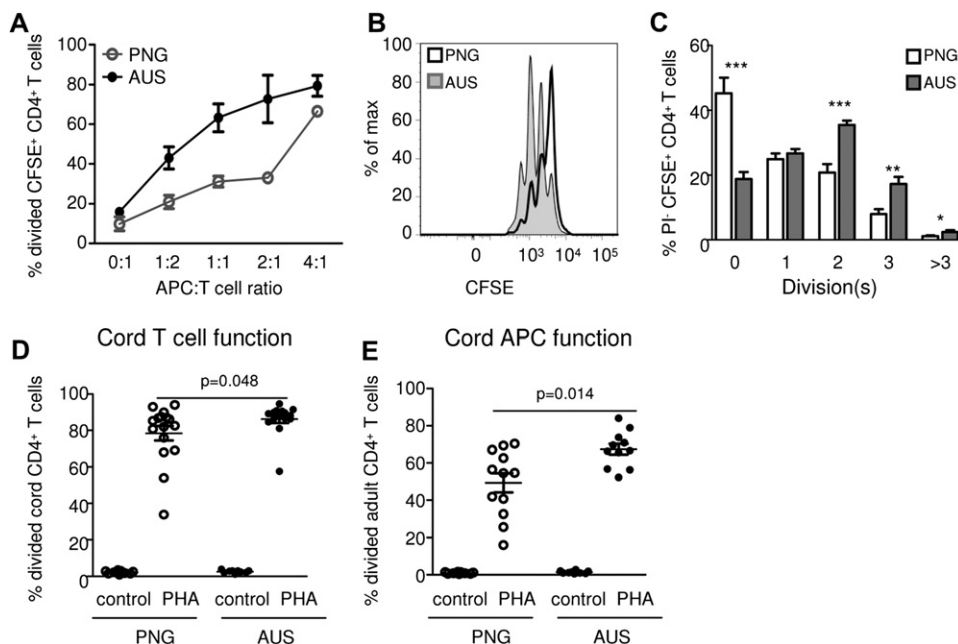


FIG 2. Neonatal T-cell proliferation in autologous and allogeneic APC culture systems. **A-C**, Autologous cultures: Neonatal T-cell proliferation under varying APC/T-cell ratios (n = 3 per group) is shown (Fig 2, **A**). Example of carboxyfluorescein succinimidyl ester (CFSE) dilution (Fig 2, **B**) and proportions of neonatal CD4⁺ T cells having undergone an increasing number of divisions when cultured at a 2:1 APC/T-cell ratio (Fig 2, **C**) are shown (white bars, PNG [n = 21]; gray bars Australia [AUS], n = 20). **D**, Cord T-cell function: neonatal CD4⁺ T cells (PNG, n = 15; AUS, n = 14) cultured with “standardized” APCs from adult donors. **E**, Cord APC function: neonatal APCs (PNG, n = 12; AUS, n = 11) cultured with “standardized” CD4⁺ T cells from adult donors. Cells were stimulated with PHA for 68 hours. Data represent means and SEMs. Significance level is indicated as follows: **P* < .05, ***P* < .01, and ****P* < .001.

PNG and Australian APCs were cultured with a standardized T-cell compartment consisting of naive adult CD4⁺ T cells to further investigate neonatal APC function. Again, there was no

evidence of a mixed lymphocyte reaction in control cultures. After PHA stimulation, adult T-cell proliferation was significantly lower in cultures containing PNG versus Australian APCs, thus

