Human Neonatal Naive CD4+ T Cells Have Enhanced Activation-Dependent Signaling Regulated by the MicroRNA miR-181a

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Compared with older children and adults, human neonates have reduced and delayed CD4+ T cell immunity to certain pathogens, but the mechanisms for these developmental differences in immune function remain poorly understood. We investigated the hypothesis that impaired human neonatal CD4+ T cell immunity was due to reduced signaling by naive CD4+ T cells following engagement of the αβ-TCR/CD3 complex and CD28. Surprisingly, calcium flux following engagement of CD3 was significantly higher in neonatal naive CD4+ T cells from umbilical cord blood (CB) compared with naive CD4+ T cells from adult peripheral blood. Enhanced calcium flux was also observed in adult CD4+ recent thymic emigrants. Neonatal naive CD4+ T cells also had higher activation-induced Erk phosphorylation. The microRNA miR-181a, which enhances activation-induced calcium flux in murine thymocytes, was expressed at significantly higher levels in CB naive CD4+ T cells compared with adult cells. Over-expression of miR-181a in adult naive CD4+ T cells increased activation-induced calcium flux, implying that the increased miR-181a levels of CB naive CD4+ T cells contributed to their enhanced signaling. In contrast, AP-1-dependent transcription, which is downstream of Erk and required for full T cell activation, was decreased in CB naive CD4+ T cells compared with adult cells. Thus, CB naive CD4+ T cells have enhanced activation-dependent calcium flux, indicative of the retention of a thymocyte-like phenotype. Enhanced calcium signaling and Erk phosphorylation are decoupled from downstream AP-1-dependent transcription, which is reduced and likely contributes to limitations of human fetal and neonatal CD4+ T cell immunity. The Journal of Immunology, 2013, 190: 000-000.
polyclonal treatment with anti-CD3 and anti-CD28 mAbs. This treatment results in activation of the tyrosine kinases Lck, ZAP-70, and phospholipase C (PLC)–γ1. Activated PLC-γ1, in turn, catalyzes production of the second messengers inositol triphosphate and diacylglycerol. Production of inositol triphosphate stimulates calcium release from the endoplasmic reticulum, which initiates an influx of extracellular calcium through the calcium release activated calcium channel of the cell membrane. This increase in the free intracellular concentration of Ca2+ ([Ca2+]i) results in the calcium-dependent activation and nuclear translocation of the NFAT family of transcription factors (17).

Diacylglycerol and other ZAP-70–derived signals activate Ras, which, in turn, activates Erk in a MAPK cascade that results in the generation of AP-1, a heterodimeric transcription factor of Fos and Jun proteins (18). The activation-dependent expression of cytokines, such as IL-2 and IFN-γ, and TNF ligand family members, such as CD154, by T cells requires de novo transcription of their cognate genes by the engagement of NFAT and AP-1 in promoter cis-elements (19–22). Considerable evidence exists that the generation of calcium flux, with the consequent NFAT activation in the absence of AP-1 activation, results in a program of gene transcription promoting anergy rather than a productive immune response (22–24).

Limitations at many steps in T cell activation–induced signaling and cytokine gene transcription may contribute to decreased Ag-induced cytokine and CD154 expression by CD4+ T cells of the fetus or neonate. In support of proximal signaling limitations, some studies of CB T cells have found reduced tyrosine phosphorylation of the tyrosine kinases, such as Lck and ZAP-70 (25) or PLC-γ1 (26), following CD3 and CD28 mAb stimulation, compared with T cells from APB. An important limitation of these studies and many others (reviewed in Ref.1) is that they did not directly compare naive (CD45RAhighCD45ROlow) CD4+ T cells of CB with those from APB, but instead used unfractionated APB T cells, which have a substantial number of memory/effector cells. Because memory/effector T cells have distinct, often enhanced, activation-dependent signaling properties, compared with naive T cells (27), this approach is not as informative for identifying true developmental limitations in signaling by naive T cell populations. Limitations imposed upon CD4+ T cell immunity in neonates by developmental changes in activation-dependent signaling in naive CD4+ T cells remain unclear.

