

ORIGINAL ARTICLE

Comparison of neonatal T regulatory cell function in Papua New Guinean and Australian newborns

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Abstract

Background: Environmental changes, including declining microbial exposure, have been linked with the rising incidence of allergic and autoimmune diseases in 'western' populations. This potentially occurs by altering early development of immunoregulatory pathways including T regulatory cells (T_{reg}). As there is now increasing evidence that such conditioning begins *in utero*

Methods: We have compared neonatal T_{reg} from children born under typical western conditions (Australia, AUS) with those of neonates born under more traditional conditions of high microbial burden (Papua New Guinea, PNG).

Results: The frequency of neonatal T_{reg}, defined as CD4⁺ Foxp3⁺ CD127⁻ CD25^{+/high} was found to be higher in the cord blood of AUS compared to PNG newborns. However, cord T_{reg} suppressive function in a small subset of children was qualitatively similar between PNG and AUS newborns in both a T_{reg} depletion assay and a T_{reg} supplementation assay.

Conclusions: These findings do not support the hypothesis that living in a 'western' versus more traditional environment leads to poor induction or suppressive function of neonatal T_{reg}. However, environmentally-induced immuno-regulation may potentially occur via alternative mechanisms in PNG newborns that should now be investigated further.

Regulatory T cells (T_{reg}) play a key role in controlling immune responses, balancing adequate immune defense against excessive and potentially harmful inflammatory responses, as well as maintaining tolerance to harmless self antigens and innocuous environmental allergens (1). Microbial exposure in early life is potentially important for the developmental maturation of these regulatory pathways; reduced levels of exposure associated with Western environmental changes have been linked to aberrant immune development and consequently a rise in allergic and auto-immune diseases in these populations (2, 3). Interestingly, there is growing evidence from both human and animal studies that the allergy-protective effects of microbial exposure begin prenatally (2, 4–6), highlighting the importance of the maternal environment in pregnancy.

T_{reg} are readily detectable in cord blood and have been reported to exhibit suppressive function (7, 8). Of immediate relevance here, prenatal environmental exposures have been recently demonstrated to influence T_{reg} development. Specifically, newborns of mothers with high microbial exposure during pregnancy (in a farming environment) showed enhanced numbers and function of T_{reg} at birth (5). Increased circulating T_{reg} were also demonstrated in newborns of parasite-infected mothers (9, 10). While links to allergic disease are less clear, our preliminary studies revealed deficiencies in T_{reg} cell number and function at birth in Australian-born children that subsequently developed allergy compared to those that did not (11). Together, these observations suggest that: (a) prenatal microbial exposure may increase T_{reg} numbers and/or enhance their suppressive function, and (b) prenatal

induction of T_{reg} may be protective against allergic disease development.

Populations in resource-poor settings are beginning to show the same trends for rising susceptibility to immune diseases, as they begin to shift from traditional towards western lifestyles (12–14). This emphasizes the strength of environmental pressures on immune development despite presumed genetic differences between populations. The aim of our study was to investigate T_{reg} cell frequency and function of neonates born under more traditional lifestyle conditions including high microbial burden (Papua New Guinea, PNG) compared with neonates born under typical western conditions in a neighboring region (Australia, AUS). The suppressive capacity of PNG versus AUS cord T_{reg} was assessed by means of both a T_{reg} depletion and a T_{reg} supplementation assay. We hypothesized that AUS newborns would show evidence of reduced T_{reg} -associated immune regulation compared with PNG newborns, compatible with the reduced maternal microbial burden and the elevated incidence of allergic disease in the AUS population.

