Plasma cell differentiation is coupled to division-dependent DNA hypomethylation and gene regulation

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The epigenetic processes that regulate antibody-secreting plasma cells are not well understood. Here, analysis of plasma cell differentiation revealed DNA hypomethylation of 10% of CpG loci that were overrepresented at enhancers. Inhibition of DNA methylation enhanced plasma cell commitment in a cell-division-dependent manner. Analysis of B cells differentiating in vivo stratified by cell division revealed a fivefold increase in mRNA transcription coupled to DNA hypomethylation. Demethylation occurred first at binding motifs for the transcription factors NF-κB and AP-1 and later at those for the transcription factors IRF and Oct-2 and was coincident with activation and differentiation gene-expression programs in a cell-division-dependent manner. These data provide mechanistic insight into cell-division-coupled transcriptional and epigenetic reprogramming and suggest that DNA hypomethylation reflects the cis-regulatory history of plasma cell differentiation.

RESULTS

Coupling of B cell differentiation to unique transcriptional states

We used an in vivo model of inducible B cell differentiation to investigate the molecular events that could be traced to a defined stimulus. C57BL/6J mice challenged intravenously with the mitogen lipopolysaccharide (LPS) exhibited splenomegaly and a threefold population expansion of splenic B220+ B cells relative to their abundance in naive mice, while activated B220+GL7+ B cells constituted 35% of splenocytes by 3 d after challenge, relative to a frequency of 2% in naive mice (Supplementary Fig. 1a–c). Extrapolation of those data indicated that there were approximately 120 x 106 new B cells in the splenic compartment (Supplementary Fig. 1d–f). Analysis of differentiating CD138+ B cells showed an admixture of cells with intermediate to low gene expression and cell division (Fig. 1a–f). B220 expression on CD138+ plasma cells is a marker of rapid cellular turnover in the spleen15 and bone marrow16, whereas B220CD138+ plasma cells represent a post-mitotic population15. Both B220CD138+ plasma cells and B220CD138+ plasma cells were substantially induced by 3 d after challenge with LPS (Fig. 1a) and are called ‘plasmablasts’ and ‘plasma cells’, respectively, here.

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Figure 1  B cell differentiation is coupled to unique transcriptional states. (a) Flow cytometry analyzing the expression B220 and CD138 in splenocytes from naive mice and LPS-challenged mice on day 3 after challenge (left), and quantification of B220⁺CD138⁺ plasmablasts (PBs) and B220⁺CD138⁺ plasma cells (PCs) in such mice (right). Numbers adjacent to outlined areas (left) indicate percent B220⁺CD138⁺ B cells (top left), B220⁺CD138⁺ plasmablasts (top right) or B220⁺CD138⁺ plasma cells (bottom right). (b) Purity of B cells, plasmablasts and plasma cells (key) after sorting. (c) Hierarchical clustering of the expression of 16,181 genes in the populations in a,b. (d) PCA of expression data in c, showing principal components 1 (PC1) and 2 (PC2). (e) Gene expression in B220⁺CD138⁺ plasmablasts or B220⁺CD138⁺ plasma cells showing genes expressed differentially (Supplementary Table 1) in plasmablasts (burgundy) or plasma cells (gold) or both (black) relative to their expression in B cells from naive mice; dashed gray lines indicate cutoff of a change in expression of twofold. (f) Gene-set–enrichment analysis of expression changes in plasmablasts and plasma cells relative to that in B cells, for genes regulated in human plasma cells 17 (PC vs B cell (left); false-discovery rate (FDR), <0.05) or the ‘reactome’ pathway ‘Mitotic M-M/G1 phases’ (right; false-discovery rate, <0.01 (for plasmablasts only)), presented as enrichment score (top); below, overlap of genes from each set with ordering of the differences in expression above. *P < 0.001 (two-tailed t-test). Data are representative of two experiments with 15 mice (a; mean and s.d.) or one experiment with three mice and two B cells, three plasmablasts and two plasma cells (c-f).

