Interleukin-8 (CXCL8) production is a signatory T cell effector function of human newborn infants

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In spite of their precipitous encounter with the environment, newborn infants cannot readily mount T helper type 1 (T_H1) cell antibacterial and antiviral responses. Instead, they show skewing toward T_H2 responses, which, together with immunoregulatory functions, are thought to limit the potential for inflammatory damage, while simultaneously permitting intestinal colonization by commensals[1–3]. However, these collective capabilities account for relatively few T cells. Here we demonstrate that a major T cell effector function in human newborns is interleukin-8 (CXCL8) production, which has the potential to activate antimicrobial neutrophils and γδ T cells. CXCL8 production was provoked by antigen receptor engagement of T cells that are distinct from those few cells producing T_H1, T_H2 and T_H17 cytokines, was co-stimulated by Toll-like receptor signaling, and was readily apparent in preterm babies, particularly those experiencing neonatal infections and severe pathology. By contrast, CXCL8-producing T cells were rare in adults, and no equivalent function was evident in neonatal mice. CXCL8 production counters the widely held view that T lymphocytes in very early life are intrinsically anti-inflammatory, with implications for immune monitoring, immune interventions (including vaccination) and immunopathologies. It also emphasizes qualitative distinctions between infants' and adults' immune systems.

Although early survival of preterm infants has improved, late-onset septicemias or severe complications such as necrotizing enterocolitis (NEC) have increased as causes of mortality and long-term morbidity[4–6]. Yet relatively little research effort has focused on neonatal immune development, particularly in highly susceptible preterm infants. To redress this situation we studied a cohort of 28 preterm babies (born between 23 and 30 weeks post-conception) whose blood was drawn weekly from 14 to 35 d post-partum.

As was previously reported[1–3], T cell receptor $\alpha\beta^+$ $(TCR\alpha\beta)^+CD4^+$ T cells from preterm infants showed very little interferon-γ (IFN-γ) production in response to stimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin as compared to $TCR\alpha\beta^+CD4^+$ T cells from adults. The same was true for IL-17A production (**Fig. 1a**). In contrast, up to 50% of $TCR\alpha\beta^+CD4^+$ T cells of preterm infants (average >27% ($n = 28$)) displayed intracellular CXCL8 production, a highly significant increase relative to adults (average 4.7% ($n = 28$, $P < 8 \times 10^{-13}$)) (**Fig. 1a**,**b**). CXCL8 production was evident for babies born as early as 23 weeks' gestational age, and was mostly stable over successive samplings post-partum (**Fig. 1c**). The frequencies of CXCL8-producing cells did not correlate with gestational age at birth (**Supplementary Fig. 1a**; see **Supplementary Data Set**) and were comparable in cord blood samples from babies at term (average > 24% ($n = 12$)) (**Fig. 1b**). A higher percentage of preterm infants' T cells produced CXCL8 rather than tumor necrosis factor-α (TNF-α), which is heretofore the most widely documented effector cytokine of neonatal $CD4^+$ T cells (**Supplementary Fig. 1b**). $CD8^+$ T cells in preterm infants also produced significantly more CXCL8 than did $CD8^+$ T cells from adults (**Supplementary Fig. 1c**, $P < 3 \times 10^{-7}$). CXCL8 production by large fractions of T cells was unexpected, as it is mostly ascribed to myeloid and epithelial cells. In contrast to αβ T cells, γδ T cells in preterm infants showed high interindividual variation in CXCL8 production and more reproducibly produced IFN-γ, as previously reported[3] (**Supplementary Fig. 1d**).

PMA and ionomycin treatment induced production of the signatory T cell growth factor IL-2 by ~70–75% of preterm infants' $CD4^+$ T cells; ~30% of these cells co-produced CXCL8, whereas ~25–35% of CXCL8-producing cells did not co-produce IL-2 (**Fig. 1d**). Thus, among cytokine-producing cells, the ratio of $IL\text{-}2^+CXCL8^-$ to $IL\text{-}2^+CXCL8^+$ to $IL\text{-}2^-CXCL8^+$ cells was ~6:3:1. In experiments detecting intracellular effector cytokine production, CXCL8-producing cells were clearly distinct from the very small numbers of neonatal $CD4^+$ T cells producing IFN-γ, IL-13, IL-17, or granulocyte-macrophage colony–stimulating factor, and most were separable from those $CD4^+$ T cells (average 10%) producing TNF-α (**Fig. 1d**). There was also negligible expression of IL-4, CCL2, CCL5, CXCL10 and CXCL2 among CXCL8-producing cells. Thus, CXCL8 is not a secondary product of cells producing other effector cytokines. In adults, the small numbers of CXCL8-producing $CD4^+$ T cells could be readily discriminated from IFN-γ producers by CD38 expression (**Supplementary Fig. 1e**).