Methods

Population characteristics

As the aim of this study was to investigate potential differences in T_{reg} cell frequency and function in neonates born under more traditional compared to typical western conditions, pregnant women were recruited through Goroka General Hospital in the Eastern Highlands Province of PNG ($n = 35$), and through private/state antenatal clinics in Perth, the capital city of Western Australia, in the neighbouring country AUS ($n = 32$), each exemplifying traditional and western environments, respectively. PNG is a comparatively low-income country with a gross national income per capita (PPP international dollars) of \$2030 compared to \$37250 for AUS. The majority of the PNG population (88%) lives in rural areas, whereas in AUS most people (89%) live in urban centres (15, 16). In PNG, the under-5 childhood mortality rate (68/1000) is considerably higher than in AUS (5/1000) (15, 16). At 773 per 100,000 births, PNG also has one of the highest maternal mortality ratios in the world (only 8/100,000 births in AUS) (16, 17). The proportion of the population using improved drinking-water sources and sanitation facilities (approximately 40% and 32%, respectively) in rural PNG is considerably lower than that in AUS (100%) (15, 16). As well as this, the PNG population has a relatively low adult literacy rate (56%) compared to the AUS population (87%) (15, 16). The Goroka study area in PNG is highly endemic for respiratory pathogens and intestinal helminthes and protozoa, but not for malaria due to its location at high altitude (1500–1900 m above sea level). The most commonly isolated respiratory pathogens include *Streptococcus pneumoniae* and *Haemophilus influenzae* (18). By 3 months of age, all children in this study area carry pneumococci in the upper respiratory tract and most (76%) are already carriers within

the first month of life (19). Comparatively, in high-income countries such as Australia, <50% of high-risk and 25% of low-risk children experience pneumococcal colonization within the first year of life (20). Furthermore, adult carriage rates are also significantly higher in PNG compared to populations of the developed world (19, 21). PNG mothers were not examined for respiratory pathogens or intestinal infections in the current study; however, in other studies conducted during the same time period in the same location, 30% of pregnant women carried pneumococci in the upper respiratory tract (19). Moreover, 82% of examined pregnant women were diagnosed with at least one intestinal infection: most commonly *Entamoeba histolytica*, *Giardia lamblia*, Hookworm and/or *Trichomonas hominis* infections (van den Biggelaar A. H. J, unpublished data). Sexually transmitted diseases (commonly *Trichomonas vaginalis* and/or *Chlamydia trachomatis* infection) are also highly prevalent (60%) amongst women from the Eastern Highlands Province of PNG (22). These discrepancies between the PNG and AUS study areas lend support to the overall difference in our chosen traditional (PNG) and western (AUS) environment (including microbial burden) and lifestyle conditions.

Inclusion criteria for the current study included uncomplicated pregnancy of at least 37 wk gestation, a birth weight of >2000 g, no acute neonatal infection and no severe congenital abnormality. HIV positive mothers were excluded from the study. In line with PNG standard clinical care, 82% of the PNG study mothers (23/28, 7 unknown) were vaccinated against tetanus in the third trimester of pregnancy. In accordance with the high prevalence of allergy in Westernized populations, 41% (13/32) of the recruited AUS study mothers were atopic as determined by a positive skin prick test to common allergens. While the atopic status of PNG mothers in this particular study was unknown, no evidence of atopy could be detected in a similar population living in a mountain village on a nearby PNG island (12). The exact number (N) of samples used in each experiment is included in the results section.

In addition, healthy laboratory volunteers ($n = 15$; mean age $\pm$ SD = 32.6 ± 7.5 yr) were recruited to collect peripheral blood mononuclear cells (PBMC) for use in functional assays, as described below.

Written informed consent was obtained from pregnant women (and in PNG 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.

Cell isolations

Blood samples were collected in sterile tubes containing an equal volume of RPMI (Invitrogen-Life Technologies, Melbourne, Australia) and preservative-free heparin (20 U/ml). Cord and peripheral blood mononuclear cells (CBMC and PBMC) were isolated by Ficoll-Hypaque (Alexis-Shield, Oslo,

Norway) gradient centrifugation and cryopreserved in 50% heat-inactivated (HI) FCS (JRH Biosciences, Lenexa, KS) and 7.5% dimethyl sulfoxide within 18 h from collection. Identical protocols and consumables were used in the PNG and AUS study location. PNG and AUS CBMC were both kept continuously in a liquid nitrogen vapor phase including during transport to Perth by dry-shipper, until used in further experiments. Cell viability was similar between PNG and AUS CBMC upon thawing.