To characterize transcriptional stages in B cell differentiation, we obtained splenic B220⁺ B cells from naive C57BL/6J mice and B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells from mice 3 d after LPS challenge (Fig. 1b), then isolated RNA from the cells and analyzed it by bead–microarray. Hierarchical clustering of all expression data stratified samples by cell type (Fig. 1c), which indicated each cell type was transcriptionally unique. That observation was supported by principle-component analysis (PCA), which separated each biological replicate by cell type (Fig. 1d). B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells upregulated 937 genes and 567 genes and downregulated 1,016 and 501 genes, respectively, relative to their expression in naive B220⁺ B cells (Supplementary Table 1). Despite the distinct gene-expression programs of each cell type, 544 genes exhibited similar regulation in plasmablasts and plasma cells that was different from their regulation in naive B220⁺ B cells (Fig. 1e). Gene-ontology annotation showed that genes commonly downregulated in both B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells encoded products involved in hematopoiesis (for example, Hhex), immune-system development (Bcl2 and Irf8) and antigen presentation (Ciita, H2-Aa, H2-Ab1, H2-Eb1). Genes upregulated uniquely in B220⁺CD138⁺ plasmablasts encoded products associated with mitosis and cellular division (Supplementary Table 2). Those observations were supported by gene-set–enrichment analysis, which indicated that the gene-expression changes in B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells were similar to those previously reported for humans 17 and identified that B220⁺CD138⁺ plasmablasts selectively had higher expression of genes encoding products associated with mitotic pathways (Fig. 1f and Supplementary Table 3). These data showed that LPS induced robust B cell differentiation characterized by transcriptionally distinct B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells.

Targeted DNA hypomethylation in plasmablasts and plasma cells

To directly measure epigenetic changes during B cell differentiation, we extracted DNA from naive splenic B220⁺ B cells and from B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells at 3 d after LPS induction and determined their DNA-methylation state by reduced-representation bisulfite sequencing (RRBS) 18. In total, 911,004 CpG dinucleotides were analyzed (Supplementary Table 4). Hierarchical clustering of the DNA-methylation data separated B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells from naive B220⁺ B cells (Fig. 2a), which indicated that DNA methylation might have an important role during B cell differentiation. PCA of the DNA-methylation data indicated that B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells were distinct from B220⁺ B cells (Fig. 2b), suggestive of a common epigenetic change in plasmablasts and plasma cells. Highly methylated loci in naive B220⁺ B cells showed a substantial shift to more intermediate methylation in B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells (Fig. 2c). Average DNA methylation was lower in B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells than in naive B220⁺ B cells (Fig. 2d), which suggested that B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells underwent global DNA hypomethylation.

Differentially methylated loci (DML) were identified by a statistical model that accounts for sequencing depth and biological variation 19. This identified 17,628 loci and 99,265 loci that were methylated differentially in B220⁺CD138⁺ plasmablasts and B220⁺CD138⁺ plasma cells, respectively, relative to their methylation in naive B220⁺ B cells.
Demethylated regions at B cell enhancers

To determine whether the demethylation in B220<sup>hi</sup>CD138<sup>+</sup> plasmablasts and B220<sup>lo</sup>CD138<sup>+</sup> plasma cells occurred ‘preferentially’ at enhancer regions, we used an odds ratio to assess the overlap of demethylated loci with active enhancers (H3K4me1<sup>+</sup>H3K27ac<sup>+</sup>H3K4me3<sup>+</sup>) or poised enhancer regions (H3K4me1<sup>+</sup>H3K27ac<sup>−</sup>H3K4me3<sup>−</sup>)<sup>30</sup>. Here, we identified enhancers in splenic B220<sup>+</sup> B cells, splenocytes, the CH12 lymphoma cell line and whole-tissue homogenates of thymus, testis and brain from C57B/6J mice using data generated by the ENCODE (Encyclopedia of DNA Elements) consortium and others<sup>31,32</sup>. We observed DML more often at enhancers than at other regions of the genome, and this was most pronounced in active enhancers of B cells, splenocytes and CH12 cells (Fig. 3a), which suggested that these regulatory regions were used during plasma cell differentiation.