In this study, we tested the hypothesis that reduced signaling in naive CD4+ T cells following engagement of the αβ TCR/CD3 complex and CD28 contributes to limitations in CD4+ T cell immunity in the human neonate. Unexpectedly, we found that TCR-induced increases in [Ca2+]i and Erk phosphorylation were increased calcium response was mediated, at least in part, by calcium and magnesium and without phenol red (Life Technologies/Invitrogen) supplemented with 1% heat-inactivated human AB serum (hAB) (Gemini Bioproducts, West Sacramento, CA), and combined with equivalent numbers of unlabeled naive CD4+ T cells. The combined labeled and unlabeled cells were then stained with allophycoerythrin-conjugated CD19 (clone SJ25-C1; Invitron), CD69 (clone 3B5; Invitrogen), and CD45RO (clone UCHL1; Becton Dickinson Biosciences or Invitrogen) mAbs. After washing, cells (1 × 10^7/ml) in HBSS with 1.0% hAB were incubated with indo-1 acetoxyethyl ester (Molecular Probes/Invitrogen) at a final concentration of 500 μM for 30 min at 37˚C. After washing, cells were incubated in HBSS with 1.0% hAB for another 30 min at room temperature and stained with 7-AAD. Indo-1 emission at 346 and 380 nm was measured using an LSR II instrument (both from Becton Dickinson Biosciences). Samples were equilibrated at 37˚C for 10 min prior to data acquisition. A 30 s baseline was collected, followed by addition of 1:1000 (v/v) of OKT3 (CD3ε) mAb–containing ascites (Cocalico Biologicals, Reamstown, PA) at 30 s. After 2 min 45 s, 30 μg goat anti-mouse Ig (Jackson Immunoresearch, West Grove, PA) or sheep anti-mouse Ig (Millipore, Danvers, MA) was added to cross-link surface-bound CD3ε mAb. After 8 min, calcinomycin (Sigma-Aldrich) was added at a final concentration of 1.0 μM, and after 10 min 15 s, acquisition was either terminated (for miRNA-transfected samples) or EGTA was added at a final concentration of 5.0 mM and acquisition was terminated at 12 min 30 s. The peak and mean 330 nm/346 nm ratios from the CD3 mAb cross-linking period were calculated using FlowJo software (TreeStar, Ashland, OR).

Protein tyrosine kinase 7 surface staining of naive CD4+ T cells

Purified APB naive CD4+ T cells were stained with rat IgG monoclonal anti-human protein tyrosine kinase 7 (PTK7) (generated by Genovac, Freiburg, Germany) or, as a negative control, purified rat IgG (Sigma-Aldrich) for 20 min at room temperature. After blocking with 10% hAB serum, cells were incubated with PE-conjugated goat–anti-rat–IgG (Jackson Immunoresearch), followed by additional blocking by incubation with unlabeled rat IgG for 15 min at 4˚C. For calcium flux assays, cells were stained with allophycoerythrin-conjugated CD19, CD69, and CD45RO mAbs, as described above. 7-AAD was added after washing, and samples were loaded with indo-1 acetoxyethyl ester and analyzed as described above. For miRNA expression analysis, PTK7+ and PTK7– cell populations were separately evaluated and confirmed by fluorescence-activated cell sorting. After isolation of CB or APB MCs by Ficoll-Hypaque density gradient centrifugation, as described above, cells were stained with CD3–Alexa 700 (clone CD3e–Alexa Fluor 750–conjugated, clone OKT3, eBioscience, San Diego, CA; or allophycoerythrin–Alexa Fluor 750–conjugated, clone S4.1, Invitrogen, Carlsbad, CA); CD4+ (allophycoerythrin–conjugated, clone S3.5; Invitrogen); CD45RA+ (FITC-conjugated, clone HI100; Becton Dickinson Biosciences); CD45RO+ (PE-conjugated, clone UCHL1; Invitrogen) and 7-aminoactinomycin D+ (7-AAD; Becton Dickinson Biosciences).

Materials and Methods

Blood mononuclear cell and naive CD4+ T cell isolation

Blood was collected from healthy adult donors with informed consent in accordance with the requirements of the Stanford University Internal Review Board. Umbilical CB was collected from the placentas of healthy births of infants that were of term gestation and normal weight, and delivered vaginally at Lucille Packard Children’s Hospital (Palo Alto, CA), in accordance with Internal Review Board requirements. CB and APB were anticoagulated with preservative-free heparin sodium sulfate (Sigma-Aldrich, St. Louis, MO) and subjected to density gradient centrifugation using Ficoll-Hypaque (GE Healthcare Biosciences, Pittsburgh, PA) to isolate the mononuclear cell (MC) fraction. Unless otherwise indicated, naive CD4+ T cells used in experiments were purified from MCs by MACS after treatment with a Human Naïve CD4+ T Cell Negative Selection Kit II and application to paramagnetic LS columns (Miltenyi Biotec, Auburn, CA). Cells for miR-181a measurements were positively selected with a CD4 Selection Kit (Miltenyi) and FACS sorted on a FACSAria or modified FACS Vantage (both from Becton Dickinson Biosciences, San Jose, CA). Naive CD4+ T cells were sorted and defined as follows: CD3+ (allophycoerythrin–Alexa Fluor 750–conjugated, clone OKT3, eBioscience, San Diego, CA; or allophycoerythrin–Alexa Fluor 750–conjugated, clone S4.1, Invitrogen, Carlsbad, CA); CD4+ (allophycoerythrin–conjugated, clone S3.5; Invitrogen); CD45RA+ (FITC-conjugated, clone HI100; Becton Dickinson Biosciences); CD45RO+ (PE-conjugated, clone UCHL1; Invitrogen) and 7-aminoactinomycin D+ (7-AAD; Becton Dickinson Biosciences).

