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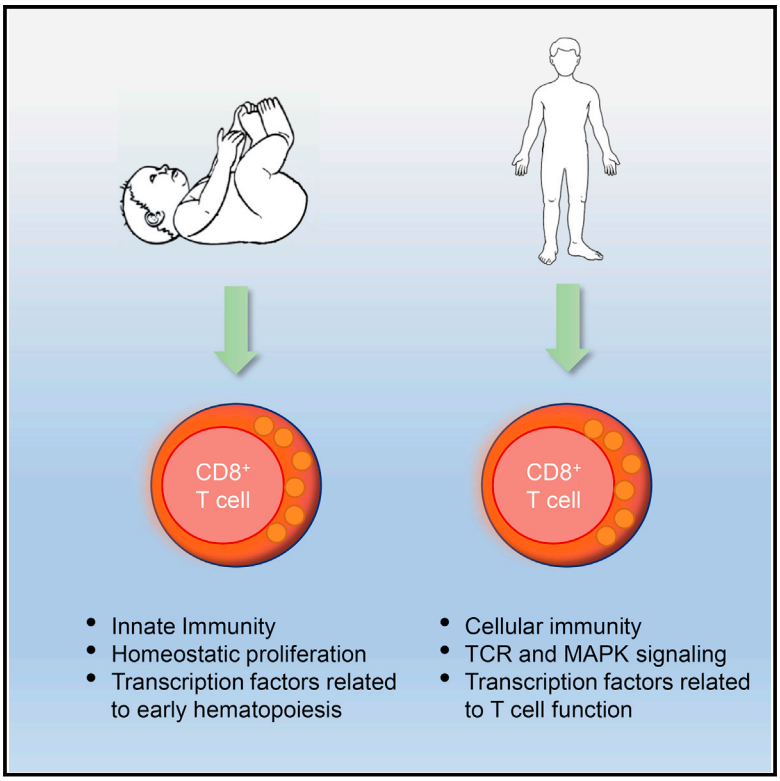
CD8+ T Cells from Human Neonates Are Biased toward an Innate Immune Response

Graphical Abstract



Highlights

- Human neonatal CD8+ T cells have a distinctive transcription and chromatin landscape
- Human neonatal CD8+ T cells are less cytotoxic than their adult counterparts
- Human neonatal CD8+ T cells are biased toward an innate immune response
- This bias could explain the sensitivity of neonates to infections and inflammation

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In Brief

Galindo-Albarrán et al. examine the distinct pattern of gene transcription and histone modification landscape in human neonatal CD8+ T cells. Their data suggest that cells are skewed toward an innate immune response and low cytotoxic function. These properties could explain the high susceptibility of neonates to infections and inflammation. Explore the consortium data at the Cell Press IHEC webportal at www.cell.com/consortium/IHEC.

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CD8+ T Cells from Human Neonates Are Biased toward an Innate Immune Response

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SUMMARY

To better understand why human neonates show a poor response to intracellular pathogens, we compared gene expression and histone modification profiles of neonatal naive CD8+ T cells with that of their adult counterparts. We found that neonatal lymphocytes have a distinct epigenomic landscape associated with a lower expression of genes involved in T cell receptor (TCR) signaling and cytotoxicity and a higher expression of genes involved in the cell cycle and innate immunity. Functional studies corroborated that neonatal CD8+ T cells are less cytotoxic, transcribe antimicrobial peptides, and produce reactive oxygen species. Altogether, our results show that neonatal CD8+ T cells have a specific genetic program biased toward the innate immune response. These findings will contribute to better diagnosis and management of the neonatal immune response.

INTRODUCTION

Infant morbidity and mortality is a major health problem worldwide. Reducing the mortality of children under 5 years old is a priority for the United Nations. Of the deaths of children under five, 37% are neonatal (from birth to 1 month of age); 25% of these are caused by infections (PrabhuDas et al., 2011). Following immune challenges, the neonatal response is often tolerant, skewed, or deficient (Levy, 2007). This leads to a poor response to infection and low immune memory in response to vaccines, which require multiple booster shots to protect infants. Murine neonatal lymphocytes are, however, capable of adult-like responses under particular conditions, suggesting full functionality but a rather high activation threshold (Opiela et al., 2008). IL-12 is necessary as a third signal to induce an efficient response of neonatal CD8+ T cells (McCarron and Reen, 2010).

In human neonates, epigenetic mechanisms, involving impaired nucleosome remodeling, keep IL-12 levels very low; IL-12 does not reach adult levels until the age of 12 (Vanden Eijnden et al., 2006). The neonatal immune system faces a particular demand during the transition from the basically sterile conditions of the womb to the outside world. This transition should permit the colonization of the mucosal flora and the recognition of non-dangerous antigens of the new environment, avoiding strong inflammation that could damage still developing tissues. Neutralizing antibodies from the mother, as well as antimicrobial peptides and lipids, help to protect the newborn. Nonetheless, no passive cellular immunity is transferred from the mother, and neonates depend on their own immune system to fight intracellular pathogens (Adkins et al., 2004; Levy, 2007).

T cell differentiation proceeds through developmental stages in which gene expression is established by signal clues that program the epigenetic landscape, affecting the functionality of the cells (Taniuchi, 2013). In this work, we have examined the transcriptome and analyzed the RNA sequencing (RNA-seq) and epigenetic profiles of neonatal and adult human naive CD8+ T cells. The expression signature of the neonatal cells is described along with functional assays that corroborated that neonatal CD8+ T cells undergo homeostatic proliferation, are less cytotoxic, and produce antimicrobial peptides and reactive oxygen species. Analysis of epigenetic marks showed correspondence between changes in specific modifications and differentially expressed genes. Furthermore, a specific set of transcription factors is associated with these cell types, with maturation and innate immunity characteristics in neonatal cells and with functional cytotoxic T lymphocytes in adult cells. Chromatin state analysis also showed a specific configuration of the neonatal cells. Altogether our data show that human neonatal

