

ALLERGY

Cord blood monocyte–derived inflammatory cytokines suppress IL-2 and induce nonclassic “T_H2-type” immunity associated with development of food allergy

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Food allergy is a major health burden in early childhood. Infants who develop food allergy display a proinflammatory immune profile in cord blood, but how this is related to interleukin-4 (IL-4)/T helper 2 (T_H2)–type immunity characteristic of allergy is unknown. In a general population-derived birth cohort, we found that in infants who developed food allergy, cord blood displayed a higher monocyte to CD4⁺ T cell ratio and a lower proportion of natural regulatory T cell (nT_{reg}) in relation to duration of labor. CD14⁺ monocytes of food-allergic infants secreted higher amounts of inflammatory cytokines (IL-1 β , IL-6, and tumor necrosis factor- α) in response to lipopolysaccharide. In the presence of the mucosal cytokine transforming growth factor- β , these inflammatory cytokines suppressed IL-2 expression by CD4⁺ T cells. In the absence of IL-2, inflammatory cytokines decreased the number of activated nT_{reg} and diverted the differentiation of both nT_{reg} and naïve CD4⁺ T cells toward an IL-4–expressing nonclassical T_H2 phenotype. These findings provide a mechanistic explanation for susceptibility to food allergy in infants and suggest anti-inflammatory approaches to its prevention.

INTRODUCTION

Immunoglobulin E (IgE)–mediated food allergy in infants and young children has emerged as a major health burden in both high- (1–3) and low-income (4, 5) countries. Immune changes associated with development of food allergy include increased mononuclear cell interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) expression (6) and deficits in natural regulatory T cell (nT_{reg}) number and function (7) in cord blood, as well as persistence of IL-4/T helper 2 (T_H2)–type immune responses in the first year of life (8). Whether these findings are related to each other and to the IL-4/T_H2–dependent process of allergen-specific IgE production by B cells (9) required for development of food allergy is unknown.

In response to microbial exposure, innate immune cells such as granulocytes, monocytes, macrophages, and dendritic cells secrete cytokines that determine the fates of naïve CD4⁺ T cells (10). For example, IL-12 promotes differentiation of T_H1 cells, and IL-6 and transforming growth factor- β (TGF- β) promote differentiation of T_H17 cells (11). IL-4, although not regarded as a classical inflammatory cytokine, induces differentiation of T_H2 cells, which by further secreting IL-4 promote class switching to IgG1 and IgE antibody production by B cells (9, 12). It is widely held that commitment by naïve CD4⁺ T cells to T_H2 differentiation in vitro requires induction and activation of the transcription factors GATA-3 and STAT-5 (signal transducer and activator of transcription 5) by IL-4 and IL-2, respectively (13). However, T_H2 differentiation independent of GATA-3 and

IL-4 has also been observed in vivo (14), and the influence of inflammatory cytokines other than IL-4 on T_H2 differentiation is poorly documented (15).

Inflammatory cytokines also regulate the function of nT_{reg}. The suppressive function and survival of nT_{reg} are known to depend on IL-2, which is secreted by activated CD4⁺ T cells (16). IL-2 expression is repressed during microbial infection, which decreases the number and impairs the function of nT_{reg} (17). In addition, high concentrations of IL-6 (18) and TNF- α (19) promote degradation of FOXP3 (forkhead box P3), the master transcription factor for nT_{reg} (20). In the case of allergy, IL-6 and IL-1 β have been shown to break allergen-specific CD4⁺ T cell tolerance in human tonsil and peripheral blood (21). The increased innate immune responsiveness of cord blood (6, 22–24) may be a clue to T_H2 differentiation associated with food allergy. Here, in cord blood from a population-derived birth cohort, we uncover a relationship between innate and adaptive immunity in relation to subsequent challenge-proven IgE-mediated food allergy.