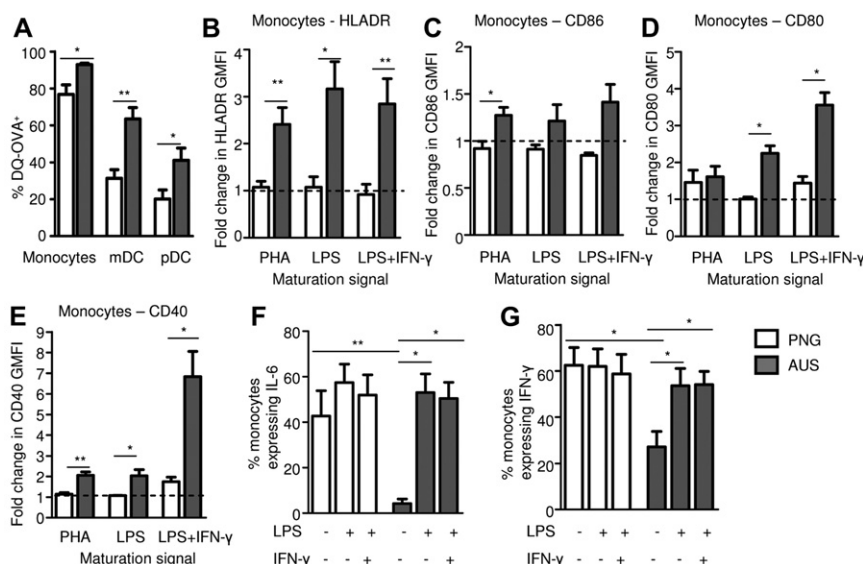


FIG 3. Neonatal APC function. **A**, Proportions of cells taking up and processing DQ-ovalbumin (DQ-OVA; white bars, PNG; gray bars, Australia [AUS]; n = 6 per group). **B–E**, Fold change in geometric mean fluorescence intensity (GMFI) of HLA-DR (Fig 3, B), CD86 (Fig 3, C), CD80 (Fig 3, D), and CD40 (Fig 3, E) on monocytes after stimulation compared with nonstimulated controls (media only, n = 4–6 per group). **F** and **G**, Percentage of monocytes expressing intracellular IL-6 or IFN-γ after culture (n = 6 per group). Data represent means and SEMs. Significance level is indicated as follows: * $P < .05$ and ** $P < .01$.

offering further support that PNG APCs limit T-cell proliferation (Fig 2, E).

Cord blood APC function

Given their differing abilities to induce T-cell proliferation, we further evaluated additional functional properties of PNG and Australian neonatal APCs.

Comparing the *in vitro* antigen uptake and processing capacity of PNG and Australian neonatal APCs, significantly fewer PNG than Australian APCs, including monocytes, mDCs, and pDCs, became DQ-ovalbumin positive, indicating reduced antigen processing in PNG APCs (Fig 3, A). Monocytes were the predominant DQ-ovalbumin-positive APC subpopulation in both PNG and Australian cultures and, for this reason, were chosen for further studies.

Adopting similar culture conditions as used in the T-cell proliferation assays, the ability of monocytes to upregulate activation markers when stimulated *in vitro* with PHA was determined. Compared with nonstimulated cultures, the expression of CD40, CD86, and HLA-DR on monocytes significantly increased in Australian but not PNG cell cultures (Fig 3, B–E, and see Fig E5, A–C, in this article's Online Repository at www.jacionline.org). After PHA stimulation, the ratio of CD80⁺ to CD86⁺ monocytes also remained higher for PNG versus Australian newborns (see Fig E5, D). Notably, other APC subpopulations did not upregulate activation markers in either Australian or PNG cultures (data not shown). Similar results for monocytes were observed when CBMCs were given alternative signals, such as LPS with or without IFN-γ (Fig 3, B–E, and see Fig E5, A–C).

LPS with or without IFN-γ was used as the maturation signal to investigate the ability of cord monocytes to produce inflammatory cytokines. In the absence of stimulation, the proportion of monocytes expressing IFN-γ or IL-6 was significantly higher in PNG compared with Australian cultures (Fig 3, F and G). PNG cytokine-positive monocytes also expressed higher levels of

HLA-DR compared with their Australian counterparts (see Fig E5, E and F). However, only Australian and not PNG monocytes demonstrated the potential for increased cytokine production when stimulated. Of note, the addition of IFN-γ to LPS-stimulated cultures did not further increase the potential for cytokine production in either group.

DISCUSSION

Consistent with recent speculation of a potentially important role for fetal APCs in mediating the disease-protective effects of prenatal microbial exposure,^{20–22} we here report significant differences in neonatal APC phenotype and function in children born under the divergent environmental conditions of PNG and Australia. The most distinct difference was that PNG neonatal APCs were comparatively quiescent: they processed less antigen, induced less robust T-cell proliferation, and were less responsive or nonresponsive to *in vitro* stimulation compared with Australian neonatal APCs, which in contrast were significantly more responsive.

We suggest that functional quiescence of APCs, as observed in PNG newborns, might be an evolutionary mechanism of immune regulation learned *in utero* to prepare newborns for the intensity and frequency of immune challenges in their postnatal environment and to protect them from pathologic inflammation. Alternatively, under reduced microbial burden, the innate immune system might not be properly programmed *in utero*, consequently altering the process of immune development and increasing the risk for allergic and autoimmune diseases in later life.³⁰ Notably, a relatively more quiescent innate immunity at birth does not necessarily translate into defective postnatal immune responsiveness, as supported by our findings that neonatal vaccination induces normal memory T-cell responses in PNG infants.²⁹

In addition to functional differences, variations in neonatal APC phenotype were observed. PNG monocytes and DCs expressed significantly more HLA-DR than their Australian

counterparts, and this, together with their reduced capacity to process antigen, suggests a higher baseline activation or maturation status. Interestingly, HLA-DR expression of APCs was reported to correlate with infection status,³¹ including in neonates exposed to malaria prenatally.³² Furthermore, enhanced baseline expression of the inhibitory markers ILT3 and ILT4 on monocytes of PNG neonates might also suggest enhanced potential for tolerogenic activity. PNG ILT3⁺ILT4⁺ monocytes coexpressed high levels of HLA-DR, suggesting that cells with potential inhibitory function were those that had been activated *in utero*. Of interest, tolerogenic APCs are known to exist in a range of maturation states,³³ with ILT3 and ILT4 expression subject to modulation by infections³⁴ and, in neonates, by certain prenatal exposures.³⁵ Furthermore, APCs from PNG compared with Australian newborns overexpressed CD80 relative to CD86, which has been associated with promotion of T_H1 rather than T_H2 responses.^{36,37} Additionally, differential roles and expression of these costimulatory molecules have been observed in states of infection/inflammation^{38,39} and in the development of allergic responses.^{40,41}