Given their distinction from other cytokine-producing T cells, we analyzed the phenotypes of CXCL8-producing $CD4^+$ T cells in infants

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Received 21 May; accepted 23 July; published online 21 September 2014; doi:10.1038/nm.3670

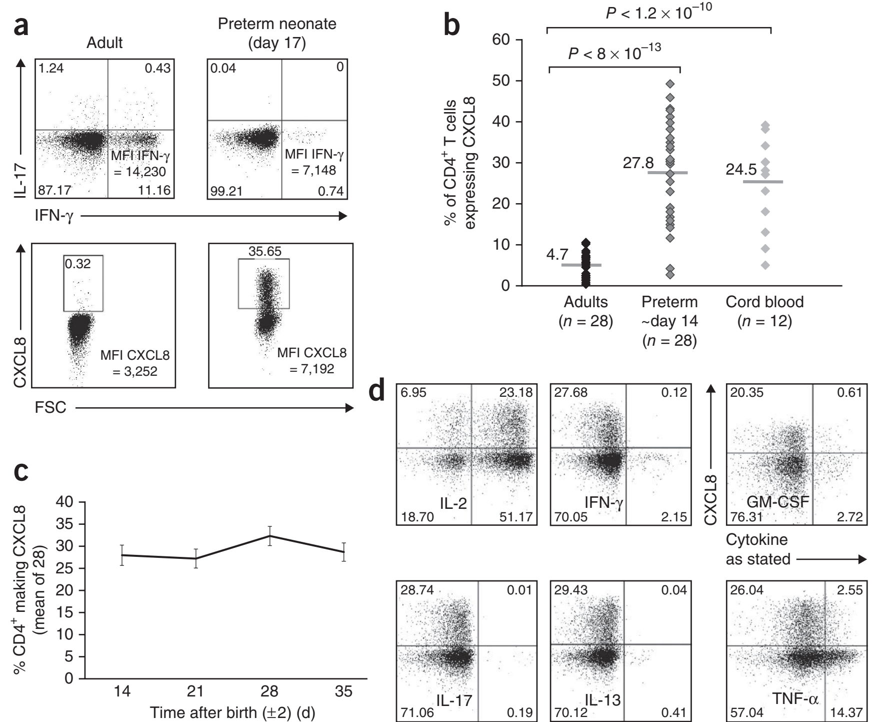

Figure 1 CXCL8-producing T cells in infants. (**a**) IFN-γ and IL-17 expression (top) and CXCL8 expression (bottom) in TCRαβ+CD4+ T cells isolated from an adult or infant (preterm day 17) following polyclonal activation. MFI, mean fluorescence intensity. (**b**) Percentage of CD4+ T cells that express CXCL8 after stimulation in adults (n = 28), preterm babies at around 2 weeks of age (n = 28) or cord blood (n = 12). Student's t-test values are indicated. (**c**) Mean percentage of preterm CD4+ T cells expressing CXCL8 over time (n = 24) following polyclonal activation. Error bars are s.e.m. (**d**) Expression of CXCL8 on y axis and other cytokines (as labeled) on x axis from TCRαβ+CD4+ T cells isolated from a representative (n = 5 preterm infants for IL-2 and GM-CSF and 28 infants for IL-17, IL-13, TNF-α and IFN-γ) preterm baby. GM-CSF, granulocyte-macrophage colony–stimulating factor.

and those few CXCL8-producing CD4+ T cells in adults. Most CXCL8-producing cells expressed the naive/immature T cell markers CD31, CD45RA, CCR7, CD38 and lacked CD95 (**Fig. 2** and **Supplementary Fig. 1e**). Of note, CD31+ T cells have relatively long telomeres, reflective of low turnover, and are enriched in infants[7,8]. However, the scarcity of CXCL8-producing CD4+ T cells in adults did not simply reflect the scarcity of naive T cells, as CXCL8 was on average produced by only 5% of adult cells expressing CD45RA, a marker of naive T cells (n = 10 adults), with the IL-2+CXCL8− to IL-2+CXCL8+ to IL-2−CXCL8+ ratio being ~15:0.75:1 in adults, compared to 6:3:1 for neonates (**Supplementary Fig. 2**). Thus, substantive CXCL8 production is a signature of naive/immature CD4+ T cells in infants.