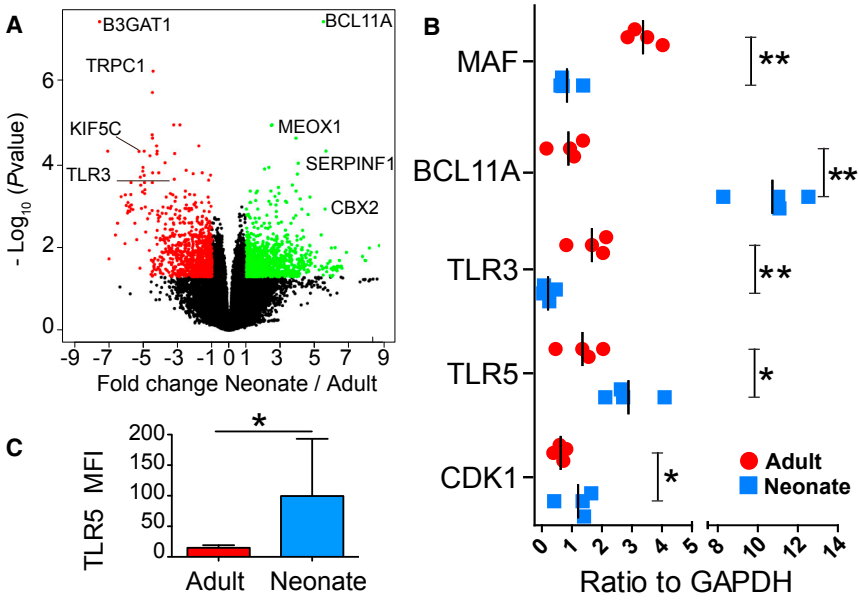

Figure 1. Neonatal and Adult CD8⁺ T Cells Have Their Own Characteristic Transcription Profile

(A) Volcano plot shows differentially expressed genes between adult and neonatal CD8⁺ T cells.

(B) Validation of a group of genes by qRT-PCR is shown.

(C) Flow cytometry analysis of TLR5 expression in four adult and five neonatal samples of CD8⁺ T cells (mean ± SEM). Statistical significance was assessed by Mann-Whitney U test (*$p < 0.05$ and **$p < 0.01$).

CD8⁺ T cells have a specific gene expression program, resulting in a bias toward innate immunity defense mechanisms.

RESULTS

Neonatal CD8⁺ T Cells Have a Distinctive Transcriptome Signature

To investigate the nature of the high sensitivity of neonates to intracellular pathogens, we assessed the transcriptome of naive CD8⁺ T cells from four human neonatal and four adult donors (GEO: GSE61570). To ensure that we were evaluating only the naive circulating populations of CD8⁺ T cells, peripheral blood mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMCs) were first submitted to negative selection to eliminate monocytes, B cells, CD4⁺ T cells, γδ T cells, and natural killer (NK) and NKT cells. PBMCs also were depleted of effector and memory populations with antibodies against CD44 and CD45RO. CBMCs were negative for these markers. Cells were then purified with anti-CD8 antibodies. The resulting populations were about 98% CD3+CD8+, over 95% negative for CD45 RO and 100% positive for CD45 RA. The percentage of TCRγδ⁺ cells was found comparable between adult and neonatal samples, and it represented less than 1% of the cells (Figure S1C).

To assess the consistency of the transcriptome data, we performed a Pearson correlation with all microarray probes, showing a clear separation between the adult and neonatal samples (Figure S1A). Figure 1A shows the volcano plot of differentially expressed genes among adult and neonatal cells, with 576 significantly overexpressed genes in the neonatal samples and 659 in the adult cells ($p < 0.05$, Limma corrected t test; Data S1). Differential expression of genes of immunological interest (Figures 1B, 3B, and 4C) or randomly chosen (Figure S1D) was validated by qRT-PCR, using independent samples. Protein expression of *TLR5* was validated by flow cytometry (Figures 1C and S5D). It has been reported that human neonatal CD8⁺ T cells respond to TLR5 enhancing the production of IFN-γ (McCarron and Reen, 2009), suggesting that the overexpressed TLR5 protein could be relevant for neonatal CD8⁺ T cell functions.

We asked whether differentially expressed genes clustered in specific pathways. Common pathways were certainly found, but each cell group also showed enrichment in their own specific signaling pathways (Figure 2) and gene ontology (GO) terms (Figure S2A). Neonatal cells were enriched in cell cycle, hematopoietic cell lineage, and homologous recombination pathways. Adult cells were enriched in T cell receptor (TCR)- and mitogen-activated protein kinase (MAPK)-signaling pathways, outlining differences in the maturity and basic functions between the two cell groups. Global analysis of our samples using the gene set enrichment analysis (GSEA) software, which calculates significance as related to the number of genes altered in a given pathway, corroborated that neonatal CD8⁺ T cells were enriched in pathways associated with cell cycle, cell maturation, and inflammation, while adult cells were enriched in TCR signaling and function (Figures S2B and S2C). Consistent with these observations, reactome network analysis showed that adult-specific transcripts formed a network of pathways related to TCR signaling, while neonate-specific transcripts formed webs associated with cell cycle, antiviral pathways, and innate immunity (Figure S3). Differential expression of genes of these main signature pathways also was validated by comparing our microarray analysis with RNA-seq data from Blueprint (neonatal CD8⁺ T cells) (Adams et al., 2012) and Roadmap (adult CD8⁺ T cells) (Kundaje et al., 2015) international consortia (Figure S4). The analysis of these pathways is described below.

Neonatal CD8⁺ T Cells Are Less Cytotoxic than Adult Cells

The signaling pathways enriched in adult CD8⁺ T cells were related to cytotoxicity and TCR and MAPK signaling. Upregulation of adult cell genes involved in these pathways is shown in Figure 3A. The genes for the effector molecules directly involved in cytotoxic death (*GZMB*, *PRF1*, and *FASL*), however, were not differentially expressed between adult and neonatal cells after Limma correction, although differences in the mean fluorescence were higher than 2-fold. We therefore examined the expression of these three genes and the other three genes

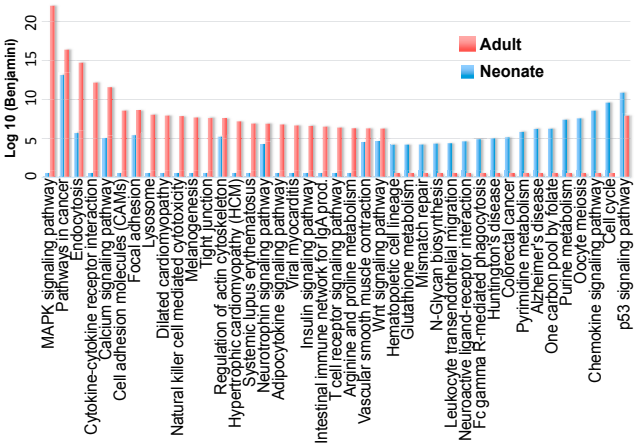

Figure 2. Differential Pathways Enriched in Neonatal or Adult T Cells

Differentially expressed genes were analyzed with DAVID software to associate genes to KEGG signaling pathways. The top 25 more significant pathways are shown.

involved in cytotoxicity that were differentially expressed (*GZMH*, *KIR2DS3*, and *ITGAL*) in four independent neonatal and adult cells samples by qRT-PCR. We validated the significant difference in the expression of IFN-γ, a signature molecule for the activation of the cytotoxic response. We observed highly significant differences in the expression of these seven genes between the two types of cells (Figure 3B).