RESULTS

Enhanced innate immunity is associated with development of food allergy

Initially, in examining birth and immune parameters, we found that infants who developed food allergy had a higher ratio of monocyte/CD4⁺ T cells in relation to duration of labor (Fig. 1A and fig. S1A). The duration of labor itself was not related to subsequent food allergy (Fig. 1C), suggesting that prolonged labor revealed a proinflammatory immune state among at-risk infants. Further analysis of stored cord blood mononuclear cells (fig. S1B) demonstrated that the proportions of CD14⁺ monocytes and CD4⁺ T cells were negatively correlated, and their ratio was increased in infants who developed food allergy (Fig. 1D). To assess the function of innate immune cells, we stimulated flow-sorted

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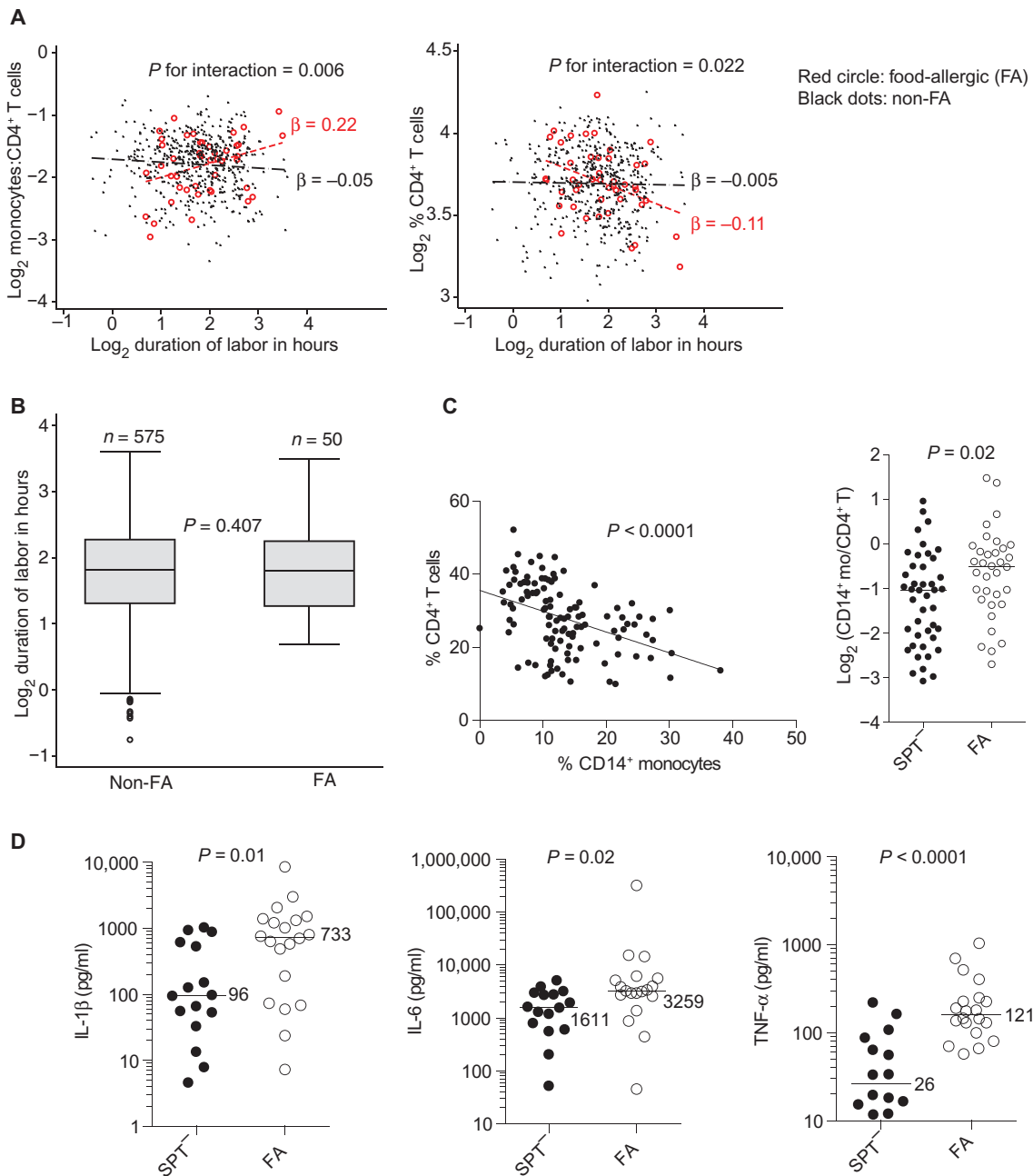


Fig. 1. Enhanced innate immunity is associated with development of food allergy. (A) In relation to duration of labor, cord blood of food-allergic (FA) infants had increased monocyte/CD4⁺ T cell ratio (left panel) and decreased CD4⁺ T cell (right panel) proportion compared to non-food-allergic infants (non-FA) ($n_{\text{non-FA}} = 630$, $n_{\text{FA}} = 54$; logistic regression with an interaction term). (B) Duration of labor was not different between FA and non-FA controls ($n_{\text{non-FA}} = 575$, $n_{\text{FA}} = 50$; *t* test). (C) In frozen cord blood mononuclear cells, the proportions of CD14⁺ monocytes (mo) and CD4⁺ T cells were inversely related