To determine which transcription factors might be active during B cell differentiation, we used HOMER software (for motif discovery and next-generation sequencing analysis) to search regions within 50 base pairs of demethylated loci for enrichment for transcription-factor-binding motifs relative to assay coverage<sup>33</sup>. Transcription-factor-binding motifs that showed significant enrichment in demethylated loci in B220<sup>hi</sup>CD138<sup>+</sup> plasmablasts or B220<sup>lo</sup>CD138<sup>+</sup> plasma cells were clustered by the similarity of their consensus binding motif; this showed that binding motifs for transcription factors of the IRF, POU homeobox domain and bZIP families were overrepresented.
in demethylated regions in both B220<sup>hi</sup>CD138<sup>+</sup> plasmablasts and B220<sup>lo</sup>CD138<sup>+</sup> plasma cells, whereas binding motifs for transcription factors of the RHD (‘Rel-homology domain’), NFATc1 and E2A families were significantly overrepresented only in demethylated regions in B220<sup>lo</sup>CD138<sup>+</sup> plasma cells (Fig. 3b). That observation was in agreement with the fact that transcription factors that bind these motifs are critical determinants of B cell fate<sup>26,34–36</sup>. These data suggested that DNA demethylation in B220<sup>lo</sup>CD138<sup>+</sup> plasmablasts and B220<sup>lo</sup>CD138<sup>+</sup> plasma cells reflected transcription-factor activity and enhancer usage.

**Coupling of DNA demethylation and differentiation to cell division**

To address the role of DNA methylation during plasma cell differentiation, we labeled splenic B220<sup>+</sup> B cells with carboxyfluorescein succinimidyl ester (CFSE) or CellTrace Violet (CTV) to track cellular division and cultured the cells for 3 d in the presence of LPS, interleukin 2 (IL-2) and IL-5 (ref. 37) and the DNA-methylation inhibitor 5-azacytidine (5-azaC). Flow cytometry showed that splenocytes cultured with 5-azaC had a higher frequency of differentiation into CD138<sup>+</sup> plasma cells than that of splenocytes in untreated cultures, and the effect of 5-azaC was dose dependent (Fig. 4a,b). Moreover, 5-azaC induced differentiation into plasma cells after fewer cell divisions than that required for such differentiation in untreated cultures (Fig. 4c).

To determine how DNA-methylation and gene-expression changes occur relative to cell division during B cell differentiation in vivo, we used an adoptive-transfer model. We isolated splenic B220<sup>+</sup> B cells from CD45.1<sup>+</sup> C57BL/6J donor mice, labeled the cells with CFSE or CTV and transferred them intravenously into CD45.2<sup>+</sup> μMT mice, which lack B cells due to a disrupted exon encoding the transmembrane domain of the immunoglobulin M heavy chain<sup>38</sup>. We challenged the host mice with LPS 1 d after the adoptive cell transfer, and 3 d after challenge we found CD45.1<sup>+</sup> donor B cells in the spleen of recipient mice (Supplementary Fig. 4a,b). CFSE dilution indicated that B cells transferred into LPS-challenged mice underwent zero to over eight rounds of division, whereas B cells transferred into mice that did not receive LPS rarely divided (Supplementary Fig. 4c,d). At 3 d after LPS challenge or mock treatment, donor CD45.1<sup>+</sup>B220<sup>hi</sup>CD138<sup>+</sup> plasmablasts and CD45.1<sup>+</sup>B220<sup>lo</sup>CD138<sup>+</sup> plasma cells were present only in the spleen of mice that received LPS (Fig. 5a). Donor CD45.1<sup>+</sup>B220<sup>lo</sup>CD138<sup>+</sup> plasma cells had divided more than CD45.1<sup>+</sup>B220<sup>hi</sup>CD138<sup>+</sup> plasmablasts had, and no differentiation was observed before division 6 (Fig. 5b). B220 expression on CD45.1<sup>+</sup> donor B cells was lost only after many rounds of division in LPS-challenged mice (Fig. 5c). These cells were viable, and a large proportion of them expressed the plasma cell marker CD138 (Supplementary Fig. 4e-h). Expression of the germinal-center marker GL7 on donor CD45.1<sup>+</sup> B cells increased without cell division and was progressively upregulated at later divisions in LPS-challenged mice (Fig. 5d). In agreement with published ex vivo experiments<sup>4,5</sup>, these data linked plasma cell differentiation to cell division and suggested that the observed changes in gene expression and DNA hypomethylation might be related to the multiple rounds of mitotic division that plasmablasts and plasma cells undergo in vivo.

