DNA methylation regulates the neonatal CD4+ T-cell response to pneumonia in mice

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Pediatric acute lung injury, usually because of pneumonia, has a mortality rate of more than 20% and an incidence that rivals that of all childhood cancers combined. CD4+ T-cells coordinate the immune response to pneumonia but fail to function robustly among the very young, who have poor outcomes from lung infection. We hypothesized that DNA methylation represses a mature CD4+ T-cell transcriptional program in neonates with pneumonia. Here, we found that neonatal mice (3–4 days old) aspirated with Escherichia coli bacteria had a higher mortality rate than juvenile mice (11–14 days old). Transcriptional profiling with an unsupervised RNA-Seq approach revealed that neonates displayed an attenuated lung CD4+ T-cell transcriptional response to pneumonia compared with juveniles. Unlike neonates, juveniles up-regulated a robust set of canonical T-cell immune response genes. DNA methylation profiling with modified reduced representation bisulfite sequencing revealed 44,119 differentially methylated CpGs, which preferentially clustered around transcriptional start sites and CpG islands. A methylation difference-filtering algorithm detected genes with a high likelihood of differential promoter methylation regulating their expression; these 731 loci encoded important immune response and tissue-protective T-cell pathway components. Disruption of DNA methylation with the hypomethylating agent decitabine induced plasticity in the lung CD4+ T-cell marker phenotype. Altogether, multidimensional profiling suggested that DNA methylation within the promoters of a core set of CD4+ T-cell pathway genes contributes to the hyporesponsive neonatal immune response to pneumonia. These findings also suggest that DNA methylation could serve as a mechanistic target for disease-modifying therapies in pediatric lung infection and injury.

Pneumonia, also known as lower respiratory tract infection (LRTI), ranks as the primary cause of acute lung injury among pediatric populations (1). Incidence estimates of pediatric acute lung injury in United States–based cohorts approach 13 per 100,000 person-years (2), a rate similar to the incidence of all childhood cancers (3), with a mortality rate of 22%. Worldwide, LRTI annually claims the lives of over 6 million children younger than 5 years of age, approximately 1 million of whom are neonates (younger than 28 days of age) (4–6). Indeed, compared with older children, neonates and prematurely born babies display a particular vulnerability to morbidity and mortality from LRTI. The mechanisms for this age-related susceptibility to poor LRTI outcomes remain unclear, although immature neonatal immune responses may lead to insufficient pathogen clearance and coordination of tissue-protective and reparative processes (7).

The neonatal respiratory system undergoes numerous dynamic changes after birth, with lung growth and immunological development continuing in the postnatal period (8, 9). In lymphoid populations, classic experiments demonstrated a failure of neonatal T helper (Th) 1–type immunity to foreign allograft antigens, instead skewing toward Th2-like tolerization (10–12). This skewed T-cell function renders neonates with an immature CD4+ T-cell response to bacterial infection with hypofunctional Th1- and Th17-coordinated protection from microbial pathogens (13). CD4+ regulatory T (Treg) cells, which express the lineage-specifying transcription factor Foxp3, play a central role in providing tissue protection as well as orchestrating resolution and repair from lung inflammation and injury in adult (14–16) and neonatal (17) mice. Studies in murine systems demonstrated that heterochronic adoptive transfer of adult Treg cells to neonatal mice at the time of lipopolysaccharide-induced acute lung injury provided tissue protection and normal developmental weight gain within 48–72 h compared with a sham administration of adult CD8+ T cells (17).

This article contains Fig. S1 and Tables S1–S4.

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T-cells (17). Additionally, experimental data show that CD4+ T-cell subsets increase in the lungs of both neonatal and juvenile mice within 48 h after aspiration of *Escherichia coli* bacteria (18). A detailed understanding of the CD4+ T-cell response to LRTI as a function of age among pediatric populations remains unknown.

Coordinated transcriptional programs determine CD4+ T-cell development and responses to a variety of sterile and infectious provocations (19–21). Epigenetic phenomena control the expression of key effector molecules and canonical transcription factors in Th1, Th2, Th17, and Treg cells, including *Tbx21*, *Gata3*, *Rorc*, *Foxp3*, and members of the *Ilkzf* family. DNA methylation, a generally repressive epigenetic mark occurring predominantly at cytosine-phospho-guanine (CpG) residues clustered into CpG islands near transcriptional start sites and gene promoter elements, represents a powerful factor that can determine T-cell skewing and reaction to stimuli (22). Moreover, locus- and lineage-specific alterations in DNA methylation patterning dynamically occur during neonatal thymic CD4+ T-cell development with important consequences for cell fate decisions (23–25). It remains unknown how DNA methylation influences the lung CD4+ T-cell response to LRTI in the very young. In this study, we hypothesized that DNA methylation patterning governs an immature lung CD4+ T-cell transcriptional program in neonatal mice with LRTI. We employed unsupervised genome-scale sequencing approaches and computational analyses in mice to define a core set of differentially methylated gene loci that regulate, at least in part, the immature neonatal lung CD4+ T-cell response to *E. coli* bacteria, a leading pathogen isolated from human neonates with LRTI (6).

Results

**Neonatal mice exhibit increased morbidity and mortality after *E. coli* lower respiratory tract infection**

We first sought to characterize the age-dependent lung injury phenotype following *E. coli* LRTI. Aspiration of phosphate-buffered saline (PBS) caused no mortality, and both neonatal (3–4-day-old) and juvenile (11–14-day-old) mice exposed to PBS gained weight as expected (Fig. 1, A and B). When aspirated with *E. coli*, both neonates and juveniles demonstrated extensive lower respiratory tract inflammation at 48 h after aspiration (Fig. 1C). However, neonates displayed significantly decreased survival after *E. coli* aspiration compared with juveniles (Fig. 1A). Percent weight gain 48 h after aspiration was also significantly less in neonates aspirated with *E. coli* compared with age-matched controls (Fig. 1B). Although juveniles aspirated with *E. coli* also had significantly less percent weight gain compared with age-matched controls, it was to a lesser extent than that which occurred in neonates.