(left panel), and food-allergic infants had an increase in the ratio of CD14⁺ monocytes to CD4⁺ T cells (right panel) compared to infants who were skin prick test (SPT)-negative (SPT⁻) ($n_{\text{SPT}^-} = 43$, $n_{\text{FA}} = 34$; linear regression on the left and Mann-Whitney *U* test on the right). (D) Sorted CD14⁺ monocytes (0.6 million/ml) were activated with lipopolysaccharide (LPS) (1 ng/ml) for 24 hours, and secreted cytokines in the media were measured by Bio-Plex assay (horizontal lines are median values). Infants who developed food allergy secreted higher amounts of inflammatory cytokines ($n_{\text{SPT}^-} = 17$, $n_{\text{FA}} = 20$; Mann-Whitney *U* test).

CD14⁺ monocytes with LPS for 24 hours. The monocytes of infants who developed food allergy secreted higher amounts of IL-1 β , IL-6, and TNF- α (Fig. 1E). Thus, infants who developed food allergy displayed an increase in the monocyte/CD4⁺ T cell ratio and an increase in monocyte responsiveness at birth.

Inflammatory cytokines decrease nT_{reg} number and promote nT_{reg}-T_H2 differentiation

The proportion of naïve nT_{reg} in the CD4⁺ T cell compartment was lower among infants who developed food allergy (Fig. 2A and fig. S1C). Consistent with this finding, demethylation at the *FOXP3* (25) and *TIGIT*