Enhanced baseline expression of activation markers but functional quiescence is also a feature of APC “exhaustion,” which is commonly described of mature APCs that become refractory to further stimulation and produce less T_H1-polarizing cytokines (even in the presence of IFN- γ) after secondary or chronic exposure to some pathogens (eg, in “endotoxin tolerance”).^{42,43} We previously reported that APCs from PNG compared with Australian newborns produce significantly lower proinflammatory cytokine responses to IFN- γ -primed BCG stimulation.¹⁸ Here we again demonstrate that PNG monocytes do not upregulate cytokine production after LPS stimulation, even in the presence of IFN- γ . Thus if the functional quiescence observed here is in fact a result of exhaustion, this further suggests that PNG APCs might be activated *in utero* under a higher maternal antigen experience. Indeed, monocytes of PNG newborns expressed higher intracellular cytokine levels in the absence of stimulation, and these cytokine-positive cells also coexpressed higher levels of HLA-DR, suggesting a previous cytokine response (activation) *in utero*.

Differences in neonatal APC phenotype and function driven by prenatal exposures potentially play a role in altering the risks for allergic disease in later life. Indeed, animal studies have demonstrated that early postnatal exposure to microbial products reduces such risks.^{11,44} Exposed animals had IL-10-producing tolerogenic DCs that, in line with our findings for PNG neonatal APCs, had enhanced expression of activation markers but a reduced ability to induce T-cell proliferation.^{11,44} Likewise, others have reported an inverse association between baseline HLA-DR expression on cord blood monocytes and development of atopic disease.⁴⁵ Furthermore, we have recently shown in Australian cohorts that reduced neonatal innate inflammatory cytokine production is associated with reduced allergy risk.^{46,47} Together with the present study, these findings collectively support the hypothesis that protection from allergic disease under conditions of high microbial burden can be mediated, at least in part, through relative quiescence of neonatal APC responses. Further studies are needed to test this longitudinal effect in human subjects and to explore the most important underlying *in utero* risk factors.

In addition to differences in neonatal APCs, we also found dissimilarities in CD4⁺ T-cell frequency and function between PNG and Australian newborns. A main difference was that proportions were significantly higher in PNG compared with Australian newborns. Interestingly, in neonatal mice increasing the

quantities of conventional CD4⁺ T cells by means of adoptive transfer reduced innate immune responses and decreased the risk for hyperinflammation.⁴⁸ A second difference was that CD4⁺ T cells of PNG compared with Australian newborns underwent less proliferation in autologous APC-dependent cultures; however, PNG neonatal T cells possessed the intrinsic ability to respond more efficiently when stimulated by APCs from an unrelated adult donor, thereby highlighting the role of PNG neonatal APCs in the reduced proliferative capacity of PNG T cells. These findings suggest that T-cell proliferation might be limited *in vivo* in PNG newborns, perhaps to an even greater extent than that observed in our experiments given the lower ratio of circulating APCs to T cells found in total CBMCs of PNG newborns. The effect of limited neonatal T-cell proliferation in the clearance of infectious microorganisms in early postnatal life is currently unknown and requires further investigation.

In addition to variation in microbial burden, differences in other environmental exposures such as maternal diet and indoor air pollution, as well as genetic and obstetric factors, might have contributed to the observed differences in neonatal APC function. For example, in our study Australian mothers were significantly older than PNG mothers at the time of delivery, which, to a certain extent, might have contributed to observed differences between the neonatal immune systems because older maternal age was associated with relatively higher proportions of monocytes in cord blood (see Tables E2 and E3 and the Results section in this article's Online Repository at www.jacionline.org). Also, 41% of the Australian study mothers delivered by means of cesarean section in contrast to none of the PNG mothers. In the current study we did not observe an effect of cesarean section on studied Australian neonatal immune readouts (see Table E4 in this article's Online Repository at www.jacionline.org); however, several other studies have investigated the effect of mode of delivery on neonatal immune responses, with variable findings.^{18,49,50} In any case mode of delivery and maternal age are 2 examples of population-differentiating factors that might be of importance for differences in neonatal immune function, and further studies are needed now that the current study has successfully demonstrated the existence and nature of differential neonatal immune function in PNG and Australian populations.