We also evaluated the mRNA levels of IL-2 and GZMB, both under basal conditions and after TCR stimulation in vitro with anti-CD3/CD28 cross-linking for 6 hr. In both cases, the basal levels of expression were lower in neonatal cells, and the adult cells responded to stimulation with a higher increase in *IL2* expression (Figure S5C). We also assessed the amounts of IFN-γ, Granzime B, the activation marker CD69, and IL-2 proteins in basal and stimulated conditions. The base level amount of CD69 was similar in both naive neonatal and adult cells, but it was much higher in the adult cells after stimulation (Figure S5A). For IL-2 and IFN-γ, changes after 6 hr of stimulation were limited in the two cell populations, but the basal levels of the two cytokines were significantly lower in the neonatal cells (Figure S5B), in agreement with a previous report (Hodge et al., 2001).

To assess whether the differential expression in the cytotoxicity-related genes leads to a functional difference, we examined the cytotoxicity induced in target cells by neonatal or adult CD8$^+$ T cell populations. To avoid misinterpretation occasioned by the use of cell lines and their increased resistance to apoptosis, we used as targets PBMCs of an allogeneic donor (the same donor in all assays). Figure 3C shows that adult T cells induced a greater extent of apoptosis in the target cells than the neonatal cells. Furthermore, we measured the amount of the degranulation marker CD107 and of Granzyme B in both cell populations. Although both neonatal and adult T cells were positive for CD107 at similar proportions, only adult cells were positive for Granzyme B; neonatal cells were basically negative for this marker (Figures 3D and S5D). The lower cytotoxicity of neonatal cells also could be related to a lower expression of the TCR and

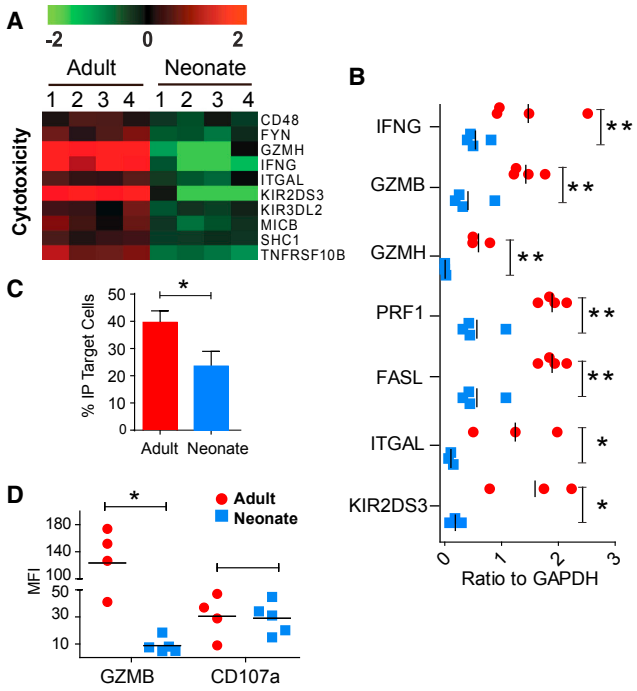

Figure 3. Adult CD8$^+$ T Cells Are Enriched in Cytotoxicity-Related Genes

(A) Heatmaps show the differentially transcribed cytotoxicity-signaling-related genes.

(B) Validation of a group of genes related to cytotoxicity function by qRT-PCR is shown.

(C) Flow cytometry of cytotoxicity assay with activated CD8$^+$ T cells. The experiments were performed in triplicate (error bars, mean ± SEM).

(D) Flow cytometry analyses of Granzyme B and CD107a expression in neonatal and adult CD8$^+$ T cells. Statistical significance was assessed by Mann-Whitney U test (*$p < 0.05$ and **$p < 0.01$).

MAPK signaling in these cells, consistent with their more immature phenotype.

Neonatal CD8$^+$ T Cells Proliferate Constitutively and Are Biased toward Innate Immunity

A prominent characteristic of the neonatal CD8$^+$ T cells was the high expression of cell cycle genes (Figure 4A). These genes included cell cycle regulators (*E2F2* and *RBBP4*) or were associated with the formation of the replicative complex (*MCM7*, *MCM3*, and *CDT1*), the maturation-promoting factor (*CCNB2*), and the cyclin-dependent kinase (*CDK1*), which promotes mitosis from the G2 phase. T cells undergo two types of proliferation: homeostatic proliferation and clonal expansion (den Braber et al., 2012). In the former, cells proliferate without differentiation and, therefore, maintain the pools of circulating cells. In the latter, during clonal expansion, antigen-recognizing clones proliferate due to TCR and co-stimulatory signals, and cells differentiate into effector and memory cells.

To investigate whether neonatal cells were actually undergoing homeostatic proliferation, we labeled the cells with carboxyfluorescein succinimidyl ester (CFSE) and evaluated cell division, without any stimulus apart from that of the fetal calf

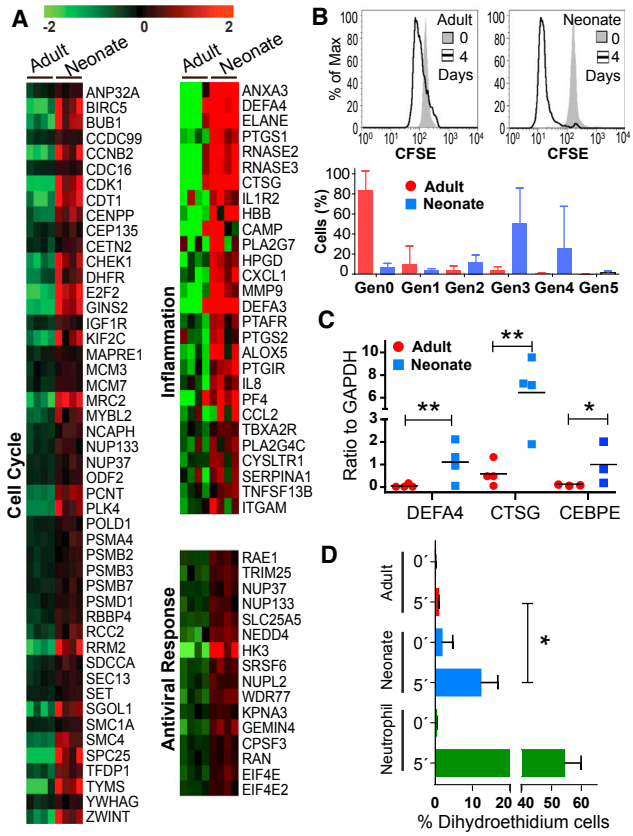

Figure 4. Neonatal CD8$^+$ T Cells Are Biased toward Innate Immunity

(A) Heatmaps show differentially transcribed genes involved in cell cycle, inflammation, and antiviral response.