Fluorescent labeling and measurement of calcium flux

Purified naive CD4+ T cells (1 × 10^5 cells per milliliter) were either incubated with 8.0 μg/ml Alexa 488 succinimidyl ester (Molecular Probes/Invitrogen) in PBS (Life Technologies/Invitrogen) or in PBS alone for 20 min at room temperature. Labeled cells were then washed twice with calcium and magnesium and without phenol red (Life Technologies/Invitrogen) supplemented with 1% heat-inactivated human AB serum (hAB) (Gemini Bioproducts, West Sacramento, CA), and combined with equivalent numbers of unlabeled naive CD4+ T cells. The combined labeled and unlabeled cells were then stained with allophycoerythrin-conjugated CD19 (clone SJ25-C1; Invitron), CD69 (clone 3B5; Invitrogen), and CD45RO (clone UCHL1; Becton Dickinson Biosciences or Invitrogen) mAbs. After washing, cells (1 × 10^7/ml) in HBSS with 1.0% hAB were incubated with indo-1 acetoxyethyl ester (Molecular Probes/Invitrogen) at a final concentration of 500 μM for 30 min at 37˚C. After washing, cells were incubated in HBSS with 1.0% hAB for another 30 min at room temperature and stained with 7-AAD. Indo-1 emission at 346 and 380 nm was measured using UV laser–equipped modified FACS Vantage or an LSR II instrument (both from Becton Dickinson Biosciences). Samples were equilibrated at 37˚C for 10 min prior to data acquisition. A 30 s baseline was collected, followed by addition of 1:1000 (v/v) of OKT3 (CD3ε) mAb–containing ascites (Cocalico Biologicals, Reamstown, PA) at 30 s. After 2 min 45 s, 30 μg goat anti-mouse Ig (Jackson Immunoresearch, West Grove, PA) or sheep anti-mouse Ig (Millipore, Danvers, MA) was added to cross-link surface-bound CD3ε mAb. After 8 min, calcinomycin (Sigma-Aldrich) was added at a final concentration of 1.0 μM, and after 10 min 15 s, acquisition was either terminated (for miRNA-transfected samples) or EGTA was added at a final concentration of 5.0 mM and acquisition was terminated at 12 min 30 s. The peak and mean 330 nm/346 nm ratios from the CD3 mAb cross-linking period were calculated using FlowJo software (TreeStar, Ashland, OR).
Naive CD4+ T cells were transfected with 2.5 μg/ml PHA (Sigma) for 19.5 h, as previously described (28), and then transfected with plasmids (2.0 μg/1 x 10^6 cells), using a Neon electroporation system (Invitrogen) set for 2400 V, 12 ms, and 2 pulses. The AP-1 reporter plasmid (a generous gift of O. Martinez, Stanford University) has five AP-1 binding sites (from pHexAP1) linked with 33 μg/ml goat anti-mouse IgG (Jackson ImmunoResearch) in 10% FBS (Atlanta Biologicals, Lawrenceville, GA) at 37°C in Maxisorb 96-well plates (Nunc; Thermofischer Scientific, Rockford, IL) previously coated overnight with 1.0 μg/ml functional grade CD3 mAb (clone OKT3; eBioscience, San Diego, CA), and 2.5 μg/ml CD28 mAb (clone CD28.2; eBioscience) in PBS for 10, 15, 30, and 60 min. Other stable RNA was isolated for analysis after 24 h and 48 h of transfection, the miRNAs were labeled with Cy3, using Label IT siRNA room temperature, according to the manufacturer’s protocol. Prior to transfection, CD4+ T cells were defined as CD3+ CD4+ CD45RA+.