In summary, we have demonstrated that under conditions typical of traditional environmental settings, neonatal APCs exhibit a more activated phenotype at baseline while being quiescent in function, potentially a protective mechanism learned *in utero*. On the other hand, neonatal APCs from children born in a more modernized environment were comparatively more responsive. Both prenatal and early postnatal exposures are thought to be critical to develop balanced immune regulatory responses protecting infants against severe and harmful inflammation in early life and possibly allergic and autoimmune diseases in later life. This study provides further evidence that these effects begin *in utero* and that fetal/neonatal innate immune pathways are involved.

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Key messages

- The function of APCs at birth varies between geographically diverse populations.
- Neonatal APCs are relatively more quiescent in children born in traditional compared with modern environments.

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RESULTS

To study whether maternal age might account for observed differences between PNG and Australian neonatal immune function, linear regression models were used, including neonatal immune readouts that significantly differed between the 2 study populations and that had sufficient power (sample sizes) for regression analysis: relative proportions of monocytes (PNG, $n = 11$; Australia, $n = 8$) and CD4 T cells in CBMCs (PNG, $n = 18$; Australia, $n = 23$) and PHA-induced percentage of divided T cells in autologous cell cultures (PNG, $n = 15$; Australia, $n = 18$) and cultures of cord APCs in the presence of adult T cells (PNG, $n = 9$; Australia, $n = 8$). Maternal age was studied as a continuous variable. To determine whether maternal age explains population differences, we first tested for associations between maternal age and immunologic readouts for the 2 populations separately (Table E2) and then performed regression analysis by using model 1, which studies the population effect alone, and model 2, which also included maternal age as a variable (Table E3). When the linear regression coefficient for the relationship between population and the immunologic readout changes considerably in model 2 compared with model 1, this indicates that the observed population difference is (to a certain extent) explained by maternal age. As demonstrated in

Table E2, within the group of Australian mothers, increasing maternal age was found to be associated with relatively higher cord CD14⁺ monocyte proportions, whereas in the PNG group older maternal age was associated with lower cord CD4⁺ T-cell proportions. Because relative proportions of cord CD4 and CD14 cells are interrelated, these observations therefore might indicate that the higher proportion of CD4 T cells in PNG cords and the relatively higher proportions of CD14 cells in Australian cords can be explained by differences in maternal age between these 2 study populations. This is in line with the observation that in the adjusted linear regression analysis (Table E3) the population difference for the percentage of CD14 cord cells disappears when adjusting for the effect of maternal age (comparing model 2 with model 1). In spite of this effect of maternal age, in the functionality experiments that follow in the presented study, we correct for the population differences in cord CD4⁺ T-cell and CD14⁺ monocyte proportions, and the maternal age effect is then no longer at play. For the proliferation capacity, no effect of maternal age was found: there was no association found with maternal age when studying the 2 populations separately (Table E2) and inclusion of maternal age as a variable in regression analysis did not change the population effect (Table E3).

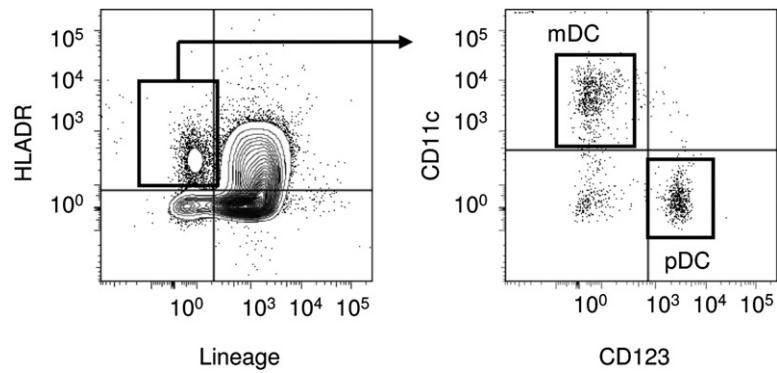


FIG E1. Cord DC gating strategy. CBMCs were stained for HLA-DR, lineage markers, CD11c, and CD123 by means of flow cytometry. pDCs were defined as lineage⁻HLA-DR⁺CD123⁺, whereas mDCs were defined as lineage⁻HLA-DR⁺CD11c⁺.