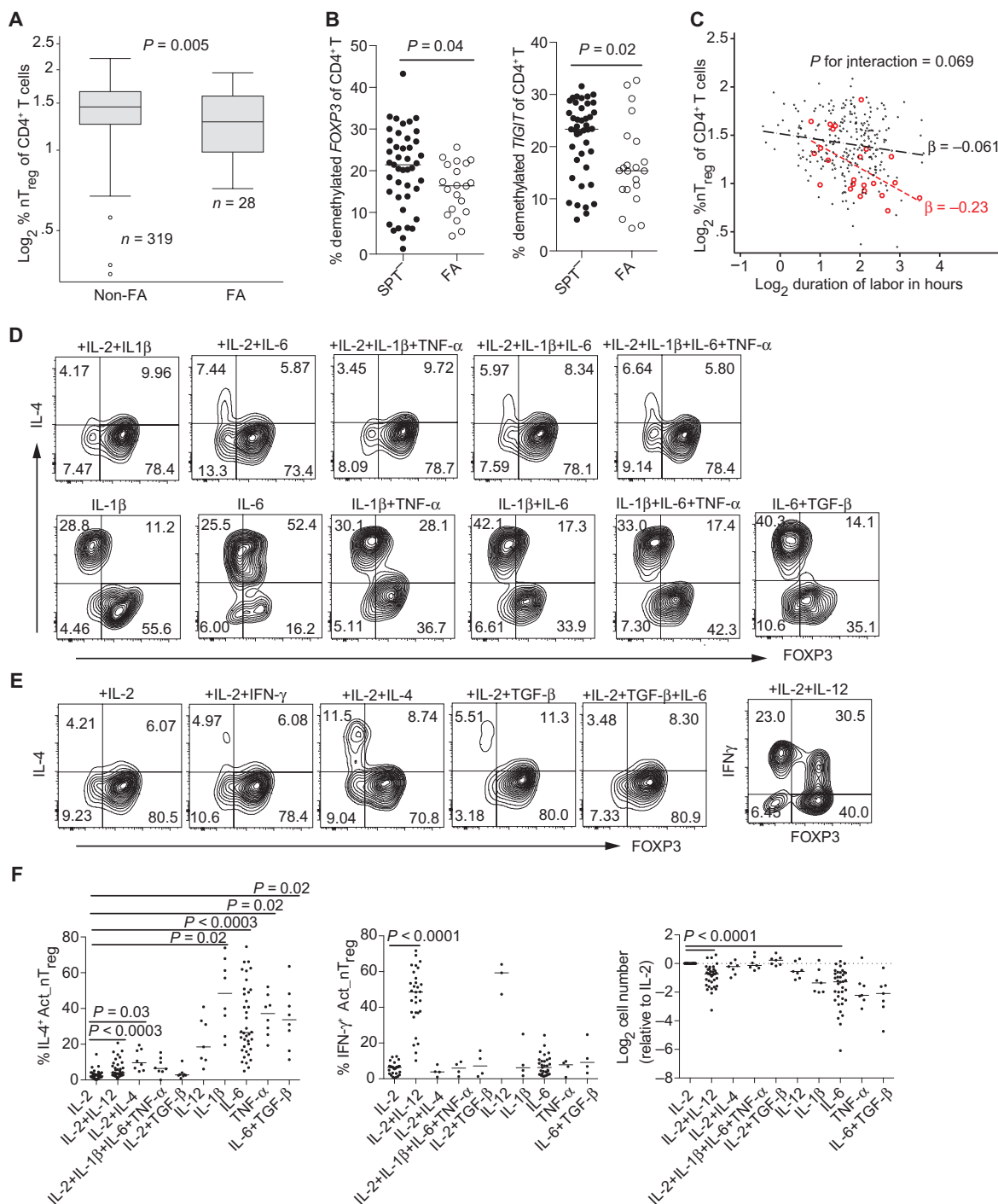


Fig. 2. Inflammatory cytokines decrease nT_{reg} number and promote nT_{reg}-T_H2-type differentiation. (A) Infants who developed food allergy (FA) had a decreased proportion of naive (CD4⁺CD45RA⁺FOXP3⁺) nT_{reg} compared to non-FA controls (geometric mean ratio, 0.17; 95% CI, 0.05 to 0.29; $n_{\text{non-FA}} = 319$, $n_{\text{FA}} = 28$; t test). (B) Demethylation of nT_{reg} signature genes *FOXP3* (left panel) and *TIGIT* (right panel) in sorted CD4⁺ T cells was decreased in food-allergic infants ($n_{\text{SPT}} = 49$, $n_{\text{FA}} = 21$; horizontal lines are median values, and P values are calculated by Mann-Whitney U test). (C) The proportion of naive nT_{reg} was decreased in relation to the duration of labor ($n_{\text{non-FA}} = 319$, $n_{\text{FA}} = 28$; linear regression). (D) Expression of IL-4 was in-

creased, and FOXP3 decreased in nT_{reg} activated in the absence of exogenous IL-2 and the presence of inflammatory cytokines \pm TGF- β . (E) Excepting T_H1 differentiation conditions (far right panel), expression of IL-4 and FOXP3 was unchanged in nT_{reg} activated in the presence of IL-2 under cord T_H differentiation conditions. (F) Activated nT_{reg} data for individual cord blood samples showing IL-4 and IFN- γ expression and cell number, modified by different cytokine combinations. ($n = 39$ for the largest number of tests performed; P value is calculated by Wilcoxon matched-pairs signed-rank test.) Multiple comparisons adjusted by the method of Benjamini and Hochberg (44). Act, activated.