To determine the relationship between DNA methylation and gene expression at successive stages of differentiation, we sorted cells on the basis of CTV dilution, representing divisions 0, 1, 3, 5 and those that divided at least eight times. We also sorted the populations of cells that divided at least eight times into those that were CD138<sup>−</sup> or CD138<sup>+</sup> (called ‘8−’ and ‘8+’, respectively, here) (Fig. 5e). Using these cell populations, we performed high-throughput sequencing technologies for cDNA (RNA-seq) in which we used synthetic mRNA ‘spike-in’ controls.
Figure 5 Transcriptional amplification and DNA hypomethylation coincide with cellular division. (a) Flow cytometry analyzing the expression of B220 and CD138 on donor (CD45.1+) splenic naive B220+ B cells after transfer into μMT (CD45.2+) host mice subsequently challenged with LPS or not (control (Ctrl)) (above plots; left), and frequency of B220CD138+ plasmablasts (middle) and B220CD138+ plasma cells (right) as in mice at left. (b) CFSE dilution by B220+ B cells, B220CD138+ plasmablasts, and B220CD138+ plasma cells (left), and frequency of B220CD138+ plasmablasts and B220CD138+ plasma cells (key) at various stages of division (horizontal axis; right). (c,d) Flow cytometry analyzing the expression of B220 (c) or GL7 (d) and staining with CFSE of cells from mice as in a (left), and frequency of B220+ cells (c) or GL7+ cells (d) at various stages of division (right). (e) Flow cytometry analyzing the expression of CD138 and CTV staining of donor (CD45.1+) cells from host mice challenged with LPS as in a (top), and purity at various stages of cellular division (key) after sorting (bottom). (f) Transcript expression (top) and DNA methylation (bottom) of the populations in e. (g) Quantification of mRNA (average per-cell values; top) and DNA methylation of 1,639,598 CpG loci (bottom), in populations as in e. (h) Probability density for mRNA expression (top) and DNA methylation (bottom) in populations as in e (key); dashed vertical line (top), 90% detection sensitivity of assay. (i) PCA of mRNA expression (top) and DNA methylation (bottom) in populations as in e (key). Each symbol (a,g) represents an individual mouse (a) or population (g); small horizontal lines indicate the mean (± s.d.). *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test). Data are from two experiments with four or six mice each (a–e; mean ± s.d. in b–d) or one experiment with biological duplicates (f–i).

from ERCC (the External RNA Controls Consortium) to determine the average number of mRNAs on a per-cell basis. In addition, we performed a dual-restriction-enzyme (MspI and TaqI) RRBS protocol that resulted in coverage of 1,639,598 CpG loci (Supplementary Table 4). The resulting data sets were consistent with data generated by reverse transcription–quantitative PCR (RT-qPCR) and by a qPCR DNA-methylation assay of independently isolated cells of the same division and CD138 expression (Supplementary Fig. 5). Heat maps of mRNA expression and DNA methylation at distinct stages of B cell division indicated higher expression and less DNA methylation for populations 8– and 8+ than for cells at division 0 (Fig. 5f). Cells at division 5 and the 8– and 8+ populations had on average more mRNA per cell than that of cells at division 0, and this corresponded with significantly less average DNA methylation for populations 8– and 8+ than for cells at division 0 (Fig. 5g). The distribution of mRNA in cells at division 5 and the 8– and 8+ populations identified a uniformly greater abundance of mRNA across a wide range of expression than in undivided cells (Fig. 5h), which suggested that the increased mRNA expression was not solely attributable to a few mRNAs with high expression, such as those encoding immunoglobulins. In addition, cells that underwent eight or more divisions had fewer hypermethylated loci than that of cells that divided zero, one, three or five times (Fig. 5h). PCA indicated that the greatest variation in gene expression was between the 8+ population and cells at all other divisions (Fig. 5i). There were gradual changes in total DNA methylation between divisions 0, 1, 3 and 5, but the largest variation in DNA methylation occurred after division 8 (Fig. 5i), which suggested that some DNA-methylation changes in the 8+ population might have preceded expression changes. Cumulatively, these data identified a division-dependent global amplification of mRNA that corresponded to DNA hypomethylation in antigen-induced differentiating B cells.