**Neonatal lung CD4+ T-cells display an attenuated transcriptional response to *E. coli* LRTI**

Because of previous work demonstrating the hypofunctional lung CD4+ T-cell response to neonatal early LRTI (17, 18), we performed comprehensive transcriptional profiling with RNA-Seq on sorted lung CD4+ T-cells from neonates and juveniles exposed to either PBS or *E. coli* via aspiration 48 h previously. Multiple group testing with a false discovery rate (FDR) q-value ≤ 0.05 revealed 3932 differentially expressed genes (DEGs). Principal component analysis showed tight clustering by group assignment, with principal component 1 reflecting the transcriptional response to LRTI and principal component 2 reflecting age (Fig. 2A). Pearson correlation distance clustering separated the samples by age and LRTI status (Fig. 2B). Biological replicates clustered together in the final branches of the dendrogram.

We then investigated differences between groups with k-means clustering of DEGs. Based on review of the associated elbow plot (Fig. S1A), we selected k = 4 (Fig. 2C). K-means cluster 1 contained genes down-regulated only among *E. coli*-exposed juveniles with gene ontology (GO) processes reflecting protein localization and nucleic acid metabolic processes (Fig.
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Immunity-based processes dominated cluster 2 with both ages up-regulating genes in response to *E. coli* LRTI, albeit more robustly among juveniles. GO processes associated with cluster 3 involved cell proliferation events. PBS-exposed neonates but not juveniles up-regulated cluster 3 (proliferation) genes, whereas both ages up-regulated these genes after *E. coli* LRTI. Cluster 4 genes (reflecting immunity-based processes similar to cluster 2) were down-regulated among neonates irrespective of LRTI, modestly up-regulated among PBS-exposed juveniles, and strongly up-regulated by *E. coli*–exposed juveniles. A tab-delimited file (Table S1) details expression and statistical values for the genes shown in Fig. 2.

Pairwise comparisons revealed up-regulation of key CD4⁺ T-cell genes, mostly restricted to juveniles, upon exposure to *E. coli* (Fig. 3A–D). Few key CD4⁺ T-cell genes were differentially expressed comparing juveniles and neonates both exposed to PBS (Fig. 3A), suggesting different epigenetic states that permit rapid induction of T-cell programs upon *E. coli* exposure in juveniles only. Indeed, compared with PBS aspiration, *E. coli*–exposed juveniles up-regulated important Th1 cell genes (including *Tbx21*, *Stat1*, and *Ifng*), Th17 cell genes (including *Il17a* and *Rorc*), and Treg cell genes (including *Foxp3*, *Il2ra*, *Ctla4*, *Il10*, *Areg*, *Pdcd1*, and *Ikzf4*) (Fig. 3B). Other important regulators, including *Ahr*, *Hif1a*, *Uhrf1*, and *Itga5* were also up-regulated in juveniles with *E. coli* LRTI. In contrast, the expression level of many key CD4⁺ T-cell genes did not achieve a statistically significant difference when comparing neonates exposed to *E. coli* versus PBS (Fig. 3C). For the comparison of juveniles and neonates both exposed to *E. coli*, juveniles up-regulated key CD4⁺ T-cell genes over neonates (Fig. 3D). Expression analysis of selected CD4⁺ T-cell response genes demonstrated a dynamic range across the data set (Fig. 3E).

Figure 2. Transcriptional profiling of lung CD4⁺ T-cells. A, principal component analysis of 3932 differentially expressed genes identified from a generalized linear model and ANOVA-like testing with FDR q-value ≤ 0.05. Ellipses represent normal contour lines with one S.D. probability. B, Pearson correlation distance clustering. C, k-means clustering of differentially expressed genes with k = 4 and scaled as Z-score across rows. D, top five gene ontology (GO) processes derived from each k-means cluster ranked by −log₁₀-transformed FDR q-value. Data are from three replicates per group, each representing pooled cells from three neonatal and two juvenile mice per replicate. ER, endoplasmic reticulum.
These unsupervised approaches suggested a set of immunity-related genes that was peculiar to juveniles, so we explored the uniqueness of this gene set (Fig. 4, A and B). Although both ages up-regulated a core set of 199 immunity-based genes, juveniles uniquely up-regulated 1634 immunity-based genes following exposure to \textit{E. coli} (Fig. 4A). Juveniles also uniquely down-regulated 1307 genes in response to \textit{E. coli} (Fig. 4B), mostly reflecting GO processes similar to cluster 1 from the \textit{k}-means analysis (Fig. 2C). Neither overlap achieved statistical significance beyond that expected by chance, emphasizing the uniqueness of each age’s transcriptional program. Collectively, neonatal lung CD4\(^+\) T-cells displayed a restricted transcriptional response to \textit{E. coli} LRTI, continuing cellular maintenance and proliferation processes rather than executing immune effector and tissue-protective programs.