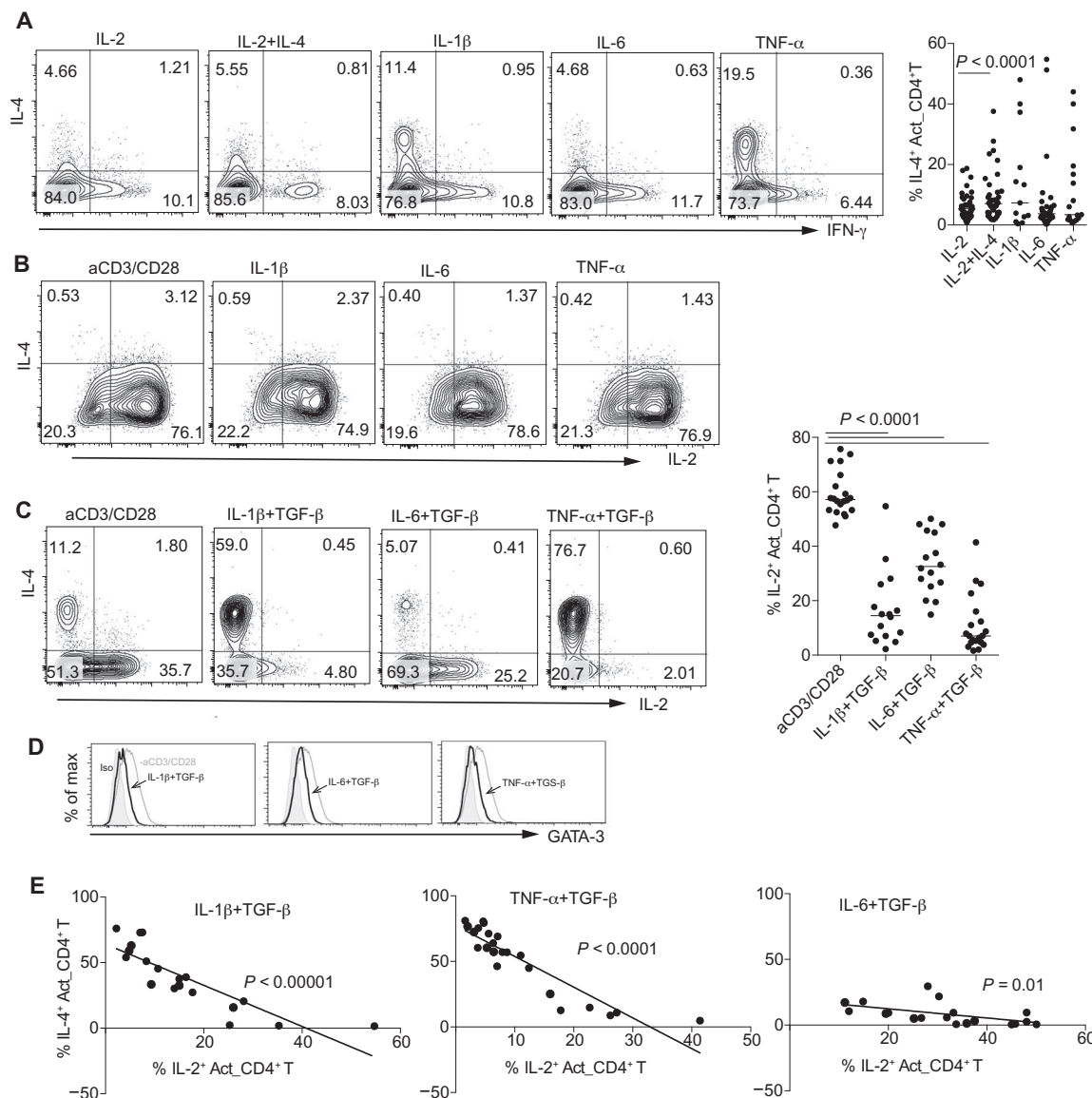


Fig. 3. Inflammatory cytokines and TGF- β repress IL-2 and induce nonclassical CD4⁺-T_{H2} differentiation. (A to C) Inflammatory conditions modified the expression of IL-4, IFN- γ , and IL-2 in activated naïve CD4⁺ T cells [$n = 40$ for (A) right-hand panel, $n = 18$ for (C) right-hand panel; Wilcoxon matched-pairs signed-rank test]. (D) GATA-3 expression was decreased in CD4⁺ T cells activated in the presence of inflammatory cytokines and TGF- β (data are representative of $n = 18$ donors). (E) IL-2 and IL-4 were negatively correlated in CD4⁺ T cells activated in the presence of inflammatory cytokines and TGF- β ($n = 19$; linear regression).

(26) loci, a specific marker of functional nT_{reg}, was less in sorted CD4⁺ T cells of food-allergic infants (Fig. 2B). The proportion of naïve nT_{reg} was decreased in relation to duration of labor, and this decrease was more pronounced in infants who developed food allergy (Fig. 2C).

Because infants who developed food allergy had increased monocyte cytokine responses to LPS, we examined the functional stability of naïve nT_{reg} upon activation in the presence of inflammatory cytokines. When activated in the presence of IL-1 β , IL-6, and/or TNF- α , naïve nT_{reg} displayed increased IL-4 and decreased FOXP3 expression, but only when exogenous IL-2 was absent (Fig. 2D). Moreover, this was also observed under conditions that promote differentiation of T_{H17} cells, that is, when IL-6 was combined with TGF- β (Fig. 2D), a cytokine

that is highly abundant in the gastrointestinal mucosa where food allergy is initiated. To further place these findings in context, we examined nT_{reg} after their activation in the presence of other cytokine combinations (Fig. 2E). Apart from decreased FOXP3 and increased interferon- γ (IFN- γ) expression observed under T_{H1} conditions (IL-2+IL-12), the expression of FOXP3 was stable. In activated nT_{reg} from individual cord blood samples, IL-4 and IFN- γ expression and cell number were variously altered by different cytokine combinations (Fig. 2F). Cytokines (IL-1 β , IL-6 \pm TGF- β , or TNF- α) that promoted skewing of nT_{reg} toward a T_{H2}-type phenotype (Fig. 2F, left panel) or cytokines (IL-12 \pm IL-2) that promoted skewing of nT_{reg} toward a T_{H1}-type phenotype (Fig. 2F, middle panel) decreased the numbers of activated nT_{reg} (Fig. 2F, right panel).