AP-1–dependent transcription reporter gene activity

Naive CD4+ T cells were transfected with plasmids (2.0 μg/1 x 10^6 cells), using a Neon electroporation system (Invitrogen) set for 2400 V, 12 ms, and 2 pulses. The AP-1 reporter plasmid (a generous gift of O. Martinez, Stanford University) has five AP-1 binding sites (from pHexAP1) linked with 33 μg/ml goat anti-mouse IgG (Jackson ImmunoResearch) in 10% FBS (Atlanta Biologicals, Lawrenceville, GA) at 37°C in Maxisorb 96-well plates (Nunc; Thermofischer Scientific, Rockford, IL) previously coated overnight with 1.0 μg/ml functional grade CD3 mAb (clone OKT3; eBioscience, San Diego, CA), and 2.5 μg/ml CD28 mAb (clone CD28.2; eBioscience) in PBS for 10, 15, 30, and 60 min. Other stable RNA was isolated for analysis after 24 h and 48 h of transfection, the miRNAs were labeled with Cy3, using Label IT siRNA room temperature, according to the manufacturer’s protocol. Prior to transfection, CD4+ T cells were defined as CD3+ CD4+ CD45RA+.

miRNA isolation and quantitative PCR analysis

RNA was isolated using a mirVana Isolation Kit (Ambion/Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol from purified naive CD4+ T cells. RNA was analyzed by quantitative PCR using a Bio-Rad (Hercules, CA) CFX384 Real-Time System or an ABI 7900 System (ABI/Life Technologies) and Taqman miRNA assays or gene-specific assays (Ambion). The fold change and relative expression were calculated using the ΔΔCt method. The miRNAs were normalized to RNU48 and mRNAs to GAPDH.

Transfection of primary CD4+ T cells with precursor miRNAs

Purified naive CD4+ T cells (1 x 10^6 cells) in RPMI 1640 with 10% FBS were transfected with 75 μM mirVana miR-181a mimic or a control, nontargeting miRNA mimic (all from Ambion), and 5% v/v siPORT reagent (Ambion) in Opti-MEM medium (Life Technologies/Invitrogen) at room temperature, according to the manufacturer’s protocol. Prior to transfection, the miRNAs were labeled with Cy3 using Label IT siRNA Tracker (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Total RNA was isolated for analysis after 24 h and 48 h of incubation, and calcium flux was measured after 48 h.

Activation of naive CD4+ T cells and total RNA isolation

MACS-purified naive CD4+ T cells were stimulated for 4 h at 37°C in 2.0 x 10^5 cells per milliliter with 2.0 μg/ml anti-CD3 (clone OKT3; eBioscience) and 10.0 μg/ml anti-CD28 (clone CD28.2; eBioscience), cross-linked with 50 μg/ml anti-human CD3 (eBioscience) and 50 μg/ml anti-human CD28 (R&D Systems). RNA was isolated from 1 x 10^6 cells per milliliter with 2.0 μg/ml anti-CD3 (clone OKT3; eBioscience) and 10.0 μg/ml anti-CD28 (clone CD28.2; eBioscience), cross-linked with 50 μg/ml anti-human CD3 (eBioscience) and 50 μg/ml anti-human CD28 (R&D Systems) in RPMI 1640 with 10% FBS. Unstimulated cells were incubated in RPMI 1640 with 10% FBS under the same conditions. After stimulation, cells were pelleted and lysed with TRI Reagent (Molecular Research Center, Cincinnati, OH). Samples were stored at −80°C prior to RNA isolation, which was performed according to the manufacturer’s protocol.

Statistical analysis

Results were analyzed using GraphPad Prism software (GraphPad, San Diego, CA), with p < 0.05 considered significant. Unless otherwise specified, a two-tailed unpaired Student t test was used.

Results

Activation-induced calcium flux is higher in CB naive CD4+ T cells than in APB naive CD4+ T cells

As activation-dependent calcium-dependent signaling is critical for inducing the de novo transcription of genes encoding cytokines and CD154 in CD4+ T cells (19, 29), we first determined if limitations in such signaling in CB CD4+ T cells might result in reduced expression of these gene products. We developed a flow cytometric assay in which naive CD4+ T cell populations were either fluorescently labeled with Alexa 488 succinimidyl ester (barcoded) or left unlabeled (Supplemental Fig. 1) and then combined, permitting the two cell populations to be simultaneously stimulated and analyzed for calcium flux under the same conditions. The labeling procedure did not significantly affect the calcium flux compared with that of unlabeled cells from the same donor (Supplemental Fig. 1). With this approach, naive CD4+ T cells from CB versus APB were directly compared after the cross-linking of surface-bound CD3 mAb. Unexpectedly, the peak (p = 0.025) and mean (p = 0.01) calcium flux following CD3 mAb cross-linking were higher in naive CB CD4+ T cells than in APB naive CD4+ T cells (n = 3) (Fig. 1A, 1B, and data not shown). The labeling did not significantly affect calcium flux (p = 0.98, Fig. 1C). In contrast, no difference was found in either the peak (p = 0.31) or the mean (p = 0.21) calcium flux after the addition of the calcium ionophore ionomycin (Fig. 1D and data not shown), indicating that the capacity for pharmacologically induced calcium flux in these two CD4+ T cell types was equivalent.