Because CXCL8 is rapidly secreted and cannot be detected at the cell surface, it was not possible to use antibodies to purify viable CXCL8-producing T cells by flow cytometry or magnetic bead–based separation. Therefore, to molecularly characterize CXCL8-producing CD4+ T cells, we used flow cytometry to purify PMA- plus ionomycin-activated cord blood and adult CD45RA+CD38+CD4+ subsets, which contain cells with CXCL8-producing potential, enabling us to compare their gene expression profiles with those of adult-derived CD45RA+CD38−CD4+ cells, which do not produce CXCL8. Validating the approach, *CD38* and *IL8* (encoding CXCL8) were among the three most (P = 0.0038 for CD38, P = 0.026 for CXCL8) upregulated genes in CD45RA+CD38+CD4+ cells (**Supplementary Table 1a**), whereas the most underexpressed genes were those encoding IFN-γ, CCL2, CCL5, XCL1, IL-22 and IL-13 (**Supplementary Table 1b**), reflecting an aggregate underexpression of networks relating to T_H1, T_H2 and T_H17 immune responses (**Supplementary Table 1c**). Of factors regulating gene expression, CD45RA+CD38+CD4+ cells most significantly overexpressed *SMAD1* (**Supplementary Table 1a**) and underexpressed *MYBL1* and *FOXP3* (**Supplementary Table 1b**), the latter result contrasting with the common perspective that neonatal CD4+ cells are disproportionately regulatory[9].

Having established that infant CD4+ T cells produce CXCL8, we determined the conditions that could induce its expression *ex vivo*. After 24 h of stimulation with beads coated in antibodies to the TCR and to CD28, a major T cell co-stimulator, cord blood CD4+ T cells produced CXCL8 (**Supplementary Fig. 3a**) and ~400 pg ml^{-1} CXCL8 was detected in cell supernatants by ELISA (**Supplementary Fig. 3b**). Expression declined by 48 h after stimulation. TCR and CD28 co-stimulation likewise induced CXCL8 RNA expression in highly purified CD4+ T cells from five preterm infants, confirming this to be an intrinsic response of neonatal CD4+ T cells (**Supplementary Fig. 3c**). Although it has previously been reported that Toll-like receptor (TLR) stimulation induces CXCL8 production from the Jurkat human T cell line[10], CXCL8 was not induced in purified cord blood CD4+ T cells by lipopolysaccharide (TLR4 ligand), poly(I:C) (TLR3 ligand) or R848 (TLR7/8 ligand), alone or in combination with beads coated in antibodies to the TCR. Likewise, neither Pam3Cys (TLR2 ligand) nor flagellin (TLR5 ligand) alone induced CXCL8, but they did co-stimulate its production by cord blood CD4+ T cells in concert with anti-TCR and anti-CD28 antibody–coated beads (**Fig. 3a**,**b** and **Supplementary Fig. 4**). However, flagellin in combination with anti-TCR and anti-CD28 antibody–coated beads did not induce substantial CXCL8 production in adult CD4+ T cells, emphasizing that infant CD4+ T cells are functionally distinct from their adult counterparts.

Having demonstrated CXCL8 production by CD4+ T cells *in vitro*, we sought evidence for it *in vivo*. Although CXCL8 is not conserved in mice, we examined neonatal mouse CD4+ T cells for the neutrophil chemoattractants Kc (keratinocyte chemoattractant, a functional ortholog of human CXCL8), Cxcl2 and Cxcl5. However, we detected negligible amounts of these products upon stimulation by ELISA (necessitated by a lack of antibodies to detect intracellular chemokines) compared to IL-2 in 2- to 3-d-old and 7-d-old mice, which mimic preterm and term human infants' T cells, respectively (**Supplementary Fig. 5**). Thus, it was impractical to use mice to assess CXCL8 production *in vivo*.

Although it is highly unusual to detect spontaneous cytokine production (for example, of IFN-γ) by CD4+ T cells from infected adults, blood from a preterm infant (26 weeks post-conception) with suspected sepsis harbored a small but discrete population of T cells constitutively expressing intracellular CXCL8 without stimulation