When naïve nT_{reg} were activated under the same neutral (IL-2) or inflammatory T_H2 (IL-6) and T_H1 (IL-2 + IL-12) conditions, no significant differences were observed between food-allergic and non-food-allergic infants (fig. S2, A and B). However, when naïve nT_{reg} were activated in the presence of autologous CD14⁺ monocytes, expression of FOXP3 was decreased and IL-4 increased. The increase of IL-4 was more prominent in food-allergic infants (fig. S2C). We deduced therefore that in infants who developed food allergy, enhanced inflammatory responses at birth may account for the lower frequency of naïve nT_{reg} and their subsequent gain of T_H2-type effector function.

Inflammatory cytokines and TGF- β repress IL-2 and induce CD4⁺ T_H2 differentiation

Because inflammatory cytokines IL-1 β , IL-6, and TNF- α promoted IL-4 expression in activated naïve nT_{reg} in the absence of IL-2, we determined whether they might have similar effects on T_H2 differentiation in cord blood naïve CD4⁺ T cells. Unlike nT_{reg} (Fig. 2D), in the absence of exogenous IL-2, inflammatory cytokines alone did not induce high IL-4 expression in most infants (Fig. 3A). We reasoned that intrinsic IL-2 expression by activated naïve CD4⁺ T cells might account for this lack of IL-4 expression (Fig. 3B and table S2). Moreover, allergic sensitization occurs at cutaneous and/or mucosal sites rich in TGF- β , which suppresses intrinsic expression of IL-2 (27). Therefore, we examined IL-4 expression when TGF- β was present under inflammatory conditions. In the presence of TGF- β , IL-1 β and TNF- α , and to a lesser degree IL-6, decreased the expression of IL-2 and increased the expression of IL-4 (Fig. 3C). The expression of GATA-3, the master transcription factor for classic T_H2 differentiation, was however reduced under inflammatory conditions (Fig. 3D). Furthermore, in contrast to the requirement of IL-2 for classic T_H2 differentiation, under inflammatory conditions, expression of IL-2 and IL-4 was inversely related (Fig. 3E and table S3). Thus, inflammatory cytokines in combination with TGF- β suppress IL-2 and promote high IL-4 expression and nonclassic T_H2 differentiation of naïve CD4⁺ T cells.

DISCUSSION

We found an enhanced inflammatory response in relation to duration of labor in cord blood of infants who developed food allergy. This was confirmed by the finding that LPS-stimulated CD14⁺ monocytes from infants who developed food allergy secreted higher amount of inflammatory cytokines IL-1 β , IL-6, and TNF- α . These inflammatory cytokines, together with TGF- β , which is abundant at cutaneous and/or mucosal sites of allergic reactions, decreased the ability of CD4⁺ T cells to express IL-2. In the absence of IL-2, the inflammatory cytokines decreased the number of activated nT_{reg} and diverted both nT_{reg} and CD4⁺ T cells toward an IL-4-secreting T_H2-type immune phenotype. These findings provide a mechanism for nonclassic T_H2 differentiation, in contrast to classic T_H2 commitment induced by IL-2 and IL-4 (13), and could explain IL-4- and GATA-3-independent T_H2 differentiation in vivo (14).

We therefore provide a mechanistic link between increased innate immune responsiveness at birth and subsequent development of IL-4/T_H2-associated food allergy. Innate cells may differ intrinsically in infants who develop food allergy or be modulated pre- or postnatally by environmental factors. Candidate gene (28) and genome-wide association studies (29, 30) have identified human leukocyte antigen (HLA), innate immune (TLR6), and cytokine signaling (IL2, IL1RL1, and

STAT6) genes associated with allergic sensitization and/or disease consistent with a genetic contribution to a hyperresponsive innate immune state. Environmental factors such as living on a farm and the consumption of nonpasteurized milk are associated with a lower risk of allergies in children (31–36). It would be of interest to determine whether these environmental conditions are associated with lower innate immune responsiveness.