**Differentially methylated CpGs cluster in regions important for transcriptional control**

We performed DNA methylation profiling with modified reduced representation bisulfite sequencing (mRRBS) to define
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The CpG methylation landscape underlying the attenuated neonatal lung CD4+ T-cell transcriptional response to E. coli LRTI. The dispersion shrinkage for sequencing (DSS) modeling procedure (26), employing a general experimental design analysis with an FDR q-value \( \leq 0.05 \), found 44,119 differentially methylated CpGs (DMCs). Principal component analysis revealed clustering by group assignment and the response to E. coli moving along a different principal component for each age (Fig. 5A). DMCs clustered by group assignment (Fig. 5B). We then examined distance from genomic elements that are biologically sensitive to transcriptional control by DNA methylation: Transcriptional start sites (TSSs) and CpG islands (CpGis). Compared with non-DMCs, DMCs preferentially clustered near TSSs (Fig. 5C) and CpGis (Fig. 5D), suggesting that differential CpG methylation impacts transcription across groups. To determine the directionality of that impact, we next performed an association analysis between tertiles of gene expression and the average CpG methylation of related promoter regions. This analysis demonstrated a statistically significant inverse correlation between gene expression and promoter CpG methylation across the data set (Fig. 5E). DMCs displayed a genome-wide distribution, and DMCs within 2 kbp of differentially expressed gene bodies (inclusive) showed a similar distribution (Fig. 5F). Venn diagram analysis with hypergeometric testing identified 2082 candidate gene loci that displayed both differential methylation (i.e., genes that contained a DMC within 2 kbp of their gene body (inclusive)) and differential expression (Fig. 5G). Altogether, transcriptional and DNA methylation profiling revealed a strong anticorrelation between gene expression and promoter methylation across the four groups.

Figure 4. Venn diagram analysis of transcriptional profiling data. A and B, Venn diagrams partitioning up-regulated (A) and down-regulated (B) genes from the pairwise comparisons of the juvenile, E. coli versus juvenile, PBS and neonate, E. coli versus neonate, PBS groups. Values for each partition represent the number of genes found within the partition. Bar graphs show the top five gene ontology (GO) processes ranked by \(-\log_{10}\) transformed FDR q-value for each partition. p-values resulting from a hypergeometric test are shown above each Venn diagram. Data are from three replicates per group, each representing pooled cells from three neonatal and two juvenile mice per replicate.

A DNA methylation difference-filtering algorithm reveals a core set of regulated genes

K-means clustering of gene expression values for the 2082 candidate gene loci that were both differentially methylated and expressed (Fig. 6A) resembled the k-means cluster structure for the total pool of DEGs (Fig. 2C). Fig. S1B shows the associated elbow plot, suggesting \( k = 4 \), and a tab-delimited file (Table S2) details expression and statistical values for the genes shown in Fig. 6A. We then performed DNA methylation differ-
ence filtering to identify gene loci with a high likelihood of differential methylation regulating expression. Informed by the inverse correlation between expression and promoter methylation (Fig. 5E), we selected for promoter CpGs with a methylation directionality (hypermethylated or hypomethylated) inverse to the corresponding gene expression directionality (down- or up-regulated) between groups. We applied this algorithm to each $k$-means cluster and obtained a pattern of promoter CpG methylation opposite to the gene expression pattern for each $k$-means cluster (Fig. 6B). One thousand three hundred fifty-one promoters did not contain any CpGs passing the filter, leaving 731 gene loci across all four $k$-means clusters.

Heat maps of average gene expression (Fig. 6C) and promoter CpG methylation (Fig. 6D) mirrored one another for the 731 gene loci passing the filter. Moreover, the data structure persisted when examining all (unfiltered) promoter CpGs (Fig. 6E). This largely unsupervised approach suggested high confidence in DNA methylation as a regulatory phenomenon for these gene loci, which contained critical CD4$^+$ T-cell control genes including Bach2, Satb1, Tet1, Notch1, Tbx21, Ikrzf4, Uhrf1, Rora, and Itgae. Homology mapping of these 731 gene loci demonstrated a substantial number of human homologues; the complete list is contained in a tab-delimited file (Table S3).

Disruption of DNA methylation produces lung CD4$^+$ T-cell plasticity

We sought to support the notion that DNA methylation regulates cell phenotype in the pediatric E. coli LRTI model and measured the effect of pharmacologic disruption of DNA methylation on lung CD4$^+$ T-cell markers. Administration of the DNA methyltransferase inhibitor decitabine (DAC) (14) for three daily doses starting 24 h after low-dose (sublethal) E. coli or PBS aspiration led to changes in lung CD4$^+$ T-cell phenotype that were more pronounced in neonates compared with juveniles. In neonates, dimensionality reduction using t-distributed stochastic neighbor embedding (tSNE) on the flow-
cytometric cellular marker profile revealed differences associated with *E. coli* LRTI that were further modified by DAC administration (Fig. 7, A and B). Juveniles, in contrast, exhibited less tSNE clustering by group assignment, suggesting a weaker effect of DAC on their lung CD4<sup>+</sup>/H11001 T-cell phenotype. We observed the most robust differences associated with DAC among the Foxp3<sup>+</sup> subset in both age groups (Fig. 7C), although juveniles exhibited smaller differences than neonates. The directionality of these differences was unexpected, with DAC administration causing decreased activation marker expression, Foxp3 expression, and expression of other Treg cell markers. Notably, DAC administration led to decreased proliferation (Ki-67 expression) among all populations. Taken together, the DAC administration experiments underscored the concept that DNA methylation regulates the lung CD4<sup>+</sup> T-cell response to LRTI in neonates.