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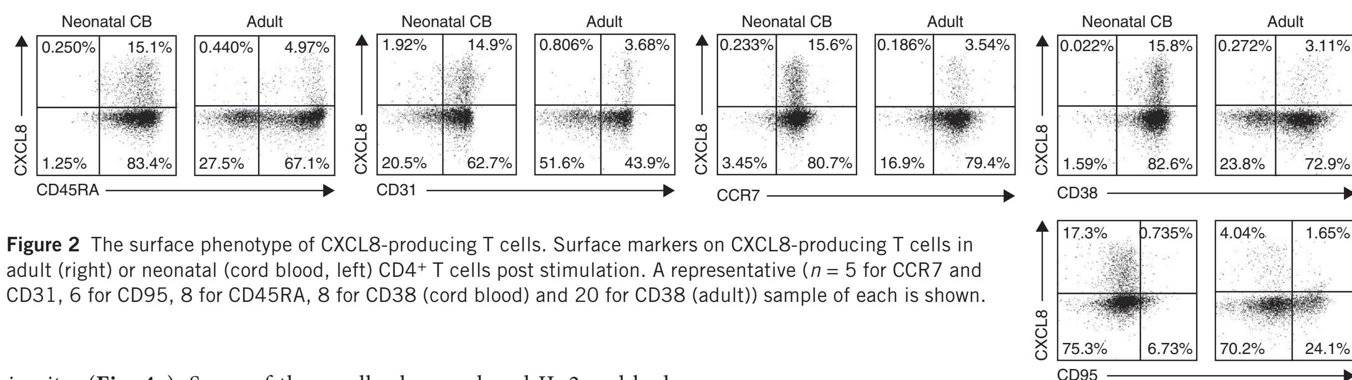

Figure 2 The surface phenotype of CXCL8-producing T cells. Surface markers on CXCL8-producing T cells in adult (right) or neonatal (cord blood, left) CD4+ T cells post stimulation. A representative (n = 5 for CCR7 and CD31, 6 for CD95, 8 for CD45RA, 8 for CD38 (cord blood) and 20 for CD38 (adult)) sample of each is shown.

in vitro (**Fig. 4a**). Some of these cells also produced IL-2 and had reduced surface CD4 levels, consistent with their having been activated *in vivo* (**Fig. 4a**). Indeed, when we evaluated CD4^{low} T cells from this infant, ~10% expressed CXCL8 (**Fig. 4a** and **Supplementary Fig. 6a**,**b**), and the ratio of IL-2$^+$CXCL8$^-$ to IL-2$^+$CXCL8$^+$ to IL-2$^-$CXCL8$^+$ cells was 4.5:2.5:1, similar to the 6:3:1 ratio of IL-2$^+$CXCL8$^-$ to IL-2$^+$CXCL8$^+$ to IL-2$^-$CXCL8$^+$ preterm CD4$^+$ T cells following stimulation *in vitro*.

Constitutive CXCL8 expression by T cells was also evident (**Fig. 4b**) in the gut mucosa from an infant with NEC. Although T cells were not the sole source of CXCL8 (**Fig. 4b**), the data confirm CXCL8 production by T cells in an infant under pathophysiologic stress. Moreover, whereas most infants showed stable longitudinal T cell expression of CXCL8 in response to PMA and ionomycin (**Fig. 1c**), there were instances of sharp, substantial perturbations, most often among infants whose C-reactive protein (CRP) levels were elevated, suggesting infection (**Fig. 4c**). Thus, one infant showed steadily increasing CXCL8 production by CD4$^+$ T cells that preceded a marked CRP rise and a clinical diagnosis of *Escherichia coli* septicemia, after which CXCL8 production plateaued as CRP declined (**Fig. 4d**). Increased frequencies of CXCL8-producing CD4$^+$ T cells coincided with and/or preceded transient increases in CRP in three further infants (**Supplementary Fig. 6c**), one of whom was diagnosed with NEC and another of whom was diagnosed with *Staphylococcus aureus* septicaemia, whereas the third remained undiagnosed. Increases in TNF-α–producing T cells were smaller and less sharp, and percentages of IFN-γ– or IL-17A–producing cells remained negligible (**Fig. 4d**; **Supplementary Fig. 6c**).

CXCL8 is a major activating chemotactic factor of human neutrophils[11,12]. Because γδ T cells precociously produce effector cytokines in newborns (as compared to αβ T cells)[3], we also examined their potential to be co-stimulated by CXCL8. Indeed, CXCL8 enhanced IFN-γ production by Vδ1$^+$ cells, which are predominant in newborns, and by Vδ2$^+$ cells, which dramatically expand in peripheral blood during infancy[13] (**Supplementary Fig. 7**).