Next, pairwise comparisons of gene expression between cells at division 0 and those at divisions 1, 3, 5 and the 8– and 8+ populations indicated that gene expression was mostly upregulated as cells divided, especially at later divisions (Fig. 6a). We identified ‘differentially expressed’ genes using criteria for both the relative change in expression and absolute change in expression; thus, only genes upregulated twofold more than the average increase in expression for any division were considered to have significantly different expression. We also performed pairwise comparisons of DNA methylation in which we identified loci methylated differentially relative to their methylation at division 0. Few DNA-methylation changes occurred at early divisions, and most changes occurred between cells at division 0 and those at division 5 and the 8– and 8+ populations (Fig. 6b). Finally, we compared the correlations between gene-expression changes and DNA-methylation changes at each division. We observed that 98% of DML associated with differentially expressed genes lost DNA methylation and gained gene expression through division 5 relative to their methylation and expression at division 0 (Fig. 6c). DML inversely correlated with gene expression in populations 8– and 8+ (with 94% and 72%, respectively, of the gene expression and DNA methylation being inversely correlated) (Fig. 6c). These data suggested that gene-expression changes had high correlation with local DNA-methylation changes in antigen-induced differentiating B cells.
Quantification revealed that 86% of division-specific differentially expressed genes were upregulated with increasing cellular division relative to their expression in undivided B cells and only 14% of genes were downregulated, with the majority of downregulated genes occurring between cells at division 0 and the 8+ population (Fig. 7a). Gene-ontology analysis indicated that genes upregulated in cells at division 5 and the 8− and 8+ populations relative to their expression in cells at division 0 encoded products involved in mitosis and metabolism, and genes downregulated in the 8+ population relative to their expression in cells at division 0 encoded products involved in mitosis and metabolism, and genes downregulated in the 8+ population relative to their expression in cells at division 0 encoded products involved in cell activation (Fig. 7b and Supplementary Table 3). Gene-set–enrichment analysis also indicated a large number of gene sets associated with genes upregulated in cells at divisions 1–8 (including the 8− and 8+ populations) relative to their expression in cells at division 0 (Fig. 7c). Genes sets upregulated in those cells encoded products associated with cell division, Myc targets, the proteasome, endoplasmic reticulum stress and processing of proteins, while genes sets regulated by the transcription factor NF-κB in response to treatment with tumor-necrosis factor and components of type I interferon signaling were downregulated in populations 8− and 8+ relative to their expression in cells at division 0 (Fig. 7c and Supplementary Table 4). Dynamic regulation of genes across cell division included downregulation of Cxcr5 and Lcost, transient upregulation of Cd83, Fas, Tlr9, Tbx21 and Aicda and upregulation of Cxcl10, Schmt2, Txndc5, Rexo2, Solk, Cxcr4 and Xbp1 (Fig. 7d). Thus, gene expression was globally upregulated with increasing division, yet annotation of genes whose expression was ‘preferentially’ increased or decreased through division stages indicated selective regulation of pathways important for B cell and plasma cell biology.