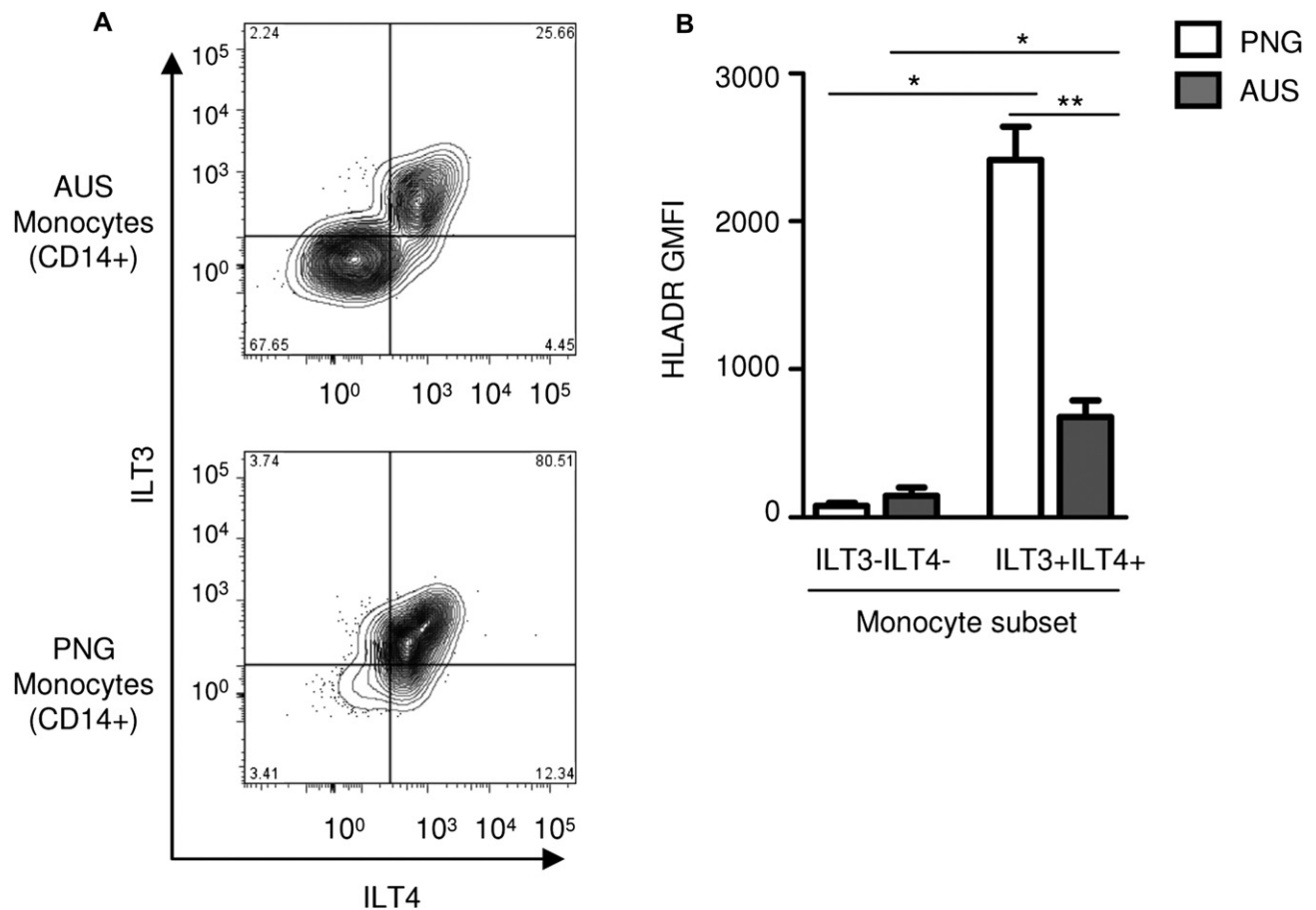


FIG E2. ILT3⁺ILT4⁺ monocytes express high levels of HLA-DR. **A**, Example of the expression of ILT3 and ILT4 on Australian (AUS) and PNG CD14⁺ monocytes. **B**, The relative HLA-DR geometric mean fluorescence intensity (GMFI) of ILT3⁻ILT4⁻ and ILT3⁺ILT4⁺ CD14⁺ monocyte subsets in PNG (white bars) and Australian (gray bars) cord samples (n = 6 per group). Data present means and SEMs. Significance level is indicated as follows: **P* < .05 and ***P* < .01.