Having shown differential production of inflammatory cytokines in the food-allergic neonates, we found that under inflammatory conditions, intrinsic IL-2 expression by CD4⁺ T cells was negatively correlated with IL-4 expression. *IL2* is one of the 10 gene loci associated with allergic sensitization (29), and *IL2RA* polymorphisms that affect IL-2R expression (37) have been identified in inflammatory diseases including allergic asthma (38). We have not determined whether naïve CD4⁺ T cell responses to inflammatory cytokines differ between food-allergic and nonallergic infants or whether these responses are modified by genetic susceptibility. It is also uncertain whether the cord blood findings described here persist into postnatal life or whether they are relevant to other allergic diseases.

We conclude that infants prone to food allergy display a hyper-responsive innate immune state at birth, which, in concert with the mucosal cytokine TGF- β , represses IL-2 expression by CD4⁺ T cells to promote nonclassic T_H2 differentiation and impair nT_{reg} function. These findings provide a mechanistic explanation for susceptibility to food allergy in infants and suggest that anti-inflammatory approaches may be beneficial in its prevention.

MATERIALS AND METHODS

Population and study design

The Research and Ethics Committee at the University Hospital Geelong, Australia, approved the research protocol, and each participating parent or legal guardian gave signed informed consent. The aims and methodology of the Barwon Infant Study (BIS) have been described previously (39). Briefly, a birth cohort of 1074 mother-infant pairs in southeast Australia was assembled using an unselected antenatal sampling frame. Infants born before 32 weeks or who developed serious illness in the first week of life or had significant congenital or genetic abnormalities were excluded. Duration of labor was defined as the interval between the onset of regular uterine contractions and the delivery of the infant. Flow cytometry analysis of immune cells was performed on freshly collected cord blood where possible, and infants were tested for food allergy at 1 year of age. Follow-up studies were performed on cryopreserved cord blood mononuclear cells from infants with food allergy compared to randomly selected infants who were SPT-negative. Of the inception birth cohort of 1074 eligible infants, 894 (83.2%) completed the 1-year review, and 697 (65%) had both flow cytometry performed on fresh cord blood immediately after birth and validated determination of food allergy status at 1 year. The demographics and size of the comparison groups are shown in table S1.

Food allergy testing

SPT was performed at 1 year according to standard guidelines (40). SPT was performed using QUINTIP skin pricks to the following allergens: cow's milk, egg, peanut, sesame, cashew, dust mite (*Dermatophagoides pteronyssinus* 1), cat, dog, rye grass, and *Alternaria tenuis* (Stallergenes). Infants with SPT wheals exceeding the negative control

by >1 mm were invited to undergo in-hospital oral food challenge (41, 42), and those with an immediate-type response to a specific food to which they were skin prick–positive were classified as allergic to that food. Infants with an SPT <1 mm greater than the negative control (SPT-negative) or an SPT >1 mm but a negative food challenge were classified as non–food-allergic.

Cord blood processing and analysis

Umbilical cord blood was collected by syringe before delivery of the placenta. About 5 ml was added to a serum clotting tube, and up to 20 ml of cord blood was added to a 50-ml Falcon tube containing 20 ml of RPMI medium (Gibco, Life Technologies) with 200 IU of preservative-free sodium heparin (Pfizer) and processed within 18 hours of collection. Where possible, an aliquot (100 μ l) of whole cord blood was stained with anti-CD3, anti-CD4, and anti-CD45 antibodies and lysed of red cells for the measurement of immune composition by flow cytometry (43), before isolation of plasma and mononuclear cells by density gradient centrifugation (Lymphoprep, Axis-Shield). Mononuclear cells (1×10^5 to 4×10^5) were stained with anti-CD4 and anti-CD45RA antibodies, fixed, and permeabilized (0.5% Tween) to stain for intracellular FOXP3. Antibodies were purchased from BD Pharmingen.