(B) Flow cytometry of CFSE dilution in CD8$^+$ T cells (top panel) at 0 day (gray shadow) and 4 days (black line). The bottom panel shows the percentage of cells in five divisions of three independent experiments (mean ± SEM).

(C) Validation by qRT-PCR of two antimicrobial peptides and CEBPE transcripts found increased in the neonatal CD8$^+$ T cells is shown.

(D) Flow cytometry of hydroethidium oxidation of CD8$^+$ T cells and neutrophils. The experiment was performed in triplicate. Statistical significance was assessed by Mann-Whitney U test (*$p < 0.05$ and **$p < 0.01$; mean ± SEM).

serum contained in the medium (5%). Within 4 days, the proliferation rate of neonatal CD8$^+$ T cells was significantly higher than that of comparable cells from the adult counterpart (Figure 4B). As a control, we measured homeostatic proliferation and clonal expansion of CD8$^+$ CBMCs and PBMCs at basal level and after CD3/CD28 cross-linking. Neonatal cells again showed a much higher homeostatic proliferation; clonal expansion after stimulation was significantly higher in the adult cells (Figure S6).

The transcriptome data also showed that neonatal CD8$^+$ T cells were enriched in gene expression signatures characteristic of innate immunity, particularly antiviral and inflammatory responses (Figure 4A). The genes enriched in the antiviral pathway participate in nucleus-to-cytoplasm transport (*RAE1*, *NUP37*, *NUP133*, and *KPNA3*) and RNA maturation (*SRSF6*, *WDR77*, and *GEMIN4*), and they are important cellular targets during viral infections (*NEDD4*) (Figure 4A).

Among the genes overexpressed in the neonatal T cells, we found several genes associated with inflammatory responses, such as those for defensins and cathelicidins. GSEA showed that neonatal CD8$^+$ T cells were significantly enriched in inflammation-related genes (Figure S2C). Because some of these genes are characteristic of the neutrophil immune response, we used the database of inflammation-induced genes in human neutrophils (Surmiak et al., 2012) to make a comparison with the genes expressed by neonatal and adult T cells. We found enrichment in 28 of the 147 genes upregulated in neutrophils during the inflammatory response, including genes encoding for antimicrobial peptides (*DEFA4*, *DEFB3*, and *CTSG*), chemokines (*CXCL8*/*IL8*, *CCL2*, and *CXCL1*), and RNases 2 and 3 (Figure 4A). It is well documented that neonatal antigen-presenting cells are high producers of antimicrobial peptides (Levy, 2007; McDonald et al., 2013), but the transcription of these genes was not documented in T lymphocytes. We therefore validated by qRT-PCR the mRNA levels of defensin A (*DEFA4*), cathepsin G (*CTSG*), and the transcription factor CEBPE, necessary for the development of granulocytes and the formation of secondary granules (Gombart and Koeffler, 2002), and we found a highly increased transcription of these genes in neonatal CD8$^+$ T cells (Figure 4C). We also validated the *CTSG* protein levels at basal and stimulated conditions, and we found increased values in neonatal CD8$^+$ T cells, particularly after CD3/CD28 activation (Figure S5B).

To see if this inflammatory signature had an impact on the cell biology of neonatal cells, we assayed the respiratory burst that is used by neutrophils as an effector mechanism to fight infections. We therefore measured dihydroethidium oxidation to measure production of free radicals by CD8$^+$ T cells. The free radical production of neonatal CD8$^+$ T cells was much higher in response to the dihydroethidium itself than was the corresponding response in adult cells, although not as high as that of neutrophils (Figure 4D). Even though this result remains preliminary and further examination is needed to clarify the meaning of the increased reactive oxygen species in neonatal cells, the higher dihydroethidium response is in agreement with a higher expression of the genes involved in ROS production, such as the proton channel *HVCV1*, NADPH oxidase subunits (*NCF*, *NCF2*, and *NCF4*), and lipoxygenase genes (*ALOX5* and *ALOX12*) in neonatal T cells (Figure S4B). Altogether, these results point to a bias of neonatal CD8$^+$ T cells toward the use of innate immunity mechanisms, which could explain the high incidence of sepsis and sterile inflammatory conditions of neonates and yet low cytotoxicity (Ghazal et al., 2013; Piantino et al., 2013).

Regulatory Pathways Involved in the Genetic Programming of Neonatal versus Adult CD8$^+$ T Cells

To gain insight into the regulatory pathways involved in the different expression profiles of CD8$^+$ T cells from neonatal and adult origins, we analyzed the transcription factors differentially expressed in the two cell types (Figure 5). Differentially expressed transcription factors were associated with different cell functions (Figure 5). We found that transcription factor genes overexpressed in the neonatal cells were involved in early T cell differentiation (*BCL11a*, *LEF1*, *MYB*, and *GFI1*) (Rothenberg et al., 2008), neutrophil differentiation (*CEBPE*) (Gombart and Koeffler, 2002), inflammation (*NF-E2*), and cell cycle (*DACH1*