This study casts a new light on the immunology of newborn babies. Whereas in newborn babies T cell production of many effector cytokines is suppressed, their production of CXCL8 was overt *in vitro* and evident *in vivo*. TCR-regulated CXCL8 production would be consistent with the regulation of *IL8* by nuclear factor of activated T cells (NFAT)[14]. As a potential means to activate γδ effector T cells and neutrophils, we suggest that CXCL8 production should be classified as a proinflammatory immunoprotective cytokine of neonatal T cells. Consistent with the importance of this function to newborn infants, they likewise display increased CXCL8 production by myeloid and epithelial cells[15–17]. However, to our knowledge, perspectives on neonatal immunoprotection have not hitherto considered CXCL8 production by primary T cells, potentially placing antigen receptor–mediated recognition upstream of neutrophil activation. This may facilitate neonatal discrimination between pathogenic and commensal bacteria, a challenging task for the small number of pattern recognition receptors (for example, TLRs) that humans possess. Thus, this newly identified neonatal T cell effector capability may be a credible target to enhance clinical support of preterm and other vulnerable infants and in childhood vaccination. Moreover, a possible failure of CXCL8-producing T cells to be reconstituted from adult bone marrow might partially explain the dysbiosis commonly seen in transplant recipients[18].

CXCL8-producing T cells may also contribute to perinatal immunopathology. However, because we did not identify any CXCL8$^+$ cells expressing memory cell markers, it is unclear whether CXCL8-producing cells can form memory T cells of the kind that perpetuate autoimmune diseases. Nonetheless, CXCL8-producing T cells may contribute to immunopathology in the form of childhood acute T lymphocyte leukemia (T-ALL)[19], for which CXCL8 production correlates negatively with prognosis. Indeed, our ongoing studies have found CXCL8 production post-stimulation by four T-ALL samples from children aged 5–15 (D.G., A.H., C. Furness and M. Greaves, unpublished data).

In addition to CXCL8, naive human T cells produce complement components[20] that may potently co-stimulate neutrophil activation.

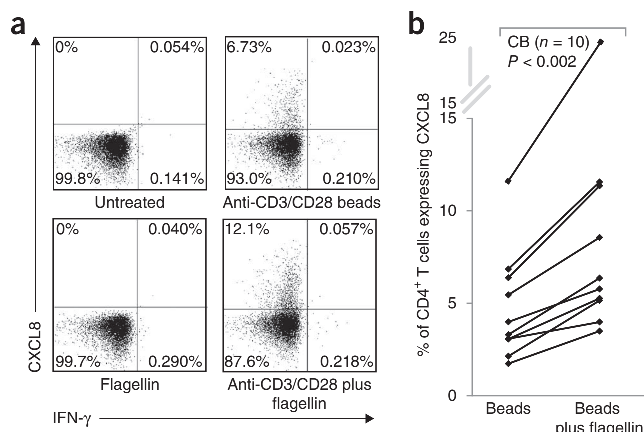

Figure 3 Flagellin co-stimulates CXCL8 production from neonatal T cells. (**a**) Expression of IFN-γ and CXCL8 from sorted cord blood CD4$^+$ T cells after stimulation with anti-CD3 and anti-CD28 beads alone (top) or in the presence of flagellin (bottom). (**b**) Expression of CXCL8 from sorted CD4$^+$ T cells isolated from cord blood (CB; n = 10) after stimulation with anti-CD3 and anti-CD28 beads with or without flagellin overnight. Means of duplicate wells are shown for each experiment. Paired Student's t-test values are indicated.

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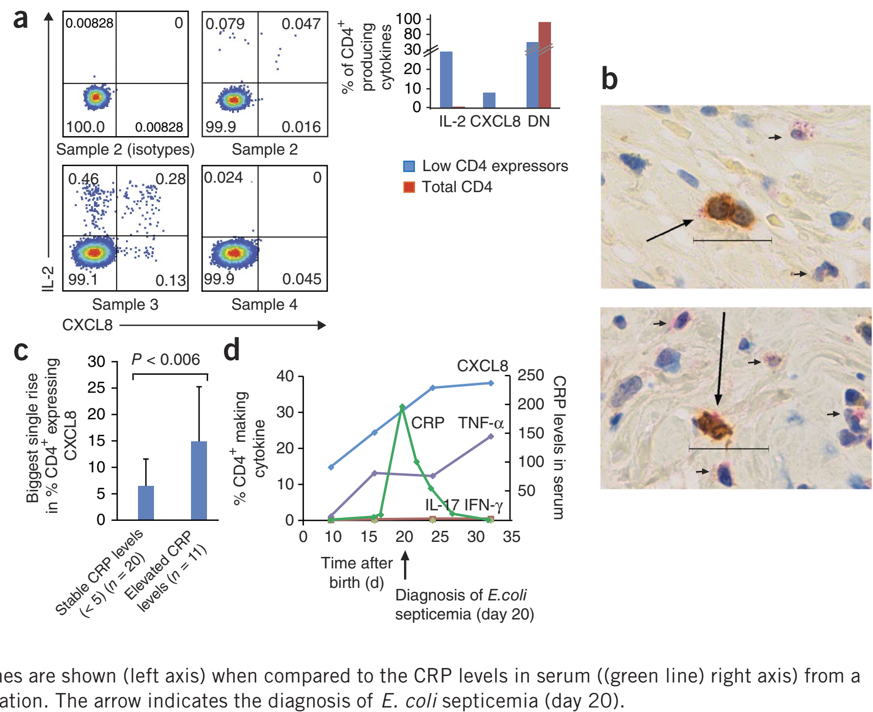