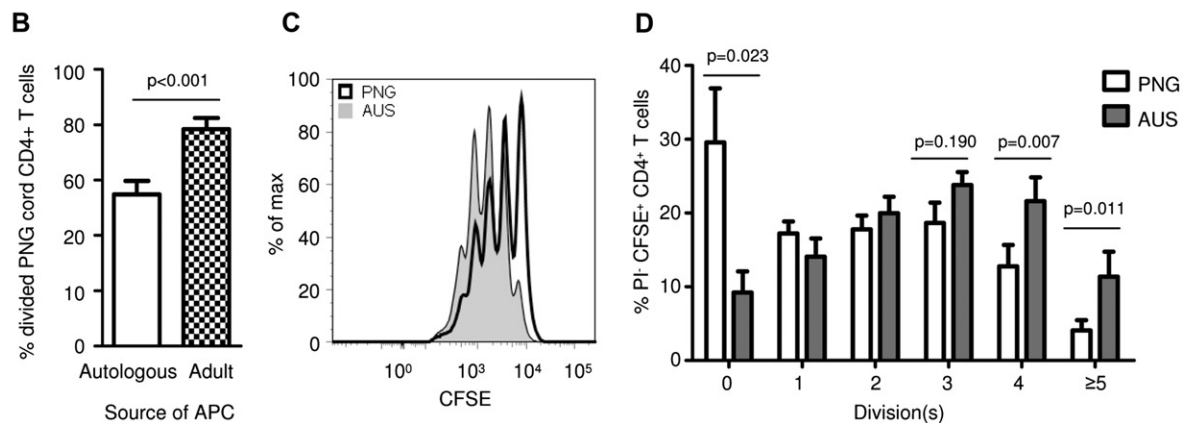
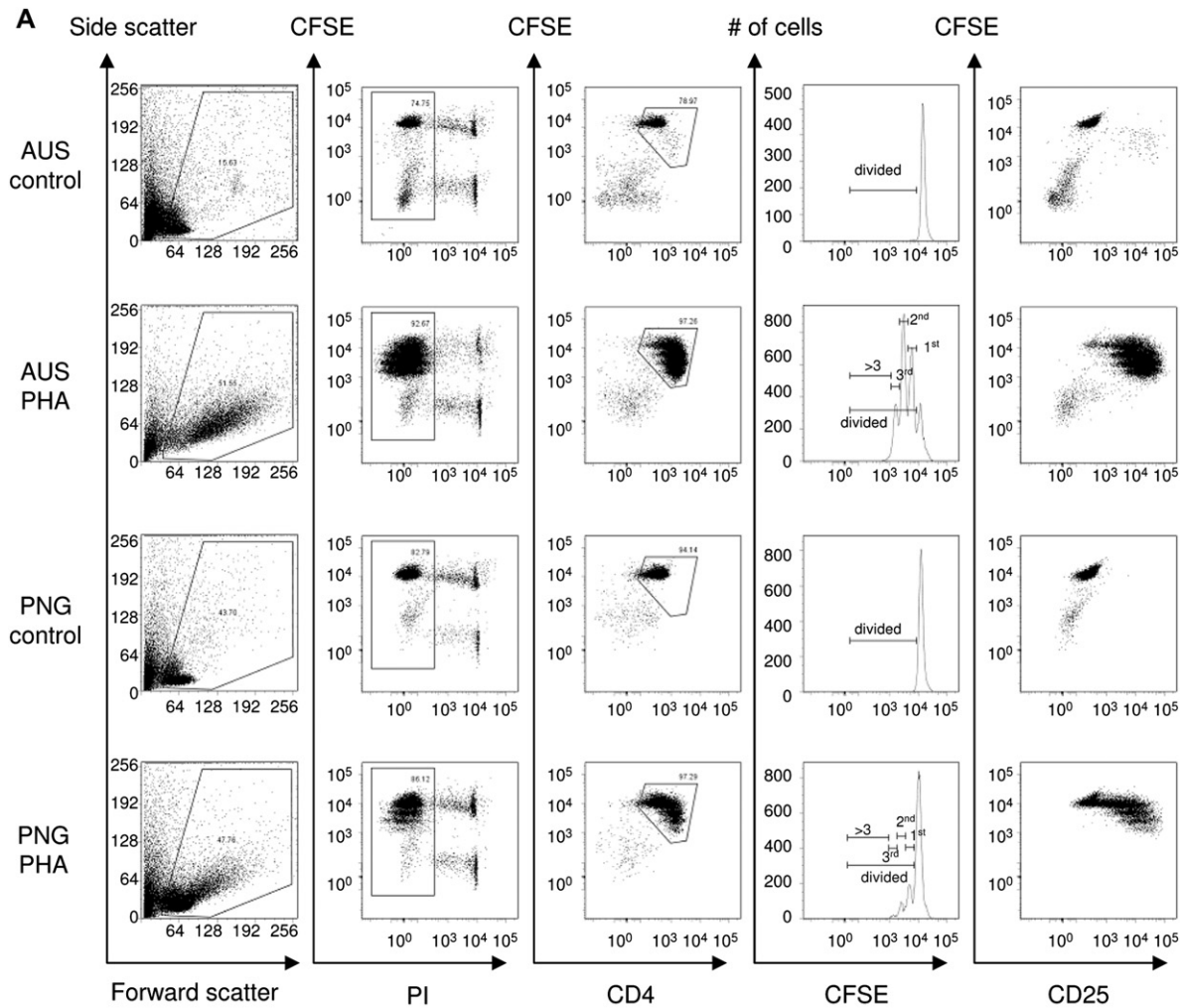


FIG E3. Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells. **A**, Gating strategy for determining proliferation of live CFSE-labeled CD4⁺ T cells in cultures supplemented with autologous APCs with or without PHA for 68 hours. **B**, PHA-induced proliferation of PNG CD4⁺ T cells after 68 hours in culture with autologous ($n = 21$) or adult APCs ($n = 16$). **C** and **D**, An example of CFSE dilution in PNG and Australian (AUS) CD4⁺ T cells (Fig E3, C) and the average proportions (mean + SEM) of CD4⁺ T cells (Fig E3, D) having undergone an increasing number of divisions in autologous cultures stimulated with PHA for 88 hours (white bars, PNG; gray bars, Australia; $n = 10$ per group). The significance level is indicated.