**Discussion**

Neonatal LRTI reflects a complex system in which lung CD4<sup>+</sup> T-cells are tasked with ongoing developmental and proliferative processes in addition to orchestrating the immune response to infection. Our data set provides a multidimensional view of lung CD4<sup>+</sup>/H11001 T-cell maturation and response to infection during the early postnatal period. With *E. coli* LRTI, neonatal mice experienced poorer outcomes associated with failure of their lung CD4<sup>+</sup> T-cells to execute a robust immune transcriptional program. Juveniles, in contrast, vigorously up-regulated multiple immune effector and tissue-protective programs while down-regulating maintenance cell functions such as protein localization and nucleic acid metabolism after aspiration with *E. coli*. DNA methylation profiling revealed a statistically likely role for promoter CpG hypermethylation in limiting the neonatal lung CD4<sup>+</sup> T-cell reaction to...
LRTI, which leads to immature physiology and ultimately poorer outcomes.

Genes that were up-regulated with LRTI only among juveniles provide insight into potential mechanisms driving a mature response to LRTI. For example, multiple integrin-encoding genes involved in lymphocyte migration and signaling processes followed this pattern, including Itgae (encoding CD103), Itgb8, and Itgal and Itgb2 (encoding products that combine to form LFA-1). Other examples include the product of Rora, which combines into complexes that have powerful effects on immune effector transcriptional programs (28), and Nt5e, which encodes the CD73 ecto-enzyme that catalyzes production of tissue-protective adenosine (29). Importantly, these genes were present among loci with high probability of methylation-regulated transcription in our data set.

The juvenile CD4⁺ T-cell response to early infection- and inflammation-induced acute lung injury involves coordination of multiple T-cell subsets, including Th1, Th2, Th17, and Treg cells (14, 15, 17, 18, 30). We elected to isolate bulk unfractionated CD4⁺ cells for our studies to provide a broad view of the CD4⁺ T-cell transcriptional and epigenetic landscape. Thus, our transcriptional and epigenetic sequencing data represent a combination of changes in cell state and dynamic fluctuations in the proportion of each subset that constitutes the CD4⁺ T-cell pool. Our experimental design limits the ability to distinguish these possibilities but does permit a comprehensive assessment of DNA methylation alterations underlying the transcriptional programs executed or maintained in the pediatric E. coli LRTI model. Future studies employing single-cell transcriptomic and epigenomic ap-

Figure 7. Lung CD4⁺ T-cell plasticity in response to decitabine administration. A, flow-cytometric characterization of lung CD4⁺ T-cells incorporating Foxp3, Ki-67, Cita-4, CD39, CD69, CD62L, CD44, CD25, and CD103 expression into tSNE analysis. B, pseudocolor density plots of the two tSNE axes. C, fluorescence histograms of selected markers for all CD4⁺ cells, CD4⁺ Foxp3⁺ cells, and CD4⁺ Foxp3⁻ cells. The y axis is scaled to the mode. Color key applies to tSNE plots in A and histograms in C. All plots represent non-down-sampled concatenated data with n = 5 mice per neonate DAC group; 4 mice per neonate DMSO group; and 3 mice per each juvenile group.
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proaches could help further define the heterogeneity displayed in these data (31).

The failure of neonatal lung CD4+ T-cells to switch from a proliferation and growth program to an immune effector and tissue-protective program suggests an evolutionary pressure to establish tissue-based immunity during a time of ongoing lung growth before poising the system to respond robustly to a pathogen. The signals driving lung tissue–specific CD4+ T-cell development remain largely unknown, but antigen exposure leading to T-cell receptor agonism could induce chromatin changes responsible for the maturation process. T-cell receptor–dependent chromatin state alterations dominate the epigenetic landscape during thymic CD4+ T-cell development (23, 25); similar processes may continue to establish tissue-specific immunity (32). Differing metabolic requirements and profiles could also contribute to the maturation phenotype with a possible mechanistic link to differential CpG methylation patterns (33).

Our experiments with the DNA methyltransferase inhibitor decitabine produced surprising results. Neonates (and to a lesser extent juveniles) displayed a hypoproliferative CD4+ T-cell phenotype in response to decitabine, which stands in contrast to observations of lipopolysaccharide- and influenza-induced acute lung injury in adult (8–10-week-old) mice (14). These findings highlight the importance of epigenetic programming in the neonatal and juvenile proliferative phenotypes and evokes proliferation machinery as a potential determinant of overall neonatal lung CD4+ T-cell phenotype. It is possible that decitabine-induced derepression of cellular proliferation inhibitors such as Cdkn1a contributed to our findings (24). Notably, Cdkn1a was one of the 731 loci with a high likelihood of methylation-regulated transcription in our data set. Decitabine’s hypoproliferative effects at higher doses may also involve a TET-dependent demethylation mechanism (34). Regardless, the DNA methyltransferase inhibitor experimental data underscore the powerful effects of DNA methylation on T-cell phenotype both during postnatal lung development as well as during E. coli LRTI. The blunted response to DAC among juveniles compared with neonates supports the hypothesis that DNA methylation regulates the neonatal lung CD4+ T-cell response to LRTI. These results also suggest caution in using broad disruptors of DNA methylation as disease therapy among the very young.

A modification to the classic RRBS protocol (35) permitted streamlined, single nucleotide–resolution, genome-scale CpG methylation analysis using low-input samples. Our mRRBS protocol utilized size selection of MspI-digested genomic DNA fragments prior to bisulfite conversion and library preparation. Following a demultiplexing, alignment, and methylation extraction pipeline, we elected to employ the DSS procedure for comparative statistics (26). Unlike the commonly used Fisher’s exact test, the DSS procedure permitted Bayesian hierarchical modeling, taking into account CpG site-specific dispersions and depth of coverage with high-fidelity type I error control (36). When combined with differential gene expression data using a methylation difference–filtering algorithm, our analysis yielded high correlative confidence in identification of loci likely to exhibit methylation-regulated transcription.