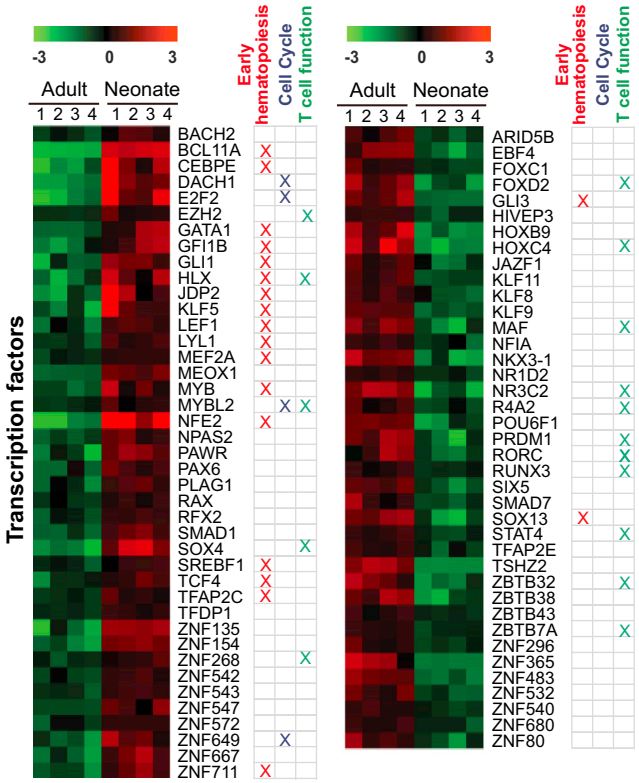

Figure 5. Neonatal and Adult CD8$^+$ T Cells Have Their Own Characteristic Transcription Factor Profile

Heatmaps showing differentially transcribed transcription factor genes between adult and neonatal CD8$^+$ T cells. The panel at the right of each heatmap shows the association with biological functions based on the literature.

and *E2F2*, among others). The overexpressed transcription factor genes in adult cells were involved in CD8$^+$ T cell function, particularly effector cell differentiation, regulation of cytotoxicity, and cytokine function (*RORC*, *STAT4*, *MAF*, and *RUNX3*) (Cruz-Guilloty et al., 2009). We concluded that the differential expression of master transcription factors might be involved in the distinct genetic programming of neonatal versus adult CD8$^+$ T cells.

Differential Epigenetic Programming of Neonatal versus Adult CD8$^+$ T Cells

We took advantage of available data from the Roadmap (Kundaje et al., 2015) and Blueprint (Adams et al., 2012) international consortia (which have generated extensive epigenomic maps of naive CD8$^+$ T cells from adult and neonatal origins) to evaluate the epigenetic regulation of these two types of samples. First, we compared the expression of genes from our adult and neonatal transcriptome signatures with the RNA-seq data obtained from the above consortia (Figures 6A and S1B). We observed a highly significant difference between the relative expression of genes from the adult and neonatal signatures (Figure 6A, left panel). We also found that the signature profile of neonatal versus adult CD8$^+$ T cells from RNA-seq data from Blueprint and Roadmap was very similar to our transcriptome data, thus cross-validating the different datasets (Figures S1B and S4). Consistently, the overexpressed genes of adult cells were associated with significantly higher levels of open chromatin marks (H3K4me3 and H4K27ac) and lower levels of the repressive mark H3K27me3 (Figure 6A, right panel). Representative examples of such epigenomic profiles are shown in Figures 6B and S7. We next quantified the enrichment of H3K27ac at distal genomic regions, which represent potentially active enhancers (Creyghton et al., 2010). Significant differences also were observed at distal peaks of H3K27ac, associated with differentially expressed genes between adult and neonatal CD8$^+$ T cells (Figure 6C). Thus, differences in gene expression levels between adult and neonatal CD8$^+$ T cells globally correlate with epigenetic differences between the two populations.

To extend our observations, we performed an independent and unbiased analysis integrating several histone modifications from three adult and two neonatal CD8$^+$ T cells. We used the ChromHMM tool (Ernst and Kellis, 2012) to define the different chromatin states of both cell populations (Figure 6D). We focused on the differential enhancer activities, that is, distal regions associated with H3K27ac and H3K4me1 (state 9, active enhancers) in one cell population and those associated with H3K4me1 only in the other cell population (state 8, poised enhancers) (Data S2). We found 858 active enhancers in adult CD8$^+$ T cells that are in a poised/inactive state in the neonatal cells and, vice versa, 439 active enhancers in neonatal cells that are in a poised/inactive state in the adult cells, thus highlighting extensive epigenetic differences at distal regulatory elements. Examples of differentially marked enhancers are provided for the T cell cytotoxic genes *GZMH* and *GZMB*, expressed only in adult CD8$^+$ T cells, and for the neonate-specific gene, coding for the transcription factor *BCL11A* (Figure 6E). Strikingly, GO terms for genes close to active enhancers specifically in adult CD8$^+$ T cells were enriched in T cell activation and function, while those specifically active in neonatal CD8$^+$ T cells were related to cell proliferation and apoptosis (Figure 6F). In conclusion, the independent analysis of specific enhancers points toward a distinct epigenomic programming of neonatal versus adult CD8$^+$ T cells, which is closely related to the observed transcriptomic and functional differences.

DISCUSSION

We have assessed the transcriptomic and epigenetic profiles of naive CD8$^+$ T cells from neonatal and adult human blood. Despite the common pathways and variations between individual samples, the neonatal cells expressed a distinctive signature of transcribed genes, including a particular set of transcription factors, and they showed a characteristic configuration of chromatin states and epigenetic marking at both proximal and distal regulatory regions, suggesting a specific epigenetic programming of the neonatal cells.

The transcriptional profile of genes enriched in the neonatal cells was associated with cell cycle and innate immune response (Figures 4 and S3). Our functional studies confirmed that neonatal CD8$^+$ T cells undergo a higher rate of homeostatic