Cell subset isolation and activation

Frozen cord blood mononuclear cells were thawed and stained with anti-TCR $\alpha\beta$ (eBioscience), anti-CD4, anti-CD45RA, anti-CD25, anti-CD14 (BioLegend), anti-CD16, and HLA-DR (HLA-antigen D related) (eBioscience). Naïve CD4⁺ T cells (CD14[−]CD16[−]TCR $\alpha\beta$ ⁺CD4⁺CD45RA⁺CD25[−]), naïve nT_{reg} (CD14[−]CD16[−]TCR $\alpha\beta$ ⁺CD4⁺CD45RA⁺CD25⁺), and CD14⁺ monocytes (CD14⁺CD16[−]HLA-DR⁺) were flow-sorted. For T cell activation, naïve CD4⁺ and naïve nT_{reg} were activated with anti-human CD3/CD28 antibody microbeads (Life Technologies) at 1:1 ratio in the presence of recombinant human cytokines in IP5 medium [Iscove's modified Dulbecco's medium (Life Technologies) supplemented with 5% pooled human serum, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 100 μ M nonessential amino acids]. On day 3, fresh cytokine-containing IP5 medium was supplemented. The final concentrations of cytokines were as follows: IL-2 (200 U/ml), IL-4 (10 ng/ml), IL-1 β (10 ng/ml), TNF- α (10 ng/ml), and IL-6 (100 ng/ml). TGF- β was used at 5 ng/ml to generate induced T_{reg} (iT_{reg}) (IL-2 + TGF- β) and at 1 ng/ml in combination with inflammatory cytokines. On days 5 and 6, aliquots of activated T cells were restimulated with phorbol myristate acetate (100 ng/ml) and ionomycin (1 μ M) in the presence of monensin (2 μ M) for 5 hours and then stained intracellularly with anti-FOXP3, anti-GATA-3, anti-IFN- γ , anti-IL-4, and anti-IL-2 antibodies. Aliquots of activated T cells were surface-stained with anti-CD4 and anti-CD25 antibodies. Microbeads were added before surface staining to calculate absolute cell numbers. All antibodies were purchased from BD unless indicated otherwise.

For activation of monocytes, LPS (1 ng/ml) (L4391, Sigma Aldrich) was added to 150 μ l of IP5 medium containing 1×10^5 monocytes for 24 hours. Cytokines were measured in media with Bio-Plex assay (Bio-Rad) according to the supplier's instructions.

Methylation studies

Genomic DNA was isolated from flow-sorted total CD4⁺ T cells and bisulfite-converted. Methylation analysis of FOXP3 and TIGIT loci was carried out as previously described (26).

Statistics

Statistical analysis of flow cytometry data on freshly collected cord blood samples was performed with Stata 13 software. Cell percentages and ratios and duration of labor were log₂-transformed to approximate normal distributions, and associations were determined by linear regression (β = regression coefficient). Comparisons between infants with and without food allergy were investigated by logistic regression. An interaction term was used to determine whether relationships between cord blood measures and food allergy were modified by birth parameters. Statistical analysis of data obtained from cryopreserved samples was performed with Prism 6 software (GraphPad Software). For paired comparisons, a *P* value was calculated by Wilcoxon matched-pairs signed-rank test. For unpaired comparisons, *P* value was calculated by Mann-Whitney *U* test. *P* values for multiple comparisons were adjusted by the method of Benjamini and Hochberg (44).

SUPPLEMENTARY MATERIALS

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Fig. S1. Gating strategy.

Fig. S2. Food-allergic cord blood CD14⁺ monocytes promote nT_{reg}-T_{H2} differentiation.

Table S1. Demographics of the comparison groups.

Table S2. Source data for Fig. 3B (right-hand panel).

Table S3. Source data for Fig. 3E.

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Cord blood monocyte–derived inflammatory cytokines suppress IL-2 and induce nonclassic "T_H2-type" immunity associated with development of food allergy

Yuxia Zhang, Fiona Collier, Gaetano Naselli, Richard Saffery, Mimi LK. Tang, Katrina J. Allen, Anne-Louise Ponsonby, Leonard C. Harrison, Peter Vuillermin and on behalf of the BIS Investigator Group (January 13, 2016)

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Editor's Summary

Fighting food allergy

For people with food allergies, a slice of pizza or a peanut butter sandwich can be deadly. Yet despite the increasing prevalence of food allergy, little is known as to the immunological causes. Now, Zhang *et al.* report that infants who later developed food allergy had altered immunity at birth. Cord blood from these infants had more monocytes compared with CD4⁺ T cells and decreased numbers of regulatory T cells. Moreover, the monocytes from food-allergic infants secreted more inflammatory cytokines than those from healthy infants. These cytokines suppressed interleukin-2 (IL-2) expression by CD4⁺ T cells and skewed differentiation of these cells to a nonclassical T helper 2 (T_H2) phenotype. Anti-inflammatory strategies should therefore be considered in preventing food allergy in these individuals.

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