Activated-induced calcium flux is higher in recent thymic emigrants than in mature naive CD4+ T cells of APB

The CB naive CD4+ T cell compartment appears to be relatively enriched in recent thymic emigrants (RTEs), T cells that have recently completed their intrathymic maturation and have emigrated to the periphery, based on relatively high content of signal joint TCR excision circles (30) and high levels of surface expression of PTK7, a human CD4+ RTE marker (31). To determine whether RTEs of APB had signaling properties similar to those of CB naive CD4+ T cells, we compared calcium flux in PTK7+ and PTK7− AB naive CD4+ T cells (Fig. 2A). Both the peak (Fig. 2B) and the mean (Fig. 2C) calcium flux after anti-CD3 mAb cross-linking were higher in PTK7+ cells than PTK7− from the same donor (n = 4) (p = 0.029 and p = 0.006, respectively). In addition, no significant difference was observed in peak calcium flux following addition of ionomycin (p = 0.41, data not shown), indicating that RTEs and more mature naive CD4+ T cells had similar capacity to flux calcium after pharmacologic stimulation. Together, these results suggest that the enhanced calcium flux of CB naive CD4+ T cells and APB CD4+ RTEs reflects the retention of a thymocyte-like phenotype.

Erk phosphorylation is higher in naive CD4+ T cells from CB than in those from APB

Phosphorylation of Erk1/2 is induced in response to TCR engagement and costimulation (32–34) and is critical for the AP-1 activation and costimulation (32–34) and is critical for the AP-1–dependent transcription of genes involved in T cell activation, so we next determined whether phosphorylation of Erk1/2 was also enhanced in CB naive CD4+ T cells compared with those of APB. Phosphorylated Erk (pErk) was detectable by 10 min after stim-
ulation with plate-bound anti-CD3 and anti-CD28 mAbs, and the percentage of cells positive for pErk (%pErk+) was significantly higher in CB after 15 and 30 min of stimulation (Fig. 3A), although %pErk+ was similar after 60 min of stimulation. The change in mean fluorescence intensity (\(\Delta\text{MFI}\)) was significantly higher \((p < 0.05)\) in CB after 15 min of stimulation (Fig. 3B). No significant difference was observed in the percentage of pErk+ cells or \(\Delta\text{MFI}\) in response to stimulation with ionomycin and PMA at 5 or 15 min after stimulation (Fig. 3C, 3D). Representative histograms for pErk in APB and CB are shown in Fig. 3E and 3F, respectively. These data indicate that the higher Erk phosphorylation observed in naive CD4+ T cells from CB compared with those from APB applied to physiologic, rather than pharmacologic, activation.

Expression of miR-181a and its positive regulation of activation-induced calcium flux in CD4+ T cells

The miRNA miR-181a has been reported to regulate calcium flux and Erk phosphorylation positively in murine CD4+ T lineage cells, including in thymocytes, which highly express miR-181a (35). As CB naive CD4+ T cells have features suggestive of an enrichment in RTEs and retention of thymocyte-like signaling properties, we next investigated whether miR-181a contributes to increased \([\text{Ca}^{2+}]_i\). Expression of miR-181a was significantly higher in naive CD4+ T cells of CB compared with those of APB (Fig. 4A, \(p = 0.0076\)), as measured by quantitative PCR. Similarly, adult PTK7+ RTEs had significantly higher miR-181a expression \((p < 0.05)\) than did PTK7- naive CD4+ T cells from the same donor (Fig. 4B), consistent with higher calcium flux in PTK7+ than PTK7- cells. To determine if miR-181a positively regulates calcium flux in peripheral naive CD4+ T cells, we chose to overexpress miR-181a in naive CD4+ T cells from APB, rather than inhibiting miR-181a in CB T cells, as others have reported difficulties in knocking down miR-181a (36). Overexpression was achieved by transient transfection with precursor miRNA (pre-miRNAs) because preliminary studies using lentiviral vectors resulted in very low infection rates and required prolonged culture (data not shown). Transfection of APB naive CD4+ T cells with...
pre–miR-181a resulted in a robust increase in mature miR-181a levels (Fig. 4C) and significantly increased the mean calcium flux (p = 0.04) after CD3 mAb cross-linking, compared with cells transfected with a nontargeting control (Fig. 4D, 4E). A trend was also observed toward an increase in peak calcium flux for cells transfected with pre–miR-181a (Fig. 4F, p = 0.11). We did not
observe decreases in mRNAs of predicted miR-181a target phosphatases previously identified using murine T-lineage cells (35) (data not shown). Peak and mean calcium flux after addition of ionomycin did not differ between pre–miR-181a and control-transfected cells (Fig. 4G and data not shown; $p = 0.63$ and $p = 0.86$, respectively), indicating that the enhancement of calcium flux by miR-181a was specific for that following ab-TCR/CD3 activation. The miR-181a overexpression did not affect the viability of the cells, as measured by 7-AAD staining of all cell events or lymphocytes (Supplemental Fig. 2).