All comparisons of PNG and AUS T_{reg} frequency and function were made side-by-side in the same laboratory (in Perth) at the same time using the same conditions/reagents. Note, it was not logistically possible to perform such comparisons of cord blood T_{reg} using fresh blood samples. While the function of cryopreserved versus fresh cord blood-derived T_{reg} may differ, comparisons of interest in our study were between PNG and AUS cord T_{reg} only; for which all cells were treated in an identical manner. Notably, the use of cryopreserved cord blood cells to study T_{reg} function in comparative studies such as ours has been validated elsewhere (11).

Cord Treg functional assays

Cord blood mononuclear cells and adult laboratory volunteer PBMC were labeled with anti-CD3 Dynabeads (Invitrogen Dynal AS, Oslo, Norway) to separate T cells from APC. The CD3⁺ APC fraction was gamma-irradiated at 3000 mCg. CD4⁺ T cells were selected from the CD3⁺ fraction using anti-CD4 detachable Dynabeads (Invitrogen Dynal AS) or, where indicated, CD4⁺CD127⁺CD25⁺ T cells and CD4⁺CD127⁺CD25⁺ T_{reg} cells were sorted from the CD3⁺ fraction using a FACSaria instrument and FACSDiva software (BD Biosciences, San Jose, CA, USA). The mean purity of CD4⁺CD127⁺CD25⁺ T_{reg} cells following cell sorting was 91.2% $\pm$ SEM of 2.2% ($n = 20$) and there were no differences between the groups ($p = 0.970$). Majority of CD4⁺CD127⁺CD25⁺ cells also expressed FOXP3 (mean % FOXP3 positive $\pm$ SD for PNG = 87% $\pm$ 8%; and AUS = 90% $\pm$ 7%, $n = 8$ /group; $p = 0.505$) (see Fig. S2).

T_{reg} depletion assay

CD4⁺ T-cells and T_{reg} -depleted CD4⁺CD127⁺CD25⁺ T-cells were collected from CBMC as described above and labeled with 11 μ M/ml carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen, Eugene, OR, USA). Per well, 5×10^4 gamma-irradiated CD3⁺ cells (APCs; autologous or obtained from adult PBMC) and 2.5×10^4 total CD4⁺ or T_{reg} -depleted CD4⁺CD127⁺CD25⁺ T cells were cultured in RPMI/5% HI-AB serum in the absence or presence of 1 μ g/ml phytohemagglutinin (PHA; Remel Europe Ltd., Kent, UK) for 68 h. In autologous assays, T cells and APC were derived from the same cord sample. In allogeneic cultures, APC were derived from adult PBMC ($n = 8$) and cultured with AUS or PNG cord T cells. Note, the same set of adult donors was used in both AUS and PNG allogeneic cultures to 'standardize' or optimally

limit any variability due to adult APC function in our comparisons.

T_{reg} supplementation assay

In order to standardize the assay and eliminate potential differences in PNG versus AUS cord APC and/or T-cell function (Lisciandro et al., manuscript in preparation), the suppressive capacity of PNG and AUS cord T_{reg} was studied in an allogeneic system in which cord T_{reg} were increasingly supplemented to cultures of adult APC and T-cells. In this system, 2.5×10^4 CFSE-labeled adult CD4⁺CD127⁺CD25⁺ responder T cells were cultured with 5×10^4 autologous gamma-irradiated CD3⁺ cells per well, and supplemented with either CD4⁺CD127⁺CD25⁺ PNG or AUS cord T_{reg} , or CD4⁺CD127⁺CD25⁺ control cells from a different adult donor (to correct for cell number) at ratios of 3:1, 2:1, 1:1 and 1:2 to adult responders. Cells were cultured in RPMI/5% HI-AB in the absence or presence of PHA (1 μ g/ml) and harvested for analysis of proliferation, phenotype and cytokine production at 68 h.

Flow cytometry

To determine T_{reg} frequency, CBMC were re-suspended in fluorescence-activated cell sorting (FACS) buffer (1×10^6 cells/tube), incubated on ice with 50 μ l of antibodies for CD4, CD25 and CD127 (BD Biosciences) (diluted 1:10, 1:5 and 1:20, respectively), washed, and then fixed, permeabilised and stained for intracellular FOXP3 using the FOXP3 staining buffer set (eBioscience, San Diego, CA, USA) as per the manufacturers instructions.