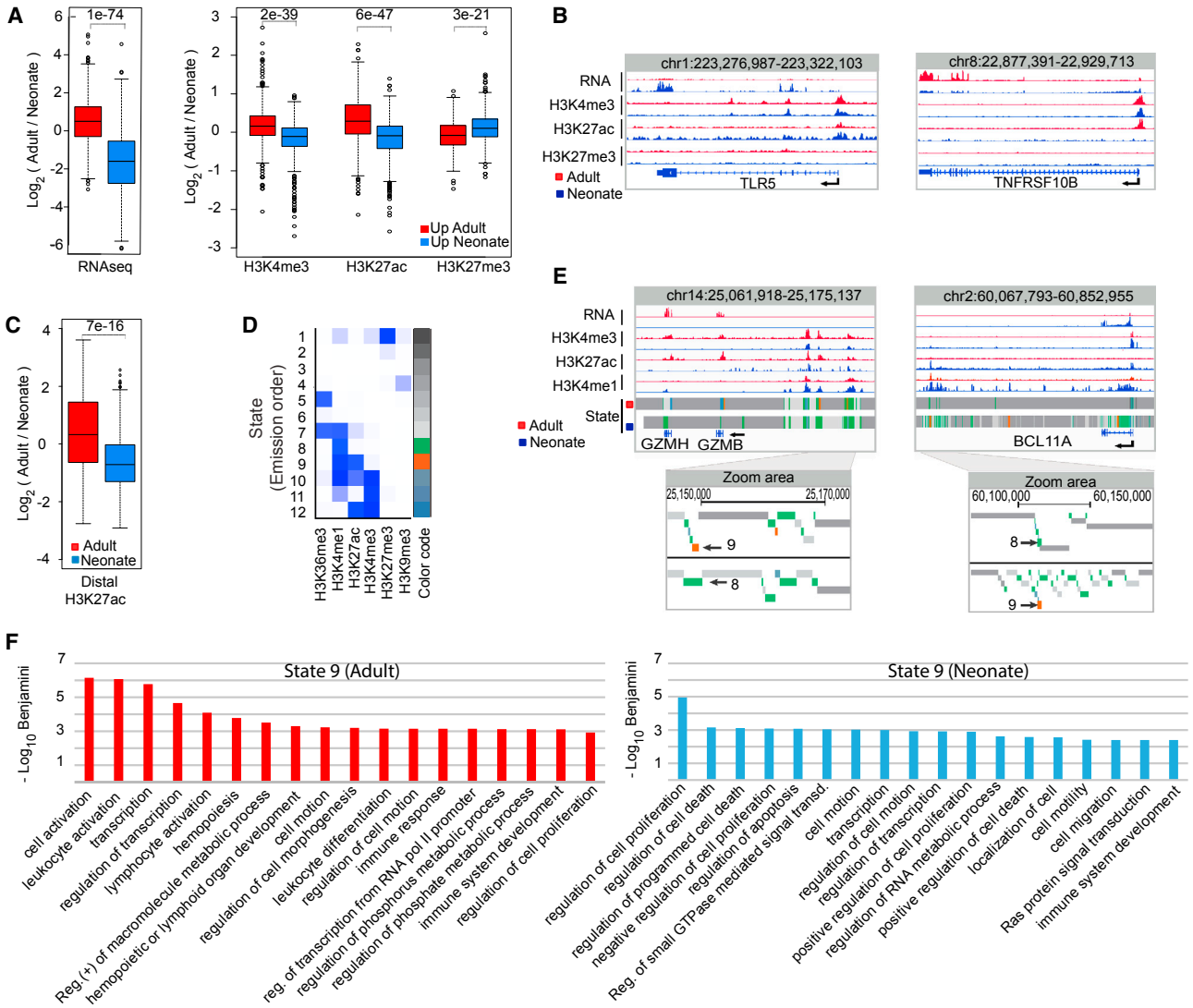

Figure 6. Epigenomic Analysis

(A) Comparison of RNA-seq (left panel) and histone marks (right panel) around the TSS between the set of genes differentially expressed in neonatal and adult CD8$^+$ T cells based on the microarray analysis. The ratio between the neonatal and adult signal is shown. Statistical significance was assessed by Mann-Whitney U test.

(B) Example of two genes overexpressed in neonate (top panel) or in adult (bottom panel). Scales were adjusted with respect to control genes shown in Figure S7A.

(C) Comparison of H3K27ac signal at distal genomic regions (>2 kb from any TSS) is shown.

(D) Chromatin states defined in CD8$^+$ T cells. The last column represents the color code for each chromatin state.

(E) Example of two loci containing dynamic regions corresponding to enhancer transition from active state (nine) in adult to poised state (eight) in neonate (left panel) and vice versa (right panel). Chromatin states are shown in the bottom of each panel. The bottom panels represent the chromatin states of a zoomed-in area.

(F) GO (biological process) analysis is shown.

cell division than do adult cells (Figure 4B). It has been described that neonatal cells divide in response to IL-7 and IL-15 (Marchant and Goldman, 2005) and spontaneously in mixed T cells populations (Schönland et al., 2003). We corroborated those results and found that purified CD8$^+$ T cells are continuously cycling. The original stimulus that triggered proliferation could have been the lymphopenic conditions of neonatal blood, in particular in relation to the CD8 compartment, given that the ratio of CD4$^+$ to CD8$^+$ T cells is higher in neonatal blood (Zhu et al., 2001).

Among the specific signatures of neonatal CD8$^+$ T cells, we found an innate immunity-related antiviral response. Neonatal cells might have an increased antiviral response to compensate for the low cytotoxicity of their CD8$^+$ T cells. It is also possible, however, that the neonatal cells have an increased expression

of these genes because of a still ongoing cell maturation process, as most of these genes are involved in nucleus-to-cytoplasm transport and RNA maturation.

We found that several genes involved in cytotoxicity were under-expressed and epigenetically silenced in the neonatal $CD8^+$ T cells (*GZMB*, *TLFRSF10B*, *RUNX3*, *NR3C2*, and *CTLA4*; Figures 6B, 6E, and S7). Functional studies showed that indeed neonatal cells do not express granzyme B; but, they expressed the degranulation marker CD107. This suggests that the granules exist in the neonatal cells, although the contents are different. It is possible that the granule content in cord blood $CD8^+$ T cells comprises antimicrobial peptides and elastase. We also show that neonatal $CD8^+$ T cells were less cytotoxic than the adult cells. Immaturity of the neonatal $CD8^+$ T cell populations was confirmed in the pathway analysis (Figures 2, S2, and S3) and in the neonate-specific set of transcription factors (Figure 5). This could be responsible for the high susceptibility of neonates to intracellular pathogen infections (PrabhuDas et al., 2011).

Neonatal $CD8^+$ T cells shared a number of transcripts with the neutrophil activation response (Surmiak et al., 2012), particularly as related to microbial peptide expression, chemokines, and RNases. This is suggestive of a role of the neonatal $CD8^+$ T cells within the innate immunity response. We found that, indeed, the neonatal $CD8^+$ T cells produce reactive oxygen species, and we also corroborated, with qPCR, the higher rate of transcription of the antimicrobial peptides cathepsin G and defensin A (Ganz, 1994). The expression of these neutrophil-related genes could be associated with the higher expression in neonatal cells of the neutrophil differentiation transcription factor CEBPE (Figure 4C) (Cloutier et al., 2009; Halene et al., 2010; Lekstrom-Himes, 2001; Ma et al., 2014).