When we compared division-specific DNA-methylation changes with methylation at division 0, we found that most such changes were hypomethylation events at later divisions (Fig. 7e). Demethylated CpG loci continued to lose DNA methylation at later divisions (Fig. 7f), and these loci were organized into contiguous regions (Fig. 7g), which identified these changes as focal epigenetic events. Division-specific DNA-methylation changes showed substantial overlap with enhancers in B cells, splenocytes and CH12 cells (Fig. 7h). This was most pronounced at earlier divisions and was considerably less for enhancers in tissue from thymus, testis and brain (Fig. 7h). We used HOMER33 to overlap DML with known transcription-factor–binding motifs and observed that demethylated loci ‘preferentially’ occurred near (within 50 base pairs or less) motifs for bZIP, IRF, MADS, POU and RHD transcription factors (Fig. 7i). Demethylated loci were present near binding motifs for the transcription factors NF-κB (RHD) and AP-1 (bZIP) starting at division 3, whereas binding motifs for members of the IRF and POU families showed enrichment only for demethylated loci specific to the 8− and 8+ populations (Fig. 7i), which suggested a hierarchy of transcription-factor use. Demethylated loci proximal to the motifs noted above lost more DNA methylation than did those not associated with a motif (Fig. 7j). Genes associated with such motifs that contained demethylated loci had higher expression at each division than that of genes that contained the motif but were not demethylated (Fig. 7k). These data suggested that the transcriptional changes were being driven by an ordered demethylation of key cis-regulatory elements.

Global gene expression and average DNA methylation were inversely correlated with each other (Fig. 8a). To understand individual correlations of gene expression with DNA methylation at each division, we annotated demethylated loci to the nearest gene and compared distinct patterns of DNA methylation and gene expression by a normalized Euclidean–distance metric, such that similar patterns had smaller distances. We used this metric to categorize gene-expression and DNA-methylation patterns into four groups by K-means clustering (Fig. 8b). Genes in clusters 1 and 4 showed negative correlation with expression, DNA methylation and division, with cluster 4 being the largest and most homogeneous group (Fig. 8b). In contrast, clusters 2 and 3 displayed a progressive loss of DNA methylation with transient gene expression in cells from division 1 to the 8+ population (Fig. 8b). Gene-ontology annotation revealed distinct functional categories for each cluster; clusters 1 and 4 showed enrichment for genes encoding products involved in endoplasmic reticulum stress, and genes encoding products involved in metabolic processes were exclusive to cluster 4 (Fig. 8c). Cluster 2 was associated with genes encoding products involved in anatomical morphogenesis and cell adhesion, and cluster 3 was associated with genes encoding products involved in leukocyte activation (Fig. 8c and Supplementary Table 2). DNA-methylation and gene-expression changes characteristic of clusters 1 and 4 were present at regions near Prdm1 and Il10 (which encode important regulators of plasma cell biology), as well as C1qbp and Rexo2 (which encode products important for mitochondrial metabolism) (Fig. 8d). Several CpG loci near Cdb80, Cdb83, Cdb86 (which encode products involved in B cell activation), Aicda (which encodes AID, a protein required for class-switch recombination and somatic hypermutation) and Abil1m1 (which encodes a molecule involved in cell adhesion that has also been linked to...
Figure 7 Dynamic gene-expression changes correspond with a hierarchy of DNA hypomethylation. (a) Quantification of differentially expressed genes (DEGs) upregulated (numbers above bars) or downregulated (numbers below bars) in cells at various stages of division (horizontal axis) relative to their expression in cells at division 0. (b) Gene-ontology results for genes upregulated (left) or downregulated (right) as in a; rows, ontology with the most significant enrichment (right margin) for genes with division-specific differential expression; columns, relationship to genes expressed differentially in other division-specific populations. (c) Quantification of positively correlated results (numbers above bars) and negatively correlated results (numbers below bars) for gene-set-enrichment analysis (left) and gene-set-enrichment analysis showing the upregulation of genes encoding components of proteosome and Myc signaling and downregulation of genes encoding components of the tumor-necrosis factor (TNF) and interferon-α (IFN-α) pathways (right; presented as in Fig. 1f). (d) RNA-seq analysis of genes (vertical axes; average mRNA per cell) in cells at various stages of division (horizontal axes). (e) Quantification of DML at various stages of division (below plot) relative to that at division 0 (presented as in Fig. 2e). (f) DNA methylation at division-specific DML. (g) Frequency of DML in contiguous blocks (presented as in Fig. 2h). (h) Odds ratio of overlap for division-specific demethylated loci with tissue-specific enhancers (presented as in Fig. 3a). (i) Enrichment for transcription-factor-binding motifs in division-specific demethylated regions (presented as in Fig. 3b). (j) Frequency of differences in DNA methylation for DML near transcription-factor-binding motifs in l, in cells at various stages of division (horizontal axis). (k) Expression of genes containing the motifs in l with (+) or without (−) demethylated DML (dDML) in cells at various stages of division (bottom). *P < 0.001 (Fisher’s exact test (b,h), permutation testing (g), ANOVA (j) or Wilcoxon rank-sum test (k)). Data are from Figure 5e (average of two biological replicates).
B cell differentiation were clusters 2 and 3 (Fig. 8d and data not shown). Thus, these analyses revealed several regions at which DNA methylation was lost, with transient gene expression occurring from division 1 to the 8− population before silencing in the 8+ population. Together these results suggested that DNA methylation reflects a 'historical footprint' of gene regulation by cell division in B cell differentiation.