To measure proliferation, cells were harvested from cultures and co-stained with antibodies to CD25, CD127 and CD4, as well as propidium iodide (PI).

Data were acquired using 4-colour FACSCalibur or 10-colour LSRII (BD Biosciences) instruments and results were analysed using FlowJo software (Tree Star Inc., Stanford, CA, USA). Dead cells were excluded from analysis using a forward versus side scatter gate or alternatively selection of PI⁺ cells in proliferation assay analysis.

Cytokine protein detection

Cytokine levels were measured in supernatants using an in-house multiplex assay. Briefly, microspheres (MagPlex, Luminex®, Austin, TX, USA) coupled to azide-free primary antibodies against IL-5, IL-6, IL-9, IL-10, TNF α (Becton Dickinson, USA), IFN- γ and IL-1 β (eBioscience, Franklin Lakes NJ, USA) in PBS-BN (PBS + 1% BSA + 0.05% Sodium Azide, pH 7.4) (1×10^6 beads/ml) were plated onto a 96-well assay plate (Costar®, Corning, NY, USA) (50 μ l/well), followed by the addition of cytokine standards, quality controls, or samples. Standards were diluted in culture media and assay buffer (PBS+1%BSA), and quality controls and samples in assay buffer. A magnetic bead separator was used to wash the plates. After addition and incubation with biotinylated-secondary antibodies, plates were incubated with streptavidin-PE (Becton Dickinson) (diluted 1 in 1000 in

assay buffer), washed and assay buffer was added before reading on a Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA, USA). Samples with concentrations below the detection limit were given the value corresponding to half the lowest concentration that could be detected in this set of samples.

Statistical methods

Differences in continuous variables were tested for significance using a student *t*-test when data were normally distributed, or the Mann–Whitney *U* test when data were not normally distributed. Wilcoxon signed rank tests were used to test for differences in related samples. Pearson chi-square and Fisher's exact tests (when cells contained numbers equal or lower than 5) were performed to test for differences between groups in categorical variables. Differences were considered to be statistically significant when *p*-values were < 0.05. All analyses were performed using the statistical package SPSS 15.0. (SPSS Inc., Chicago, IL, USA).

Results

Population characteristics

The characteristics of PNG and AUS mothers and their newborns are shown in Table 1. AUS mothers were significantly older and delivered by caesarean section more often (38%). PNG newborns weighed significantly less and had smaller head circumferences than AUS newborns.

Cord T regulatory cell frequency

T_{reg} are commonly defined as $CD4^{+}$, $FOXP3^{+}$, $CD127^{-}$ and $CD25^{+}$ or $CD25^{high}$ cells (1). Therefore, in order to cover multiple definitions of T_{reg} , both $CD4^{+} FOXP3^{+} CD127^{-} CD25^{+}$ and $CD4^{+} FOXP3^{+} CD127^{-} CD25^{high}$ cellular populations were selected for flow cytometry analysis (gating strategy demonstrated in Fig S1). The frequency of T_{reg} was found to be significantly higher within the total CBMC population of AUS compared with PNG newborns (Fig. 1). Simi-