**AP-1–dependent transcriptional activity is reduced in CB naive CD4+ T cells**

CB and neonatal naive CD4+ T cells have previously been shown to have features of anergic cells, such as reduced IL-2 production and proliferation in certain contexts, as in following exposure to bacterial superantigen (15, 16). As anergy induction can result when intact calcium-dependent activation of NFAT occurs in the absence of CD28 engagement or transcriptional activity of AP-1 (23, 37–39), we evaluated AP-1 activity by performing transient transfection of an AP-1–dependent luciferase reporter gene. We found that CB naive CD4+ T cells had significantly lower levels of AP-1–dependent transcription than did APB naive CD4+ T cells after CD3 and CD28 mAb stimulation (Fig. 5A, $p = 0.010$). In contrast, no significant difference was seen between these cell types for the low level of AP-1–dependent transcription that was detected in the absence of stimulation (Fig. 5A, $p = 0.12$) or for the high levels of activity that were induced by ionomycin and PMA treatment (Fig. 5B, $p = 0.92$). In addition, no significant difference was found between CB and APB CD4+ T cells for a β-actin promoter–driven luciferase signal (Fig. 5C, $p = 0.89$), indicating that differences in transfection efficiency did not account for these findings. Collectively, these results argued for the impairment of AP-1–dependent transcription in CB naive CD4+ T cells specifically following ab-TCR/CD3 and CD28 engagement rather than a limitation in the amount or functional competence of AP-1.

**Upregulation of anergy genes in activated CB naive CD4+ T cells**

Because reduced AP-1 induction is implicated in establishment of anergy, we examined the upregulation of genes characteristic of anergy in CB compared with APB naive CD4+ T cells. GRAIL and Cbl-b are E3 ubiquitin ligases induced after activation of cells fated to become anergic (38–40). We measured induction of these two mRNAs after 4 h of stimulation with CD3 and CD28 mAbs.
Cbl-b was induced in CB naive CD4+ T cells following activation, whereas mRNA levels decreased in APB naive CD4+ T cells following activation (Fig. 5D). The difference was statistically significant (p = 0.024). GRAIL mRNA was undetectable in all conditions assayed (data not shown). Thus, the induction of Cbl-b in CB naive CD4+ T cells provided more molecular evidence for the tendency of these cells to become anergic following activation.

Discussion

Human neonates have functionally decreased and delayed CD4+ T cell immunity, including Th1 responses, which likely contributes to their vulnerability to more severe infection by viruses and certain intracellular bacteria (1). The de novo CD4+ T cell response to neoantigens requires full activation of naive CD4+ T cells after engagement of the αβ-TCR/CD3 complex and the CD28 costimulatory molecule and their proliferation and differentiation into effector and memory cells. In the current study, we investigated whether limitations in naive CD4+ T cell activation could contribute to impaired neonatal CD4+ T cell immunity. Unexpectedly, we found that CB naive CD4+ T cells had enhanced rather than decreased calcium responses in response to engagement of CD3, and this phenotype also applied to the RTE subset of adult naive CD4+ T cells. Similarly, phosphorylation of Erk was higher in CB naive CD4+ T cells. In contrast, CD3 and CD28 mAb-induced activation of AP-1–mediated transcription, which is Erk dependent, was impaired in CB naive CD4+ T cells compared with those of APB. As expression of CD3, CD4, and CD28 is similar between APB and CB T cells (1), differences in surface receptor or coreceptor expression are unlikely to account for our results. Taken together, these findings indicate that CB naive CD4+ T cells have a striking combination of both enhanced and decreased signal transduction events with activation, and that enhanced signaling is consistent with the retention of a thymocyte-like phenotype by CB naive CD4+ T cells and circulating adult CD4+ RTEs.

The enhanced calcium signaling by CB naive CD4+ T cells was accounted for by increased levels of miR-181a; overexpression of miR-181a in APB naive CD4+ T cells resulted in enhanced CD3 mAb–induced calcium flux. In murine T-lineage cells, miR-181a expression is highest in double-negative thymocytes, followed by a progressive decline as these cells mature into CD4+CD8+ thymocytes, CD4+CD8+ thymocytes, peripheral naive CD4+ T cells, and, finally, effector CD4+ T cells. Assuming that this pattern of miR-181a expression applies to human CD4+ T-lineage maturation, our finding of greater calcium flux by adult CD4+ T cells, compared with more mature naive CD4+ T cells of APB, suggests a role for miR-181a in the enhanced calcium flux of RTEs, although this remains to be directly shown. Higher anti-CD3 mAb–induced calcium flux has been observed in circulating adult naive CD4+ T cells than in memory CD4+ T cells (41), which may be explained by higher miR-181a expression in naive CD4+ T cells.