However, causality remains a concern for many studies that explore epigenetic mechanisms, including ours. Our data integration study falls into the “inferred function” hierarchical level described in a recent review of functional epigenomics (37). The sequencing and computational approaches used in our study support the hypothesis that DNA methylation regulates an immature transcriptional program in neonates with LRTI but can only provide statistically strong associative, not causal, evidence. Future studies exploiting novel CRISPR-based DNA methylation–editing systems could elucidate causal proof of these associations (38).

Altogether, unsupervised parallel transcriptional and DNA methylation profiling paired with association analysis demonstrated that CpG methylation regulates, at least in part, the failure of neonates to execute a mature transcriptional program in response to E. coli LRTI. The juvenile transcriptional program, characterized by up-regulation of key T-cell pathway components, associated in a locus-specific manner with differential CpG methylation as a function of both age and reaction to E. coli LRTI. Pharmacologic disruption of DNA methylation with decitabine produced plasticity mostly within the neonatal lung CD4+ T-cell population, highlighting the powerful role epigenetic programming may play in lung CD4+ T-cell maturation and response to infection. These studies open up new avenues to consider the lymphocyte epigenetic machinery as mechanistic targets to improve outcomes for pediatric pneumonia.

Experimental procedures

Mice

Timed pregnant C57BL/6NJ mice were obtained from Charles River Laboratories. Adult animals were maintained on an AIN 76A diet and water ad libitum and housed at a temperature range of 20–23 °C under 12-hour light/dark cycles. All pups of both sexes were used in the reported experiments. All experiments were conducted in accordance with the standards established by the United States Animal Welfare Act set forth in National Institutes of Health guidelines and the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee.

Intrapharyngeal aspiration of E. coli

Pups were lightly sedated with isoflurane prior to aspiration with E. coli bacteria (Seattle 1946, serotype O6, ATCC 25922). Neonatal (3–4-day-old) and juvenile (11–14-day-old) mice were randomized by cage to receive either PBS alone or E. coli in PBS (2.8 × 10⁶ cfu). Forceps were used to gently retract the tongue, liquid was deposited in the pharynx, and aspiration of fluid was directly visualized as previously described (18). Neonatal mice were aspirated with 10 μl of fluid and juvenile mice received 15 μl of fluid. Blinded assessment was not possible because of obvious size and health differences between groups.

Quantitative microbiology of E. coli bacteria

E. coli was streaked on an LB agar plate and grown overnight at 37 °C. Bacteria were transferred to LB medium, agitated at 250 rpm, and incubated at 37 °C for 3–4 h. Bacterial growth was determined using optical density (OD) measured at 600 nm. Serial dilutions were performed and plated overnight at 37 °C to assess the accuracy of the OD measurement.
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Decitabine administration

Similar to previous work in adult mice (14), with a slight modification, neonatal or juvenile mice received three daily intraperitoneal injections of 5-aza-2’-deoxycytidine (decitabine, Sigma) 1 mg/kg in 30 µl beginning 24 h after aspiration of 300,000 cfu of E. coli or PBS as described above. Mice were euthanized 72 h after aspiration for flow-cytometric analysis of lung single-cell suspensions.

Processing of mouse lungs

Preparation of lung tissue for histology and processing to create single-cell suspensions were performed as previously reported (14, 17, 30). Briefly, to create single-cell suspensions, lung was minced in a Petri dish with 1 ml of buffer containing DNase and collagenase I (concentration, 0.001 g DNase and 0.005 g collagenase I in 1 ml RPMI) and incubated at 37 °C for 30 min. The lung sample was passed through an 18-gauge needle several times and then through a 70-µm cell strainer. PBS was added, and the mixture was spun at 300 × g for 10 min. ACK lysing buffer was added to the pellet, incubated at room temperature for 5 min, and then PBS was added to stop the reaction. The lung sample was filtered, spun at 300 × g for 5 min, and MACS buffer (PBS + 0.5% BSA and 2 mM EDTA) was added.

CD4⁺ T-cell positive selection from mouse lungs

CD4–phycoerythrin (PE) conjugated antibody (BD Biosciences) was added to ACK-lysed lung cells. Cells were incubated at 4 °C for 10 min, washed with MACS buffer, and spun at 300 × g for 10 min. Cells were resuspended in MACS buffer, and 20 µl of anti-PE microbeads (Miltenyi Biotec) per 10⁶ cells was added. Cells were then incubated at 4 °C for 15 min, washed with MACS buffer, and spun at 300 × g for 10 min. Cells were resuspended in 500 µl MACS buffer per 10⁶ cells and magnetically separated by placing an MS column (Miltenyi Biotec) in a MACS separator. The column was rinsed with MACS buffer, and the cell suspension was applied to the column. Unlabeled cells were collected and washed with 500 µl MACS buffer three times. The column was removed from the separator and placed on a 1.5-ml Eppendorf tube. Sorting medium (PBS + 0.5% BSA, 0.5% fetal bovine serum, 1 mM EDTA, and 25 mM HEPES) was added, and the mixture was spun at 300 × g for 10 min. Sorting medium (PBS + 0.5% BSA, 0.5% fetal bovine serum, 1 mM EDTA, and 25 mM HEPES) was added to ACK-lysed lung cells. Cells were incubated at 4 °C for 10 min, washed with MACS buffer, and spun at 300 × g for 10 min. ACK lysing buffer was added to the pellet, incubated at room temperature for 5 min, and then PBS was added to stop the reaction. The lung sample was filtered, spun at 300 × g for 5 min, and MACS buffer (PBS + 0.5% BSA and 2 mM EDTA) was added.