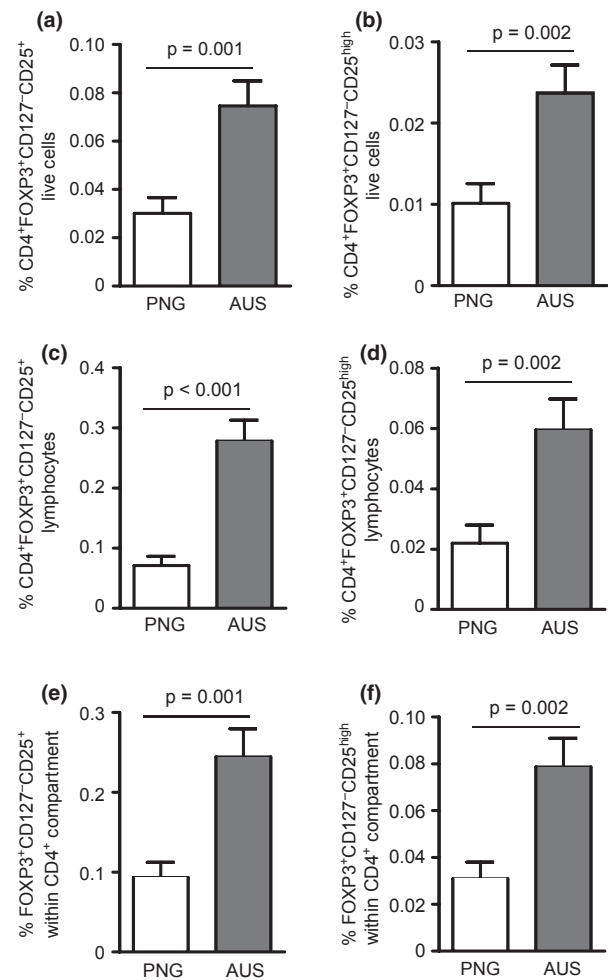


Figure 1 Cord T regulatory cell frequency. The frequency of $CD4^{+} FOXP3^{+} CD25^{+} CD127^{-}$ and $CD4^{+} FOXP3^{+} CD25^{high} CD127^{-}$ T cells in (a, b) total cord blood mononuclear cells; and within (c, d) the lymphocyte and (e, f) $CD4^{+}$ T-cell compartments of cord blood ($n = 19/\text{group}$). Data represent mean and SEM. Significance level is indicated.

Table 1 Obstetrics and newborn characteristics

	Papua New Guinea	Australia	p-value
Mean age mother, years	26.5 (7.2)	32.3 (5.0)	<0.001
Primigravida	14/31 (45%)	10/27 (37%)	0.531
Delivery by caesarean	0/31 (0%)	8/21 (38%)	<0.001
Antibiotic use in pregnancy	0/25 (0%)	2/27 (7%)	0.491
Male newborn	18/32 (56%)	13/26 (50%)	0.635
Mean gestational age, wks	39.4 (1.5)	39.3 (1.0)	0.839
Mean birth weight, kg	3.4 (0.4)	3.6 (0.3)	0.025
Mean birth length, cm	49.7 (4.6)	50.9 (2.1)	0.158
Mean head circumference, cm	33.5 (1.8)	35.1 (1.5)	0.001

Continuous variables are expressed as mean with standard deviation.

lar findings were observed when the T_{reg} frequency was determined within the lymphocyte and $CD4^{+}$ T-cell compartments of CBMC (Fig. 1).

Effects of T_{reg} 'depletion' on effector cell responses

Total $CD4^{+}$ neonatal T-cells or T_{reg} -depleted counterparts were cultured with autologous APC and PHA-induced T-cell proliferation was measured. Depletion of T_{reg} resulted in a significantly increased proliferation capacity of neonatal $CD4^{+}$ T cells from AUS newborns (Fig. 2a). However, this was not seen in the PNG neonates under the same conditions. The lack of a significant difference in proliferation between total and T_{reg} -depleted $CD4^{+}$ T-cells from PNG newborns could be interpreted as a limited suppressive capacity of PNG neonatal T_{reg} . However, we also noted a