Although the kinetics of the thymic production and peripheral turnover of CD4+ T cells in the neonate are not known, it is likely that CB peripheral CD4+ T cells are highly enriched in RTEs because of the high activity of the thymus during fetal gestation through 6 mo of age (42). In previous studies, CB naive CD4+ T cells and CD4+ RTEs of APB have been shown to share a number of phenotypic features, including the expression of high levels of PTK7, signal joint TCR excision circles, and CD31, as well as robust proliferative responses to IL-7 (31, 43). The finding of hyperstimulable calcium flux by CB naive CD4+ T cells and adult RTEs, which for both cell types is likely mediated, at least in part, by miR-181a, is also consistent with the idea that this signal transduction pattern represents the retention of a functional thymocyte-like phenotype. These results also suggest that, as for PTK7 surface expression (31), a decrease in TCR-induced calcium signaling may be part of the normal postthymic maturation of the circulating neonatal naive and adult CD4+ T cell compartments (31, 44, 45). Consistent with our results, Gorozny and colleagues (46) have found that miR-181a expression and Erk phosphorylation are reduced in naive CD4+ T cells isolated from elderly people, who, compared with younger adults, have a markedly reduced number of RTEs.
In mice, miR-181a regulates calcium flux in CD4+ T cells by downregulating a number of phosphatases that dephosphorylate Lck and Erk (35). In an MHC class II–restricted αβ TCR transgenic murine system, miR-181a has been shown to enhance αβ TCR/CD3–mediated calcium signaling and Erk phosphorylation, resulting in increased positive and negative selection of CD4+ CD8+ (double-positive) thymocytes and increased activation of mature CD4+ T cells for IL-2 production and proliferation (35).

Our data support a similar mechanism in humans, as we have found increased Erk phosphorylation in CB naive CD4+ T cells. In addition, the finding that miR-181a is highly expressed in adult CD4+ PTK7+ RTEs is consistent with the pattern of expression of miR-181a in murine T-lineage cells.

Robust initial responses by CB CD4+ T cells, followed by apoptosis of effector cells, have been previously reported (47). Our data are consistent with these findings, as CB naive CD4+ T cells have high calcium flux, due, in part, to high expression of miR-181a. Proapoptotic effects of miR-181a have been reported in cancer cell lines (48–50), and overexpression of miR-181a sensitizes cell lines to a variety of apoptosis-inducing chemotherapy agents and increases their activation of caspases 3 and 9 (49, 50). In addition, miR-181a has been shown to target multiple antiapoptotic Bcl2 family members (51). Although some antiapoptotic effects of miR-181a have been reported (52), the preponderance of published results suggests that high expression of this miRNA may mediate both the enhanced calcium flux and the high frequency of apoptosis of activated CB CD4+ T cells. As shown in Supplemental Fig. 2, we did not see effects of miR-181a overexpression on survival of APB naive CD4+ T cells, but the effect of miR-181a on modulating postactivation apoptosis remains to be determined.

The observation that CB naive CD4+ T cells have increased αβ TCR/CD3–induced calcium flux but decreased AP-1–dependent transcription after CD3 and CD28 engagement is consistent with a signaling pattern that induces anergy rather than cell proliferation and production of IL-2 and other cytokines. Anergic murine CD4+ T cell clones or αβ TCR transgenic primary CD4+ T cells result from the presence of calcium–dependent activation of transcription by NFAT in the absence of Erk phosphorylation and transcription by AP-1; this signaling profile has been achieved using calcium ionophore treatment alone, or APCs displaying antigenic peptide/MHC complexes but lacking ligands for CD28 (23, 53–56). In support of this hypothesis, we found that Cbl-b, an E3 ubiquitin ligase necessary in early induction of anergy (39), was modestly upregulated in CB naive CD4+ T cells after stimulation with anti-CD3 and anti-CD28, but was downregulated in APB naive CD4+ T cells under the same conditions. The anergy-like signal transduction profile described in this article may account for the greater tendency of TSST-1–reactive Vβ2+ CB naive CD4+ T cells than of APB cells to become anergic in vitro or in vivo following exposure to bacterial superantigen TSST-1 (15, 16). A tendency toward the induction of anergy rather than full effector function may also account for the observation that hematopoietic transplants with umbilical CB cells elicit fewer instances of Th1–mediated GVHD than do transplants with adult-derived cells (6, 7).