Flow cytometry analysis

For the decitabine administration experiments, a lung single-cell suspension was prepared for flow cytometric analysis as previously described (39) and as above. The following antibody conjugates were purchased from BioLegend, eBiosciences, or BD Biosciences: CD103-FITC, CD25-PE, KI-67–PerCPeFluor710, CD39-PE-Cy7, Foxp3-APC, CD69–Alexa Fluor 700, CD62L–APCeFluor780, Crla-4–BV421, CD44–BV510, and CD4-BUV395. Antibody and cytometer setup details are provided in Table S4. Acquisition was performed using a custom BD FACSAria II instrument with FACSDiva software (BD Biosciences). Analysis was performed with FlowJo v10.4.1, including tSNE analysis with the FlowJo tSNE plug-in using a perplexity value of 20.

RNA-Seq

Approximately 50–200 ng of total RNA was isolated using the AllPrep DNA/RNA Micro Kit or RNeasy Plus Mini Kit (Qiagen), and cDNA was generated with the NuGen Ovation RNA-Seq System V2. Fragmentation was performed on a Covaris S2. Illumina-compatible adapter ligation and indexing was followed by PCR amplification. A high-sensitivity chip on an Agilent Bioanalyzer 2100 was used to measure the size distribution and quality of amplified libraries. Library quantification was performed with the qPCR-based KAPA Library Quantification Kit or by Bioanalyzer. Equimolar concentrations of each library were pooled. Cluster generation and sequencing were performed on an Illumina HiSeq 2500 instrument employing 100 × 100 paired-end sequencing with the TruSeq Rapid PE Cluster Kit and TruSeq Rapid SBS Kit (200 cycles).

CASAVA v1.8.4 was used to convert bcl files to fastq files (default parameters). Rsem v1.2.09 (STAR option) was used for running alignments to the GRCh38/mm10 mouse reference genome using the iGenomes annotation. Counts data for uniquely mapped reads over exons was obtained using SeqMonk v1.38.2 (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/) and filtered to protein-coding genes and genes with at least one count per million in at least two samples. Differential gene expression analysis was performed with the edgeR v3.16.5 R/Bioconductor package using R v3.3.1 and v3.4.2 with RStudio v0.98.1103 and v1.1.383.

Modified reduced representation bisulfite sequencing

Genomic DNA, isolated using the AllPrep DNA/RNA Micro Kit (Qiagen), was quantified with a Qubit 3.0 instrument. Approximately 50–200 ng of genomic DNA was then digested with the restriction endonuclease MspI (New England Biolabs) per the manufacturer’s recommendations. Resulting fragments underwent size selection for fragments ~100–250 bp in length using solid phase reversible immobilization (SPRI) beads (MagBio Genomics) and subsequent bisulfite conversion using the EZ DNA Methylation-Lightning Kit (Zymo Research) per the manufacturer’s protocol. Bisulfite conversion efficiency averaged 99.4% (S.D. 0.13%) as estimated by the measured percent of unmethylated CpGs in λ-bacteriophage DNA (New England Biolabs, N3013S) added at a 1:200 mass ratio to each sample. Libraries for Illumina-based sequencing were prepared with the Pico Methyl-Seq Library Prep Kit (Zymo Research) using Illumina TruSeq DNA methylation indices. Libraries were run on a high-sensitivity chip using an Agilent TapeStation 4200 to assess size distribution and overall quality of the amplified libraries. Fluorometric quantification and TapeStation size distribution estimates permitted equimolar pooling, and six

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pooled libraries per run were sequenced on an Illumina NextSeq 500 instrument using the NextSeq 500/550 V2 High Output reagent kit (1 × 75 cycles).

Indexed samples were demultiplexed to fastq files with bcl2fastq v2.17.1.14. After standard quality filtering, reads were then trimmed of 10 bp from the 5’ end with Trim Galore! v0.4.3 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequence alignment to the GRCh38/mm10 reference genome and methylation extraction ignoring one base at the 3’ end (after reviewing the M-bias plots) were performed with Bismark v0.16.3 (40). Bismark coverage (counts) files for cytosines in CpG context were analyzed with respect to differential methylation with the DSS v2.26.0 R/Bioconductor package (26) and quantified using the SeqMonk platform (v1.38.2 and v1.40.1) with the bisulphite feature methylation pipeline. Transcriptional start sites were obtained from the Ensembl Genes 90 database and filtered for those with a Consensus CDS ID. CpG islands were identified from the MG1 database. Homology mapping was performed using the biomaRt v2.32.1 R/Bioconductor package and the getLDS function.

Statistical analysis

Indicated sample sizes were chosen to obtain a minimum of 10^6 unique CpGs per biological replicate; observed average ± S.D. CpGs per sample was 2.4 × 10^6 ± 1.0 × 10^6. Principal component analysis was performed with the prcomp base R statistical function. Pearson correlation distance clustering was generated in SeqMonk. Functional enrichment analysis using gene ontologies (GO biological processes) was conducted using the Molecular Signatures Database (MSigDB) (The Broad Institute) (41). K-means clustering and heat maps were generated using the Morpheus web interface (https://software.broadinstitute.org/morpheus/). K was selected using the elbow method. Manhattan plotting was performed with the qqman v0.1.4 R package. Venn diagrams were created with the VennDiagram v1.6.18 R package, and the phyper base R function was used to calculate p-values from hypergeometric testing using a total population size of 11,672 genes. All indicated tests were two-tailed unless otherwise stated. Computational analysis was performed using “Genomics Nodes” on Quest, Northwestern University’s High-Performance Computing Cluster. Specific statistical testing procedures are elaborated in the text and figure legends, performed either in R using packages and functions specified above or in GraphPad Prism v7.04.