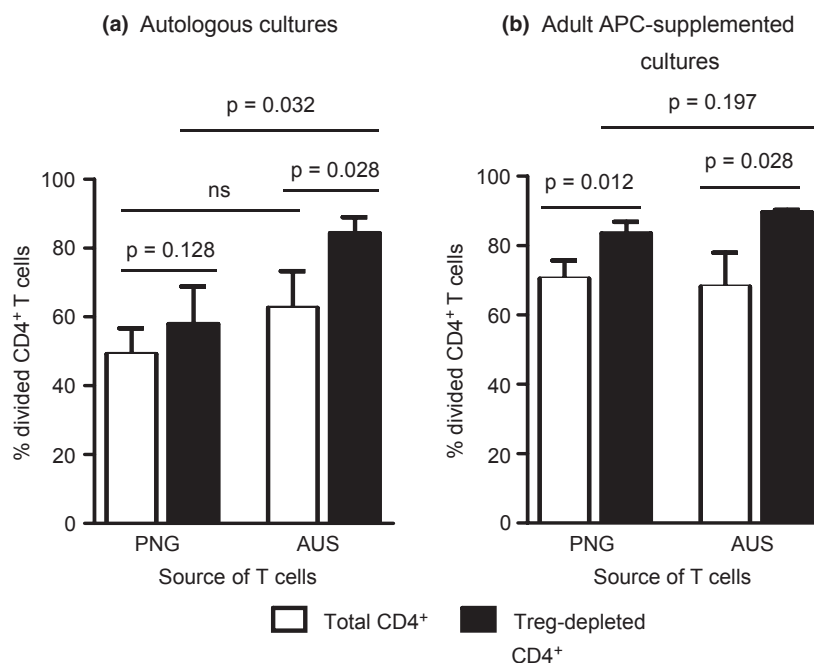


Figure 2 Suppression of proliferation in autologous and allogeneic cultures following cord T_{reg} depletion. Proliferation was assessed for cord CFSE-labeled CD4⁺ or Treg-depleted CD4⁺ T cells (CD4⁺ CD25⁻ CD127⁺) co-cultured with either (a) autologous (PNG n = 7; AUS n = 6) or (b) adult APCs (PNG n = 8; AUS n = 6) stimulated with PHA. Data represent mean and SEM. Significance level is indicated.

comparatively reduced overall proliferative response of PNG compared to AUS naïve CD4⁺ T-cells. This response is also consistent with a reduced capacity of PNG APC to induce proliferation (Lisciandro et al., manuscript in preparation) and potentially confounds the determination of T_{reg} functional activity, particularly if T_{reg} are acting via APC or *vice versa*, as has been previously reported (23).

To overcome differences in neonatal APC populations and focus more directly on the question of T_{reg} functionality, we repeated this experiment using 'standardised APC' derived from adult PBMC instead of autologous APC. In these experiments, depletion of T_{reg} resulted in a significant increase in T-cell proliferation that was comparable for PNG and AUS cells (Fig. 2b), suggesting that the suppressive activity of cord T_{reg} is similar for PNG and AUS newborns after controlling for any differences in APC-induced lymphoproliferation.

Effects of T_{reg} 'supplementation' on effector cell responses

We next analysed the functional capacity of cord T_{reg} using adult-derived CFSE-labelled CD4⁺CD25⁻CD127⁺ responder T-cells and APCs (γ-irradiated CD3⁺) that were supplemented with PNG versus AUS cord-derived CD4⁺CD25⁺CD127⁺ T_{reg} or 'control' CD4⁺CD25⁻CD127⁺ T cells derived from a different adult donor (to adjust for the total number of cells in culture). Compared to 'control' cultures, supplementation with cord-derived T_{reg} at a 3:1 ratio of T_{reg} to responders resulted in a significant reduction in proliferation (Fig. 3a,b) and activation (as measured by CD25 expression) of responder T-cells (Fig. 3c). Some suppressive effects were also observed when culturing at the 2:1 ratio of T_{reg} to responders, but were lost thereafter (Fig. 3b–

c). Importantly, no significant differences were observed between PNG and AUS cord-derived T_{reg} in their ability to suppress proliferation or activation of responders (Fig. 3a–c, and Fig. S3). Reduced proliferation was also associated with lower production of T-cell associated cytokines (IFN-γ, IL-13, IL-10, TNF-α and IL-9) in the presence of T_{reg} at a 3:1 ratio to responder cells; an effect that became less evident when supplementing cultures with lower numbers of T_{reg} to responders, but was not different between AUS and PNG neonatal samples (Fig. 3d and Fig. S3C). These data further support the finding that on a per cell basis, the suppressive function of T_{reg} from PNG and AUS newborns is similar.

Discussion

Declining microbial exposure in early life has become a popular explanation for the increasing incidence of allergies and autoimmunity. Deficient maturation or induction of regulatory pathways including T_{reg} under conditions of low prenatal microbial burden may be involved (5). To investigate this hypothesis further, we have compared the frequency and function of cord-derived T_{reg} cells in neonates born in a high-income (AUS) versus a resource-poor setting (PNG), representing a low-microbial and high-microbial environment, respectively. Interestingly, our findings of a higher T_{reg} frequency and similar suppressive function in AUS compared to PNG newborns do not support the hypothesis that T_{reg}-associated immune regulation is impaired in the more 'westernized' (AUS) population.