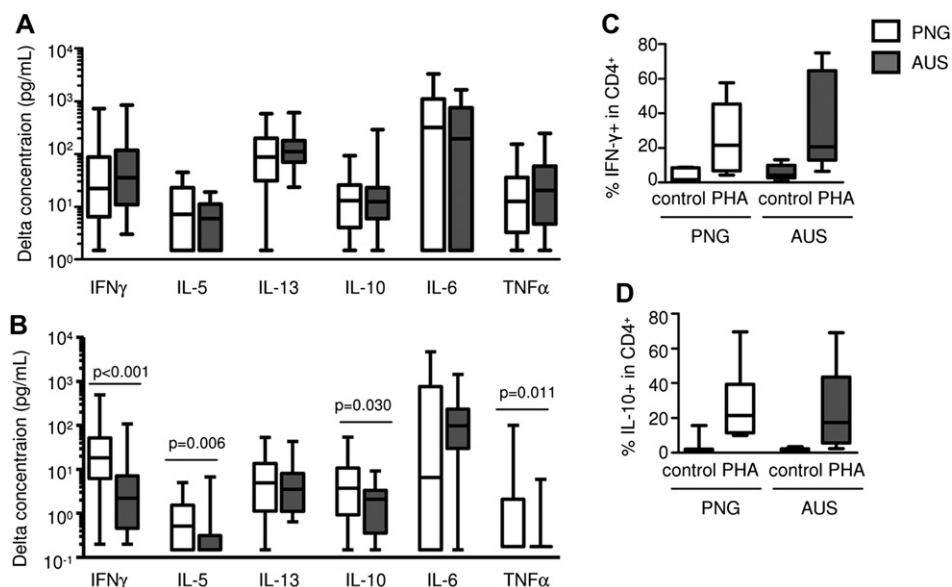


FIG E4. Cord T-cell cytokine responses to PHA. **A**, CD4 $^{+}$ T cells and autologous CD3 $^{-}$ APCs were cultured at a 1:2 ratio in the presence or absence of PHA for 68 hours (*white bars*, PNG [$n = 17$]; *gray bars*, Australia [$n = 16$]; Fig E4, *A*), or **B**, unfractionated CBMCs (1×10^6 cells/mL) of PNG (*white bars*, $n = 36$) and Australian (AUS) newborns (*gray bars*, $n = 31$) were cultured *in vitro* with medium alone (control) or with PHA for 48 hours. Net changes in PHA-induced cytokine responses are presented as box plots (medians, interquartile ranges, and minimum/maximum responses). **C** and **D**, Percentages of PNG and Australian cord CD4 $^{+}$ T cells positive for intracellular IFN- γ (Fig E4, *C*) and IL-10 (Fig E4, *D*) in unfractionated CBMC cultures with medium alone (control) or PHA is shown. The significance level is indicated.