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References

1. Flori, H. R., Glidden, D. V., Rutherford, G. W., and Matthay, M. A. (2005) Pediatric acute lung injury: Prospective evaluation of risk factors associated with mortality. Am. J. Respir. Crit. Care Med. 171, 995–1001 CrossRef Medline
2. Zimmerman, J. J., Akhtar, S. R., Caldwell, E., and Rubenfeld, G. D. (2009) Incidence and outcomes of pediatric acute lung injury. Pediatrics 124, 87–95 CrossRef Medline
3. Institute of Medicine (U.S.) and National Research Council (U.S.). National Cancer Policy Board (2003) The Epidemiology of Childhood Cancer, National Academies Press, Washington, DC
4. Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J. E., Cousins, S., Mathers, C., and Black, R. E. (2015) Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: An updated systematic analysis. Lancet 385, 430–440 CrossRef Medline
5. Rubenfeld, G. D., Caldwell, E., Peabody, E., Weaver, J., Martin, D. P., Neff, M., Stern, E. J., and Hudson, L. D. (2005) Incidence and outcomes of acute lung injury. N. Engl. J. Med. 353, 1685–1693 CrossRef Medline
6. Duke, T. (2005) Neonatal pneumonia in developing countries. Arch. Dis. Child. Fetal Neonatal Ed. 90, F211–F219 CrossRef Medline
7. Zhi, X., Zhivaki, D., and Lo-Man, R. (2017) Unique aspects of the perinatal immune system. Nat. Rev. Immunol. 17, 495–507 CrossRef Medline
8. Thurlbeck, W. M. (1982) Postnatal human lung growth. Thorax 37, 564–571 CrossRef Medline
9. Misharin, A. V., Morales-Nebreda, L., Reyfman, P. A., Cuda, C. M., Walker, J. M., Quattrocchi-Pimentel, A. C., Chen, C. I., Anealla, K. R., Joshi, N., Williams, K. N., Abdala-Velena, H., Yacoub, T. J., Chi, M., Chiu, S., Gonzalez-Gonzalez, F. J., et al. (2017) Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. J. Exp. Med. 214, 2387–2404 CrossRef Medline
10. Schurmans, S., Heusser, C. H., Qin, H. Y., Merino, J., Brighouse, G., and Lambert, P. H. (1990) In vivo effects of anti-IL-4 monoclonal antibody on neonatal induction of tolerance and on an associated autoimmune syndrome. J. Immunol. 144, 854–859 Medline
11. Abramowicsz, D., Vandervorst, P., Bruins, C., Toutouropoulou, J. M., Vandenabeele, P., and Goldman, M. (1990) Persistence of anti-donor allohelper T cells after neonatal induction of allotolerance in mice. Eur. J. Immunol. 20, 1647–1653 CrossRef Medline
12. Levy, O. (2005) Innate immunity of the human newborn: Distinct cytokine responses to LPS and other Toll-like receptor agonists. J. Endotoxin Res. 11, 113–116 CrossRef Medline
13. Singer, B. D., Mack, J. R., Aggarwal, N. R., Garibaldi, B. T., Sidhaye, V. K., Florez, M. A., Chau, E., Gibbs, K. W., Mandke, P., Tripathi, A., Yegnasu-
bramanian, S., King, L. S., and D’Alessio, F. R. (2015) Regulatory T cell DNA methyltransferase inhibition accelerates resolution of lung inflammation. *Am. J. Respir. Cell Mol. Biol.* **52**, 641–652 CrossRef Medline

15. D’Alessio, F. R., Tsushima, K., Aggarwal, N. R., West, E. E., Willeit, M. H., Britos, M. F., Pipeling, M. R., Brower, R. G., Tudor, R. M., McDyer, J. F., and King, L. S. (2009) CD4+CD25+Foxp3+ Treg cells resolve experimental lung injury in mice and are present in humans with acute lung injury. *J. Clin. Invest.* **119**, 2898–2913 CrossRef Medline

16. Mock, J. R., Garibaldi, B. T., Aggarwal, N. R., Jenkins, J., Limjunyawong, N., Singer, B. D., Chau, E., Rabold, R., Files, D. C., Sidhaye, V., Mitzner, W., Wagner, E. M., King, L. S., and D’Alessio, F. R. (2014) Foxp3+ regulatory T cells promote lung epithelial proliferation. *Mucosal Immunol.* **7**, 1440–1451 CrossRef Medline

17. McGrath-Morrow, S. A., Lee, S., Gibbs, K., Lopez, A., Collaco, J. M., Nepune, T., Soloski, M. J., Scott, A., and D’Alessio, F. (2015) Immune response to intrapharyngeal LPS in neonatal and juvenile mice. *Am. J. Respir. Cell Mol. Biol.* **52**, 323–331 CrossRef Medline

18. McGrath-Morrow, S. A., Ndeh, R., Collaco, J. M., Poupore, A. K., Dike-man, D., Zhong, Q., Singer, B. D., D’Alessio, F., and Scott, A. (2017) The innate immune response to lower respiratory tract E. coli infection and the role of the CCL2-CCR2 axis in neonatal mice. *Cytokine* **97**, 108–116 CrossRef Medline

19. Hwang, E. S., Szabo, S. J., Schwartzberg, P. L., and Glimcher, L. H. (2005) T regulatory T cells. *Immunity* **23**, 785–799 CrossRef Medline

20. Obata, Y., Furusawa, Y., Endo, T. A., Sharif, J., Takahashi, D., Atarashi, K., Nakayama, M., Onawa, S., Fujimura, Y., Takahashi, M., Ikawa, T., Otsubo, E., Kohwi-Shigematsu, T., and Sakaguchi, S. (2017) Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment and antagonizing RORγt function. *Nature* **543**, 236–240 CrossRef Medline