Given that prenatal microbial exposure is presumed to be higher in the PNG compared to the AUS setting, our results do not support other studies showing enhanced cord T_{reg} number and function with increased prenatal microbial/para-

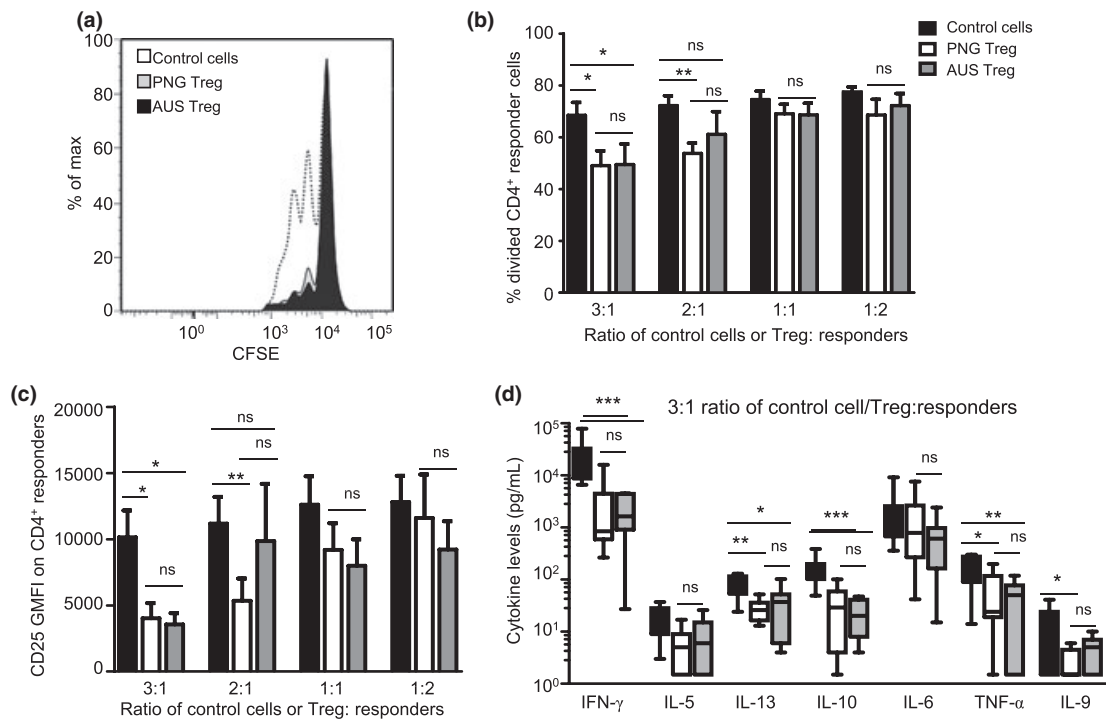


Figure 3 Effect of cord T_{reg} supplementation on activation, proliferation and cytokine production of CD4⁺ adult responders. (a–d) Cord T_{reg} cells were co-cultured with CFSE-labeled naïve CD4⁺ responder T cells and γ -irradiated CD3⁺ APCs derived from the same adult donor in the presence of PHA. Unlabelled CD4⁺ CD127⁺ CD25⁺ derived from an unrelated adult donor were added to control cultures in order to correct for cell number when comparing to T_{reg}-containing cultures (control cells, black, n = 11) (PNG T_{reg}, white, 3:1 ratio of T_{reg}: responders, n = 9; 2:1 ratio, n = 8; 1:1 and 1:2 ratio, n = 7) (AUS T_{reg}, grey, 3:1 ratio, n = 7; 2:1 ratio, n = 4; 1:1 and 1:2 ratios, n = 6). (a) An example of CFSE dilution of adult responder T-cells when supplemented with either control cells, PNG T_{reg} or AUS T_{reg} (b) Proportion (mean + SEM) of CD4⁺ responder T cells having undergone proliferation and (c) CD25 expression (geometric mean fluorescence intensity, GMFI + SEM) of CD4⁺ responder T cells. (d) Cytokine levels in PHA-stimulated cultures (3:1 ratio of control cells/Treg: responders; presented as box plots with median, interquartile range and minimum/maximum response) are shown (control n = 11, PNG n = 9, AUS n = 7). Significance at the 5% level is indicated (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant).