Figure 4 Infants with illness display CXCL8-producing T cells. (**a**) Spontaneous expression of IL-2 (*y* axis) and CXCL8 (*x* axis) from TCRαβ+CD4+ cells isolated at weekly intervals from 3 weeks after birth (sample 2) from a preterm baby, born at gestational age 26 weeks. Top left, staining observed in the presence of the isotype controls for the cytokine antibodies. The graph at right shows the percentage of CD4+ T cells from sample 3 making either IL-2 or CXCL8 or neither (DN) when gated on total CD4+ or those cells with reduced CD4 expression (activated CD4+ T cells). (**b**) Immunohistochemistry of NEC sections stained for CD3 (brown) and CXCL8 (red). CXCL8-producing T cells are indicated by long arrows and non-T cells making CXCL8 are indicated by short arrows. Scale bars, 25 µm. (**c**) Preterm babies were separated into those whose CRP levels remained <5 throughout the sampling period ($n = 20$) and those in whom CRP levels became elevated ($n = 11$). The *y* axis shows the biggest increase in the percentage of CD4+ cells expressing CXCL8 noted between two consecutive samples upon stimulation. Student's *t*-test value is indicated. (**d**) The percentage of CD4+ T cells making individual cytokines are shown (left axis) when compared to the CRP levels in serum ((green line) right axis) from a preterm infant (GA 30 weeks) after polyclonal stimulation. The arrow indicates the diagnosis of *E. coli* septicemia (day 20).

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Whereas mouse neutrophils are activated by IL-17, some reports show that human neutrophils are relatively refractory to IL-17 because of defective IL-17 receptor expression[21,22]. Thus, the roles of CXCL8-producing T cells in human neonates might be assumed in newborn mice by T_H17 cells.

The *IL8* gene was originally cloned from a T cell[23], yet there have been only sporadic reports of rare CXCL8-producing natural killer T cells[24], γδ T cells[25,26] and T regulatory cells[27]. Likewise, there are reports of CXCL8-producing cloned T_H17 cell lines[21] but none of primary CXCL8-producing T_H17 cells, consistent with the distinction between CXCL8-producing cells and T_H1, T_H2 and T_H17 cells shown here. Within adult peripheral blood, CXCL8-producing cells were reported to comprise ≤1 in 40 CD4+ T cells[28], which is consistent with our results. It is possible that CXCL8-producing CD31+ T cells are produced throughout life but in adults very rapidly transition to more mature CD31− T cells[8] that would be CXCL8 negative. Alternatively, CXCL8-producing cells (like Langerhans cells and dendritic epidermal γδ T cells[29,30]) may arise from fetal progenitors absent from adults and be subsequently outcompeted by newly arising T cells. This would be consistent with the discontiguity of fetal and adult hematopoiesis and with gene profiling data showing that *IL8* gene expression was greatly enriched in fetal naive CD4 T cells[31], although this was not commented upon by the authors of the study. Similarly, a functional dissimilarity of fetal and adult T cells seems evident in the production by neonatal T cells of a variant IL-4 isoform rarely seen in adults[2].

Our finding of a potential protective mechanism against bacterial infection in newborn infants is timely given that attempts to enhance antibacterial immunity have largely failed[32]. The existence of CXCL8-producing T cells in babies born very prematurely strongly suggests an autonomous fetal defense against infection. Thus, our characterization of this T cell function and the capacity to quantitate it within peripheral blood cells offer several possibilities, including a novel biomarker, for anticipating infection and/or response to therapeutic interventions. Prior attempts to use IL-6 in this regard highlight the need for additional metrics of neonatal T cell activity[33].