Master transcription factors could be involved in the differential programming of the neonatal cells. As expected, the transcription factors of adult $CD8^+$ T cells were mostly associated with T cell function and activation, and this could be due to the higher expression in adult cells of the master transcription factor RUNX3 (Cruz-Guilloty et al., 2009). On the contrary, neonatal T cells express master regulators of early leucocyte differentiation like BCL11A (Yu et al., 2012) and CEBPE involved in neutrophil differentiation (Gombart and Koeffler, 2002). This is indicative of a particular genetic programming of neonatal $CD8^+$ T cells, with immature characteristics and biased toward innate immunity. Supporting the idea that neonatal cells are biased toward innate immunity, it has been shown that one of the major responses of human neonatal T cells is the production of IL-8, a pro-inflammatory molecule associated with innate immunity, and that these cells respond to the TLR5 ligand, flagellin, further inducing IL-8 (Gibbons et al., 2014). Additionally, in a transcriptome study of neonates with sepsis, the authors found that the major response of neonates was innate immunity-associated gene expression (Smith et al., 2014). The authors concluded that neutrophils were the main responsive cells. Our data suggest, however, that it is also possible that $CD8^+$ T cells were responding with innate immunity mechanisms, stressing the need of neonatal immune cell expression signatures for whole-blood transcription analysis involving neonates.

Altogether our data suggest that neonatal $CD8^+$ T cells have a transitional program to deal with the birth-specific immunological demands, while they complete their maturation from recent thymus emigrants to become mature naive $CD8^+$ T cells (Fink and Hendricks, 2011; Opiela et al., 2009; Yang and Bell, 1992). It has been estimated that 90%–95% of naive $CD4^+$ T cells from human cord blood cells are recent thymus emigrants (RTEs), and, in the age groups of our adult donors (22–55 years old), recent RTEs are expected to represent a very small proportion of the naive T cell pool (den Braber et al., 2012). Therefore, part of the differences encountered in this analysis could be explained by this fact. However, in a transgenic mice model where RTEs were labeled, it was reported that neonatal and adult $CD4^+$ T cell RTEs have phenotypic and functional differences. Adult cells did not proliferate in response to IL-7 and produced less of the Th1/Th2 cytokines as compared to neonatal RTEs (Opiela et al., 2009). Our work suggests a function of neonatal cells within the innate immune response. The consistency between our observations and the transcriptomic and epigenetic data from the Roadmap and Blueprint consortia strengthens our conclusions. Further analysis will determine whether the innate immunity role of $CD8^+$ T cells could be either an ancient role of these cells, possible pointing to an evolutionary functional origin of $CD8^+$ T cells, or an acquired role adapted to the transition of birth in mammals.

EXPERIMENTAL PROCEDURES

Cell Preparations

Adult T cells were obtained from leukocyte concentrates of healthy adult donors who donated to Centro Estatal de la Transfusión Sanguínea in Cuernavaca. Neonatal blood was collected from the cord vein of full-term vaginal deliveries of healthy babies at Hospital General Parres in Cuernavaca. Blood was collected just after the delivery and before placenta expulsion. All samples for the transcriptome evaluation were from male donors. The average age of the adult blood donors was 29 ± 6.44 years old (range, 22–55), and 73% were male donors. The procedure was approved by the Hospital Parres Ethical Committee, and it was performed on informed mothers that consented to donate their babies' cord blood.

Total blood was separated through a ficoll-hypaque gradient and plastic adhesion. PBMCs or CBMCs were further depleted of unwanted cell populations with a cocktail of antibodies followed by G protein-magnetic bead (88847, Invitrogen) binding and depletion with a magnet. Antibodies used were CD57 (NK1, Zymed), CD19 (HD37, Santa Cruz Biotechnology), CD16 (3G8, BioLegend), anti-TCRγ/δ (B1, BioLegend), and CD4 (MT310, Santa Cruz Biotechnology). To eliminate memory and effector cells in PBMCs, antibodies against CD45RO (UCH-L1, Santa Cruz Biotechnology) and CD44 (BJ18, BioLegend) also were used. Then $CD8^+$ T cells were obtained with an anti-CD8 (SK1, BioLegend) antibody. This method allowed cell purities >98%. Cells were used immediately or were maintained in RPMI supplemented with glutamine, antibiotics, and 5% fetal calf serum under 5% CO_2 at 37°C.

RNA Extraction and Integrity Evaluation

The purified cells were centrifuged and placed in Trizol (15596-018, Invitrogen) before being shipped on dry ice to the TGML platform in Marseille. RNA was extracted according to the manufacturer's instructions. The integrity of the RNA was determined with an Agilent Bionalyzer, and only samples with RNA integrity (RIN) over 8.0 were used for transcriptome evaluation.

Transcriptome Analysis

The labeled RNA was hybridized and scanned on an Agilent platform in an 8X60K human Agilent microarray. Raw data are available at GEO: GSE61570.

For bioinformatic analysis, first the raw fluorescence data were normalized by the R package (AGIND). Later, analysis was performed using software Limma, Multiple Experiment Viewer (MEV) and R program, with an alpha of 0.05 to obtain the significantly changed genes. The heatmaps were obtained with the software MEV, with base 2 logarithmic transformations and analysis of arithmetic average for each gene.

GSEA (Subramanian et al., 2005) was performed regardless of Limma analysis. Reactome analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and GO analysis was performed with the ClueGO plug-in under Cytoscape 3.0 (Shannon et al., 2003), with the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (Huang et al., 2009).

Flow Cytometry

Extracellular staining was performed to evaluate purity, double positive for CD3-phicoeritrin (Hit3a, BioLegend), CD8-FitC (Hit8a, BioLegend), and TCRγ/δ-FitC (B1, BioLegend). The naive state of the cells was evaluated by low CD45RO (UCHL1, Tonbo) and by high CD45RA (T6D11, Miltenyi Biotec). For cytokine staining, we used IL-2-APC (MQ1-17H12BD, Pharmingen) and IFN-γ-FITC (45-15, Miltenyi Biotec). To quantify TLR5 (85B152.5, Abcam), extracellular staining was performed. For cytotoxicity markers, GZMB (GB11, BioLegend) and CD107a (H4A3, Miltenyi Biotec) intracellular staining was performed. For assays of cell division, PBMCs were specifically evaluated by double staining with CFSE (21888, Sigma) 50 nM and CD8-Alexa Fluor 405 (MHCD0826, Life Technologies) or only CFSE in purified $CD8^+$ T cells. For assays of oxidative burst, non-fixed $CD8^+$ T cells were stained with 50 nM dihydroethidium (D7008, Sigma) for 20 min at 37°C, then analyzed immediately on the cytometer.