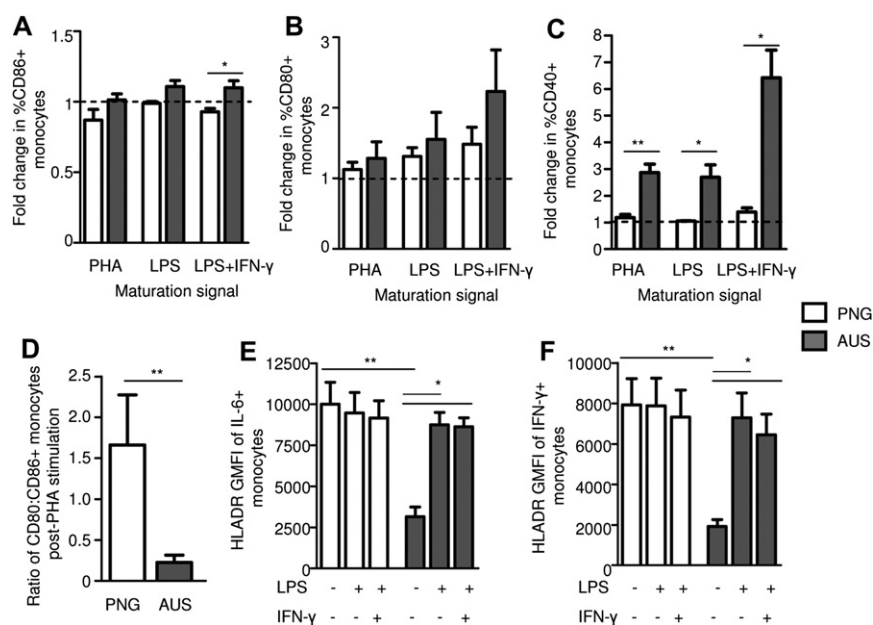


FIG E5. Expression of activation markers on cord monocytes. **A-C**, Fold change in the percentage of monocytes (CD14⁺) expressing CD86 (Fig E5, **A**), CD80 (Fig E5, **B**), and CD40 (Fig E5, **C**) after 48 hours of PHA stimulation or 24 hours of stimulation with LPS with or without IFN- γ (white bars, PNG [$n = 6$]; gray bars, Australia [$n = 5$]). **D**, Relative ratio of CD80-expressing to CD86-expressing cord monocytes after 48 hours of stimulation with PHA (PNG, $n = 6$; AUS, $n = 5$). **E** and **F**, HLA-DR geometric mean fluorescence intensity (GMFI) of IL-6⁺ (Fig E5, **E**) and IFN- γ ⁺ (Fig E5, **F**) monocytes after 24 hours of LPS with or without IFN- γ stimulation. Data represent means and SEMs. Significance level is indicated as follows: * $P < .05$ and ** $P < .01$.

TABLE E1. Obstetric and newborn characteristics

	PNG	Australia	<i>P</i> value
Mother's age (y)	26.2 (6.8)	32.6 (4.2)	<.001
Primigravida	20/54 (37%)	12/39 (31%)	.530
Delivery by cesarean section	0/54 (0%)	15/37 (41%)	<.001
Antibiotic use in pregnancy	0/54 (0%)	5/47 (11%)	.014
Male newborn	35/55 (64%)	21/46 (46%)	.070
Gestational age (wk)	39.5 (1.5)	39.3 (1.2)	.496
Birth weight (kg)	3.4 (0.5)	3.5 (0.4)	.029
Mean birth length (cm)	50.7 (3.2)	50.6 (2.1)	.987
Mean head circumference (cm)	33.7 (1.8)	34.9 (1.2)	<.001

Continuous variables are expressed as means with SDs.

TABLE E2. Linear regression analysis to study association between maternal age and selected neonatal immune outcomes for the 2 populations separately

	PNG (maternal age)		Australia (maternal age)	
	B ± SE	P value	B ± SE	P value
CD14 in CBMCs (%)	0.36 ± 0.27	.218	0.33 ± 0.82	.048
CD4 in CBMCs (%)	−0.33 ± 0.14	.045	−0.04 ± 0.11	.752
Proliferation, % T cells divided: autologous cord APCs + T cells	1.42 ± 1.61	.412	−0.02 ± 0.50	.908
Proliferation, % T cells divided: cord APCs plus adult T cells	0.12 ± 1.07	.914	−0.37 ± 0.53	.507

For more information, see the [Results](#) section in this article's Online Repository. B is the linear regression coefficient.

TABLE E3. Linear regression analysis to study whether population differences in neonatal immune function can be attributed to differences in maternal age

	Model 1		Model 2	
	B ± SE	P value	B ± SE	P value
CD14 in CBMCs (%)				
Population (PNG is reference, 1)	5.03 ± 2.69	.074	1.57 ± 3.76	.682
Maternal age	—		0.51 ± 0.31	.118
CD4 in CBMCs (%)				
Population (PNG is reference, 1)	−5.94 ± 2.65	.029	−5.39 ± 3.23	.103
Maternal age	—		−2.14 ± 0.23	.364
Proliferation, % T cells divided: autologous cord APC plus T cells				
Population (PNG is reference, 1)	30.24 ± 9.45	.005	23.90 ± 11.47	.058
Maternal age	—		0.75 ± 0.85	.399
Proliferation, % T cells divided: cord APCs plus adult T cells				
Population (PNG is reference, 1)	17.95 ± 6.05	.008	16.98 ± 7.88	.050
Maternal age	—		−0.11 ± 0.59	.855

For more information, see the [Results](#) section in this article's Online Repository. B is the linear regression coefficient.

TABLE E4. Linear regression analysis studying the association of neonatal immune outcomes with cesarean section for the Australian population

	Australia (cesarean section)	
	B ± SE	P value
CD14 in CBMCs (%)	−1.84 ± 4.26	.681
CD4 in CBMCs (%)	6.78 ± 3.21	.073
Proliferation, % T cells divided: autologous cord APCs plus T cells	−11.28 ± 7.59	.197
Proliferation, % T cells divided: cord APCs plus adult T cells	4.31 ± 10.42	.696

Linear regression analysis (B is the linear regression coefficient) was used to study associations between delivery by means of cesarean section and relevant neonatal immunologic readouts within the Australian population (dependent variables; ie, relative proportions of monocytes [n = 10] and CD4 T cells in CBMCs [n = 19] and PHA-induced percentage of divided T cells in autologous cell cultures [n = 15] and cultures of cord APCs in the presence of adult T cells [n = 7]). No significant differences were found. The observation that cord CD4⁺ T-cell proportions tended to be higher in Australian children born by means of cesarean section might imply that the observed difference in CD4 T-cell proportions between PNG and Australian newborns might be even greater than reported.