sitic (9, 10) and/or farm exposure (5). However, consistent with our findings, Kohler et al. have previously demonstrated significantly higher numbers of CD4⁺CD25^{hi} cells in the cord blood of Austrian (low microbial) compared to Gabonese (high microbial) newborns (24); though T_{reg} function was not compared in that study. Notably, both the study of Kohler et al. (24) and our own (Lisciandro et al., manuscript in preparation) indicate a lower plasmacytoid DC (pDC) frequency in children born under high-microbial (Gabon and PNG) versus low-microbial conditions (Austria and Australia), which is interesting considering that pDC have been shown to induce IL-10 producing T_{reg} (25). While this phenomenon has not been investigated in neonates, it can be hypothesized that a reduced proportion of cord pDC may explain the lower T_{reg} frequency observed in cords of PNG and Gabonese study infants. Alternatively, as measuring the frequency of cell types in cord blood only provides a snapshot of circulating cells at the time of birth, it is possible that the lower frequency of circulating T_{reg} cells in PNG versus AUS cords is explained by an increased sequestration of T_{reg} in distant sites controlling tissue inflammation.

Despite the comparability of PNG and AUS cord T_{reg} function in a standardized, allogeneic culture system, PNG T_{reg} did not suppress T-cell proliferation in an autologous culture system. Moreover, in the presence of autologous APC, proliferation of PNG cord CD4⁺ T cells was lower than that of Australian counterparts and unaffected by the presence or absence of T_{reg}. Replacement of autologous cord APC with APC sourced from an adult donor enhanced the proliferative response of PNG cord T-cells and restored the T_{reg} suppressive effect (further details on differences in PNG and AUS cord APC function will be described elsewhere: Lisciandro et al., manuscript in preparation). These observations suggest that control of T cell proliferation in PNG newborns is not solely dependent on the presence of T_{reg}, but instead likely occurs at multiple levels including that of innate immune pathways. Indeed, there is increasing evidence that the prenatal (6, 26) and early postnatal innate immune systems play important roles in initiating the immuno-regulatory and potentially disease-protective effects of early life microbial exposure (27, 28). Furthermore, others have previously reported that innate

immune responses were overall lower in newborns that remained free of allergies compared to those that continued to develop allergic disease in later life (29, 30). Therefore, reduced pro-inflammatory innate immune responsiveness in early life may be another mechanism of prenatal immune regulation in a developing world setting and deserves further exploration.

It is possible that differences in the diversity and types of microbial flora present in the European farming versus developing world setting, as well as other environmental and lifestyle factors may have contributed to the discrepancies between studies. Furthermore, potential genetic differences between the PNG and AUS populations (which, to our knowledge, has never been studied) and other maternal factors such as vaccinations or atopic status may have also influenced cord T_{reg} function. Due to the relatively small sample size, we did not have the statistical power to adjust for such confounding factors; nor was it our intention to study specific associations in the current study. Larger sized and statistically-powered studies that address the contribution of confounding factors are ideally needed to dissect out the independent effect(s) of specific environmental exposures and other maternal risk factors on prenatal immune development and susceptibility to immune disease.

In summary, our findings do not support the hypothesis that T_{reg} are enhanced in frequency or function in neonates born in the developing (traditional) compared to the devel-

oped ('western') world setting. Further investigation into alternative pathways of immune regulation in populations undergoing transition to a more western lifestyle may provide further insight into how prenatal environmental exposures modulate early immune development and susceptibility to inflammatory disease.

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Supporting Information

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

Figure S1. T regulatory cell gating strategy.

Figure S2. FOXP3 expression of CD4⁺ CD25⁺ CD127[−] cord lymphocytes.

Figure S3. Suppression of activation, proliferation and cytokine levels in T_{reg} supplemented cultures.

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