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data have been deposited at NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO accession number GSE52129.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank P. Hunter for helpful discussions, T. Hayday for flow cytometry, K. Rouault-Pierre (London Research Institute, Cancer Research UK) for cord blood, Pierre Vantourout (London Research Institute, Cancer Research UK) for γδ T cell lines, M. Greaves for advice on T-ALL, P. Chakravarty for microarray analysis and M. Leite-de-Moraes for support of mouse studies. P.F. was funded by a strategic research grant from the Barts and the London Charity, A.V. and N.J.S. by the UK National Institute for Health Research (NIHR) Great Ormond Street Hospital (GOSH) Biomedical Research Centre (BRC), N.J.S. partly by GOSH Children's charity, and D.G., R.C. and A.H. by the Guy's and St. Thomas', charity, the NIHR Biomedical Research Centre at Guy's and St. Thomas', Hospital and King's College, and by a Wellcome Trust Programme Grant to A.H.

AUTHOR CONTRIBUTIONS

D.G. co-designed the study, undertook all experiments with human materials, evaluated the results and co-wrote the manuscript; P.F. co-designed the study, was attending physician to the clinical trial to which the study is annexed, provided human samples, evaluated clinical data and edited the manuscript; A.V. undertook the immunohistology; M.-L.M. undertook the animal model experiments; N.J.S. evaluated immunohistology and provided samples; K.C. designed and supervised the clinical trial to which the study is annexed and edited the manuscript; R.C. co-formulated the study as an annex to a clinical trial and edited the manuscript; N.K. co-supervised the analysis of pathology and edited the manuscript; A.H. co-designed the study, evaluated the results and co-wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Human samples. Informed consent was obtained from all human subjects (or their parents). Protocols were approved by the East London and City Research Ethics Committee (cord blood samples, HREC: O6/Q0604/110); the South London Research Ethics Committee 2 (cohort of preterm babies, HREC: 10/H0802/40); the Wandsworth Research Ethics Committee (adult controls, HREC: 07/H0803/237); and the Bloomsbury Research Ethics Committee (NEC sections, HREC: 11/LO/0495). Human cord blood was obtained from St. Bartholomew's and the Royal London Hospitals and was positively selected for $CD34^+$ stem cells (Easysep Human CD34 selection, StemCell Technologies), and the $CD34^-$ fraction was used for the experiments. Peripheral blood was sampled from prematurely born infants every week from two weeks post-partum, and adult and preterm peripheral blood mononuclear cells (PBMCs) were extracted by Ficoll gradient centrifugation (GE Healthcare).

Cell stimulation. Primary cells were stimulated with either PMA and ionomycin or anti-CD3+CD28 beads as described below. Cells were stimulated in RPMI 1640 medium (Invitrogen) containing 10% (vol/vol) FCS (StemCell Technologies), 2 mM L-glutamine (Sigma), 100 U penicillin and 100 μg ml^{-1} streptomycin (Invitrogen) (complete medium, CM) containing 10 ng ml^{-1} phorbol 12-myristate 13-acetate (Sigma) and 1 μg ml^{-1} ionomycin (Sigma) for 4 h at 37 °C, 5% CO_2 in the presence of 20 μg ml^{-1} brefeldin A (Sigma). For spontaneous cytokine production, cells were incubated in CM in the presence of brefeldin A only. Alternatively, cells were stimulated in CM in the presence of human T cell activator beads (Life Technologies) at a ratio of 1 bead to 1 cell for 4 h before the addition of brefeldin A (5 μg ml^{-1}) overnight.

Where CD4 cells were stimulated directly, they were sorted from PBMCs by negative selection using the CD4 T cell isolation kit (Miltenyi Biotec) followed by staining with streptavidin-PE (Biolegend) and flow sorting on a FACSAria II (Becton Dickinson) to a purity of >90%. Sorted CD4 cells were stimulated with PMA plus ionomycin or T cell activator beads (as above) or with anti-CD3 (LEAF purified OKT3) coated at 1 μg ml^{-1} on a 96-well round-bottomed plate together with soluble anti-CD28 (LEAF purified CD28.2). In some cases, TLR ligands were also added at the start of the incubation, as indicated.

Human polyclonal γδ T cell lines were generated from healthy donor PBMCs grown in complete medium in the presence of plate-bound LEAF anti-pan γδ TCR (clone B1, BioLegend) at 0.1 μg ml^{-1} for Vδ2 cells or 3 μg ml^{-1} for Vδ1 cells. 24 h after seeding, 100 U ml^{-1} IL-2 was added. Fresh medium containing 100 U ml^{-1} IL-2 was added every 2–3 d for 3 weeks. Cells were then frozen before stimulation on 1 μg ml^{-1} plate-bound anti-γδ in the presence or absence or recombinant CXCL8 (BioLegend) for 6 h at 37 °C in the presence of brefeldin A (20 μg ml^{-1}).