The observation that αβ TCR/CD3 engagement simultaneously induces higher levels of calcium flux and Erk phosphorylation, but reduced AP-1 activity, indicates that a decoupling of Erk activation from AP-1 activity has taken place. Erk is an immediate upstream activator of Fos, a component of the AP-1 heterodimer (57), and Erk activity is negatively regulated by death-associated protein kinase (DAPK), which sequesters pErk in the cytoplasm and prevents its translocation to the nucleus, where it directly activates Fos (58). As we have found that DAPK mRNA is more highly expressed in CB naive CD4+ T cells compared with those of APB (A. Palin and D. Lewis, unpublished observations), it is plausible that DAPK may contribute to decreased AP-1–dependent transcription (57) as well as NF-κB–dependent transcription (59). Thus, these inhibitory effects of DAPK could explain the discrepancy between the increased Erk activation and decreased AP-1 activity we have observed.

Following T cell activation, AP-1 and NFAT proteins cooperate for the transcription of genes encoding CD154 (19, 60), IL-2 (22, 24), IL-4, IFN-γ (21), and many other cytokines. Impaired AP-1 induction in CB naive CD4+ T cells may, therefore, be an important contributor to impaired CD154, IL-2, and IFN-γ production following stimulation with superantigen or allogeneic dendritic cells (9, 15, 16). In addition to the signaling pattern, we have found that Cbl-b is induced in CB naive CD4+ T cells, providing more molecular evidence that these cells may become anergic upon stimulation. Importantly, our results do not exclude other factors contributing to impaired IFN-γ gene expression, such as increased DNA methylation of the IFN-γ gene (61), reduced levels of NFATc2 in unfraccionated CB CD4+ T cells (62), and altered APC function (63, 64).

As we have previously shown that adult CD4+ RTEs also display relatively impaired responses after αβ TCR/CD3 engagement for IL-2 and IFN-γ production, compared with more mature APB naive CD4+ T cells (31), it will be of interest to determine to what extent adult RTEs and CB naive CD4+ T cells share impairments in signal transduction involving the Erk/AP-1 pathway and a tendency for anergy rather than full activation. In the mouse, single-positive thymocytes do not produce IL-2 upon stimulation under conditions in which peripheral CD4+ T cells do (65), suggesting that a decoupling between TCR engagement and effector cytokine may be a characteristic of the late stage of thymocyte development. Thus, including human CD4+ single-positive mature thymocytes (CD4+ CD8+CD3δδCD45RA+) (15) in an analysis of signaling in peripheral naive CD4+ T cell populations may also help determine to what extent alterations in signaling reflect the retention of a thymocyte phenotype by these peripheral CD4+ T cell populations.

In summary, we have observed a distinct activation-induced signal transduction profile in CB naive CD4+ T cells, with an increased calcium response, Erk phosphorylation, and expression of the Cbl-b anergy-promoting gene, but decreased AP-1–dependent transcription. The increase in calcium flux is mediated, at least in part, by increased expression of the miRNA miR-181a, which regulates calcium flux in murine CD4+ T cells, and, as we have shown in this article, in primary human CD4+ T cells and, on the basis of studies by others, may also contribute to CB naive CD4+ T cells being prone to undergoing apoptosis. These features of increased activation-induced calcium flux and miR-181a levels are also found in APB naive CD4+ RTEs, a finding consistent with the likely high proportion of CB naive CD4+ T cells that are RTEs.

Together, these results indicate that CB naive CD4+ T cells have a unique signaling pattern that may contribute to their impaired IFN-γ secretion capacity, as well as their tendency to become anergic in response to stimulation with superantigen. CB naive CD4+ T cells are not broadly hyporesponsive, despite impaired effector cell function. These findings also highlight, and provide evidence for, the need for peripheral maturation and regulation of TCR sensitivity during the late stages of intrathymic and early stages of postthymic T-lineage cell development.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Fluorescence labeling for measurement of calcium flux.

Samples were unlabeled or labeled with Alexa 488 succinimidyl ester, an amine-reactive dye for 20 minutes. A labeled and an unlabeled sample were then combined, loaded with indo-1, stained with anti-CD8, anti-CD19, and anti-CD45RO, and assayed for calcium flux. A kinetics plot is shown for a labeled (green) and unlabeled (black) aliquot from the same donor. The labeled and unlabeled aliquots overlay. For all experiments, a dye swap was performed with an unlabeled adult sample combined with a labeled umbilical cord blood sample and an unlabeled umbilical cord blood sample combined with a labeled adult sample.
Supplemental Figure 2. Transfection with pre-miR-181a does not affect viability of naive CD4\(^+\) T cells after 48h in culture.

APB naive CD4\(^+\) T cells were transfected with pre-miR-181a or a control, non-targeting pre-miRNA. Viability was assessed by staining for 7AAD after 48h in culture at 37°C. (A) Percent live cells (7AAD\(^-\)) was not significantly different in total events. (B) Percent live cells (7AAD\(^-\)) was not significantly different within the lymphocyte population.