21. Djuretic, I. M., Levanon, D., Negreanu, V., Groner, Y., Rao, A., and Ansel, K. M. (2007) Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat. Immunol.* **8**, 145–153 CrossRef Medline

22. Makar, K. W., and Wilson, C. B. (2004) DNA methylation is a nonredundant repressor of the Th2 effector program. *J. Immunol.* **173**, 4402–4406 CrossRef Medline

23. Kitagawa, Y., Ohkura, N., Kidani, Y., Vandenbon, A., Hirota, K., Kawakami, R., Yasuda, K., Motooka, D., Nakamura, S., Kondo, M., Taniuchi, I., Kohwi-Shigematsu, T., and Sakaguchi, S. (2017) Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment and antagonizing RORγt function. *Nature* **543**, 236–240 CrossRef Medline

24. Eltzschig, H. K., Sitkovsky, M. V., and Robson, S. C. (2012) Purinergic signaling during inflammation. *N. Engl. J. Med.* **367**, 2322–2333 CrossRef Medline

26. Feng, H., Conneely, K. N., and Wu, H. (2014) A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. *Nucleic Acids Res.* **42**, e69 CrossRef Medline

27. Hogg, N., Patzak, L., and Willenbrock, F. (2011) The insider’s guide to leukocyte integrin signalling and function. *Nat. Rev. Immunol.* **11**, 416–426 CrossRef Medline

28. Cook, D. N., Kang, H. S., and Jetten, A. M. (2015) Retinoic acid-related orphan receptors (RORs): Regulatory functions in immunity, development, circadian rhythm, and metabolism. *Nucl. Receptor Res.* **2**, 101185 CrossRef Medline

29. Eltzschig, H. K., Sitkovsky, M. V., and Robson, S. C. (2012) Purinergic signaling during inflammation. *N. Engl. J. Med.* **367**, 2322–2333 CrossRef Medline

30. D’Alessio, F. R., Craig, J. M., Singer, B. D., Files, D. C., Mock, J. R., Garibaldi, B. T., Fallica, J., Tripathi, A., Mandke, P., Gans, J. H., Limjunyawong, N., Sidhaye, V. K., Keller, N. M., Mitzner, W., King, L. S., and Aggarwal, N. R. (2016) Enhanced resolution of experimental ARDS through IL-4-mediated lung macrophage reprogramming. *Am. J. Physiol. Lung Cell Mol. Physiol.* **310**, L733–L746 CrossRef Medline

31. Kelsey, G., Stegle, O., and Reik, W. (2017) Single-cell epigenomics: Recording the past and predicting the future. *Science* **358**, 69–75 CrossRef Medline

32. Delacher, M., Imbusch, C. D., Weichenhan, D., Breiling, A., Hotz-Wagenblatt, A., Träger, U., Hofer, A. C., Kagbein, D., Wang, Q., Frauhammer, F., Malml, J. P., Bauer, K., Herrmann, C., Lang, P. A., Brors, B., Plass, C., and Feuerer, M. (2017) Genome-wide DNA-methylation landscape defines specialization of regulatory T cells in tissues. *Nat. Immunol.* **18**, 1160–1172 CrossRef Medline

33. Stricker, S. H., Köferle, A., and Beck, S. (2017) From profiles to function in epigenomics. *Nat. Rev. Genet.* **18**, 51–66 CrossRef Medline

34. Liu, X. S., Wu, H., Ji, X., Stelzer, T., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A., and Jaenisch, R. (2016) Editing DNA methylation in the mammalian genome. *Cell* **167**, 233–247.e17 CrossRef Medline

35. Meissner, A., Ngnirke, A., Bell, G. W., Ramsahoye, B., Lander, E. S., and Hauberli, L., Huck, C., Turka, L. A., Wood, K. C., Hale, L. P., et al. (2015) Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J. Clin. Invest.* **125**, 194–207 CrossRef Medline

36. Wang, X., Wang, J., Yu, Y., Ma, T., Chen, P., Zhou, B., and Tao, R. (2017) Decitabine inhibits T cell proliferation via a novel TET2-dependent mechanism and exerts potent protective effect in mouse auto- and allo-immunity models. *Oncotarget* **8**, 56802–56815 CrossRef Medline

37. Stricker, S. H., Köferle, A., and Beck, S. (2017) From profiles to function in epigenomics. *Nat. Rev. Genet.* **18**, 51–66 CrossRef Medline

38. Zhang, Y., Baheti, S., and Sun, Z. (2016) Statistical method evaluation for differentially methylated CpGs in base resolution next-generation DNA sequencing data. *Brieif. Bioinform.* **19**, 374–386 CrossRef Medline

39. Singer, B. D., Mock, J. R., D’Alessio, F. R., Aggarwal, N. R., Mandke, P., Johnston, L., and Damarla, M. (2016) Flow-cytometric method for simultaneous analysis of mouse lung epithelial, endothelial, and hematopoietic lineages. *Am. J. Physiol. Lung Cell Mol. Physiol.* **310**, L1796–L1801 CrossRef Medline

40. Krueger, F., and Andrews, S. R. (2011) Bismark: A flexible aligner and methylation caller for bisulfit-seq applications. *Bioinformatics* **27**, 1571–1572 CrossRef Medline

41. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 15545–15550 CrossRef Medline

42. Ilingworth, R. S., Gruenewald-Schneider, U., Webb, S., Kerr, A. R., James, K. D., Turner, D. J., Smith, C., Harrison, D. J., Andrews, R., and Bird, A. P. (2010) Orphan CpG islands identify numerous conserved promoters in the mammalian genome. *PloS Genet.* **6**, e1001134 CrossRef Medline