Flow cytometry. Flow cytometry was carried out using a BD FACS Canto II. Single-cell suspensions were prepared in FACS buffer (PBS plus 2.5% (vol/vol) FCS and 2 mM EDTA). Initially, cells were incubated on ice with Live-Dead fixable Aqua stain (Invitrogen) to allow live/dead discrimination, and cells were then surface stained on ice with the following antibodies: PeCy7-labeled anti-TCRαβ (IP26), Pacific Blue–labeled anti-CD4 (OKT3), APC-labeled anti-CD45RA (H5100); Alexa-fluor 647–labeled anti-CD31 (WM59), APC/Cy7-labeled anti-CD8α (HIT8α); and PeCy5-labeled anti-CD95 (DX2), all from BioLegend and used at 1:40 dilution; and PE-labeled anti-CD38 (HIT2) from eBioscience (used at 1:50) and PC5-labeled anti-pan γδ TCR from Beckman Coulter (used at 1:20). Staining with Brilliant Violet 421–labeled anti-CCR7 (GO43H7) was performed at 37 °C at a dilution of 1:40.

For intracellular staining, cells were fixed (Cell Fix, BD) after surface staining and then permeabilized using Perm buffer (BioLegend) and stained with the following antibodies (all from BioLegend and used at 1:40 dilution unless otherwise stated): FITC-labeled anti-CXCL8 (E8N1) or PerCPCy5.5-labeled anti-CXCL8 (BH0814), PerCPCy5.5-labeled anti-IFN-γ (4S.B3) or PE-labeled anti-IFN-γ (B27), APC-labeled anti-GM-CSF (BVD2-21C11); FITC-labeled anti-IL-17 (BL168, used at 1:20); PerCPCy5.5-labeled anti-IL-2 (MQI-17H12); PeCy7-labeled anti-TNF-α (MAb11); PerCPCy5.5-labeled anti-IL-13 (JES10-5A2). Gates were defined though isotype and fluorescent minus one (FMO) stains. Data were analyzed using FlowJo software.

RNA expression analysis. Total RNA was isolated with the RNeasy microkit (Invitrogen) as per manufacturer's instructions. For real-time RT-PCR analysis, RNA was reverse transcribed with Superscript II (Invitrogen) using oligo-dT. Quantitative PCR was performed with Power SYBR green PCR master mix on ABI 7900 equipment (Applied Biosystems). Relative expression is displayed in arbitrary units normalised to GAPDH via the $\Delta\Delta C_t$ method.

For microarray analysis, samples were first amplified using the NuGEN amplification kit, and the Affymetrix Human gene 1.0 ST array was utilized.

Immunohistochemistry. Paraffin-embedded sections of NEC (Great Ormond Street NHS Trust) were stained for CXCL8 (Invitrogen, clone 893A6G8) and CD3 (Leica, clone LN10) using a Leica Bond-Max automated immunostainer following dewaxing with Leica dewax solution and pretreatment for 20 m (HIER with ER2 at pH9). Staining was detected with Bond polymer refine detection.

Mice. Male and female C57BL/6 mice were from Harlan or Charles River laboratories and were bred and maintained specific pathogen free in the animal facility of the Cancer Research UK London Research Institute. All experiments involving animals were undertaken in compliance with relevant laws and guidelines and were approved by the Cancer Research UK animal ethics review board (PPL 80/2480). Spleens from different-aged mice were homogenized and washed in RPMI 1640 medium containing 10% (vol/vol) FCS. Mononuclear cells were stained using anti–mouse TCRβ APCeFluor780 (H57-597, eBiosciences, used at 1:50) and anti–mouse CD4 Brilliant Violet 421 (GK1.5, BioLegend, used at 1:500) and $CD4^+$ T cells sorted by flow cytometry. Cells were incubated with PMA and ionomycin, and supernatants were removed after 48 h. Kc, Cxcl2, Cxcl5 and Il-2 levels were measured by ELISA (R&D Systems) as per the manufacturer's instructions.

Statistical analyses. Student's *t*-test (2-tailed) is used throughout the manuscript, and *P* values are indicated. All tests were unpaired unless otherwise indicated. Error bars represent s.d. unless otherwise specified. Center values on graphs represent the mean value. No statistical method was used to predetermine sample size, the experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

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 doi:10.1038/nm.3670