Cytotoxicity Assays

Naive $CD8^+$ T cells were stimulated with 5 μl anti-CD3-CD28 beads (11161D, Invitrogen) or 1 μg/ml each anti-CD3 (OKT3, 70-0037-U100, Tonbo Biosciences) and anti-CD28 (CD28.2, 70-0289-U100, Tonbo Biosciences) antibodies, followed by 1 μg/ml goat anti-mouse antibody (405301, BioLegend) for 6 hr. Meanwhile, PBMCs from the same donor were stained with CFSE (50 nM) for 15 min. Finally, activated cytotoxic cells were co-cultured with the labeled target cells for 2 hr, and apoptosis was measured by propidium iodide staining (33-1200, Invitrogen). Only the CFSE-positive cells were assessed with propidium iodide, as a measure of target cell death.

Respiratory Burst Assay

Naive $CD8^+$ T cells from neonatal and adult donors, as well as neutrophils, were cultured in phenol red-free RPMI containing 5% fetal bovine serum (FBS). Cells were treated with 50 nM dihydroethidium (D7008, Sigma-Aldrich) for 20 min, and they were compared to cells treated with the same compound immediately before reading (time zero). Changes in fluorescence due to oxidation of the dye with reactive oxygen species were evaluated by flow cytometry.

qRT-PCR

The cDNA was synthesized from total RNA using the Superscript Vilo cDNA synthesis kit (11755050, Life Technologies). The qPCRs were performed using SYBR Green PCR Master Mix (4309155, Life Technologies). The mRNA levels were calculated from standard curves for each gene and relative to those of the GAPDH gene, used as a standard.

Processing of ChIP-Seq and RNA-Seq Data

RNA-seq and chromatin immunoprecipitation sequencing (ChIP-seq) (H3K4me3, K3K27ac, and H3K27me3) samples from adult naive $CD8^+$ T cells (Donor_101_8) were obtained from the ROADMAP project. The RNA-seq (donor C002YM) and ChIP-seq (H3K4me3, K3K27ac, and H3K27me3; donor C0066P12) samples from neonatal naive $CD8^+$ T cells were obtained from the Blueprint project (see Table S1 for more information). Gene expression was assessed by quantifying RefSeq gene exons signal from RNA-seq BigWig files using BigWig-Tools extract option (Pohl and Beato, 2014); then, for each gene, the corresponding exon signal was summed up and normalized using the Normalizer package (Glusman et al., 2013) with the quartiles option. Histone modifications within promoter regions (related to Figure 6A) were assessed by extracting the average of the top 25% signal from ChIP-seq BigWig files from H3K4me3, H3K27ac, and H3K27me3 histone marks using BigWig-Tools, in a region around 1.5 kb to the transcription start site (TSS) RefSeq genes, then the signal was normalized as described above. For distal H3K27ac analysis (related to Figure 6C), peak-calling for H3K27ac ChIP-seq from neonatal $CD8^+$ T cells was obtained directly from the Blueprint database. Peak-calling for H3K27ac ChIP-seq from adult $CD8^+$ T cells (Roadmap) was performed with MACS2 (Zhang et al., 2008), using the Blueprint parameters. The unique no-overlapping peaks between the two populations (each ChIP-seq data) were selected using Bedops (Neph et al., 2012); the signal was extracted from BigWig files using BigWig-Tools and normalized as described above. Each peak was assigned to one gene using GREAT software (McLean et al., 2010) with default settings. H3K27ac peaks were defined as distal if they were located more than 2 kb upstream and 1 kb downstream the TSS of any annotated gene.

Analysis of Epigenomic and Transcriptomic Signatures

We computed the ratio between the adult and neonatal RNA-seq and ChIP-seq data for each set of adult and neonate-specific genes. Statistical significance between the two sets of genes was computed using the Mann-Whitney U test. Heatmaps were obtained with the software MEV, with a base 2 logarithmic transformation, and compared to microarray heatmaps (Figure S4). Examples of genomic profiles were obtained using the IGV 2.3 software (Robinson et al., 2011).

Chromatin Segmentation

CD8 neonatal segmentations and visualization with 12 states for samples C002YMH1, C0066PH1, and S00C2FH1 were obtained from the BLUEPRINT project (2015-01-28 release, more details are given in Table S1). CD8 adult samples (naive primary cells: Donor_100_7 and Donor_101_8) were obtained from ROADMAP. ChIP-seq data for six histone modifications (H3K4me3, H3K4me1, H3K27ac, H3K36me3, H3K27me3, and H3K9me3) were used (see Table S1). For the segmentation, we used the implementation described in the ChromHMM software (version 1.10) (Ernst and Kellis, 2012). The input data were the ChIP-seq bed files with the genomic coordinates and strand orientation of mapped sequences (after remove duplicate reads). The genome was divided in consecutive 200-bp non-overlapping intervals and independently assigned present (1) or absent (0) for each of the six chromatin modifications. The assignment was based on the count of tags mapping to the interval and on the basis of a Poisson background model using a threshold of 10-4, as explained (Ernst and Kellis, 2012), after binarization, and for segmentation we used the 12 states model established by the Blueprint Consortium (2015-01-28 release, see Table S1). Further, we computed the probability that each location is in a given chromatin state, and then we assigned each 200-bp interval to its most likely state for each sample. In addition, visualization files were generated with ChromHMM software (version 1.10).

Differential Chromatin States between Adult and Neonatal $CD8^+$ T Cells

For each cell type, the chromatin state assignment at each 200-bp interval was compared. Intervals with differential chromatin states were defined as follows: all CD8 adult samples must share the same state but must be different from CD8 neonatal samples; in addition, all CD8 neonatal samples must have the same state. Lastly, we computed the frequency of segments that changed in chromatin states between adult and neonatal CD8 (Figure 6D). The GO biological process chart was performed using the DAVID Bioinformatics Resources (Huang et al., 2009).

ACCESSION NUMBERS

The accession number for the transcriptome of naive $CD8^+$ T cells from four human neonatal and four adult donors reported in this paper is GEO: GSE61570.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and two data files and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.056.

AUTHOR CONTRIBUTIONS

A.O.G.-A., S.S., and M.A.S. conceived experiments, analyzed the data, and wrote the manuscript. S.S. and M.A.S. conceived the project, revised the manuscript, and secured funding. A.B., B.L., H.H., and J.I. performed microarray hybridization and processed the raw data. A.O.G.-A., O.H.L.-P., D.Y.G.-R., O.R.-J., J.A.S.-V., O.R.-P., and A.H.-M. performed experiments. J.I. and P.F. provided expertise and feedback. E.C.d.S.P. and A.V. contributed to epigenomic analyses.

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