DISCUSSION
Here we have provided insight into dynamic gene-expression and epigenetic changes during B cell differentiation in vivo. Plasmablasts and plasma cells had distinct gene-expression programs, and both underwent focal DNA-methylation changes at up to 10% of their DNA methylome. More than 99% of DNA-methylation changes were demethylation events. Differentiating B cells increased their global mRNA expression by more than fivefold in a division-dependent manner. This increase was attributable to transcriptional amplification of thousands of mRNAs, not just immunoglobulin-encoding mRNAs, and might be required for maintenance of the B cell fate program, while the cells are undergoing massive proliferation and differentiation. Pairing gene-expression analysis with DNA-methylation data from the same division-specific, differentiating B cells provided critical insight into the functional categories of gene-expression and DNA-methylation changes and indicated that demethylation occurred at transiently expressed genes encoding products involved in B cell activation and at genes expressed specifically in plasma cells. Transcriptional changes showed high correlation with cellular division and DNA demethylation of transcription-factor-binding motifs, which would provide a mechanism by which DNA-methylation changes control enhancer accessibility and programmatic fate in differentiating B cells.

DNA-methylation studies of human steady-state plasma cells have shown that 60–90% of DNA-methylation differences are hypomethylation events relative to the methylation status of B cells, whereas the changes we reported here were 99.7% hypomethylation events relative to the methylation status of B cells. Such a difference in observed DNA hypomethylation might reflect the kinetics of the process, as the plasma cells we analyzed here were less than 3 days old, whereas the half-life of steady-state plasma cells can range from several days to more than 100 days depending on the subpopulation. If this were indeed the case, then our data would suggest that an abrupt and targeted loss of DNA methylation occurs during the initial stages of differentiation but this may be followed by gradual gains in DNA methylation as plasma cells age.
Demethylated loci in differentiating B cells were overrepresented at B cell enhancers and binding motifs for transcription factors required for B cell differentiation. This suggests that certain B cell enhancers are used upon activation and that as the cells divide and differentiate, they make use of plasma cell regulatory elements not currently defined in public data sets. That hypothesis was supported by the co-localization of transcription-factor-binding motifs and demethylated regions, such that differentiating cells showed enrichment for binding motifs for NF-kB and AP-1 (which are directly induced by signaling via LPS and Toll-like receptor 4) as early as division 3, whereas only in cells that divided eight times or more were Oct-2- and IRF-binding motifs enriched at demethylated loci. Thus, the data indicated that the DNA hypomethylation observed were Oct-2- and IRF-binding motifs enriched at demethylated loci. It is likely that a passive process partially accounted for the DNA demethylation we observed here. That proposal was supported by the cell-division-specific DNA methylation that showed that the majority of changes occurred in cells that had undergone many rounds of division. However, DNA-methylation changes were focal at regulatory regions, suggestive of a targeted process. One possibility is that DNA demethylation is facilitated by the binding of transcription factors that block replication-coupled DNA-methyltransferase activity. The observed demethylation could also have resulted from 5-hydroxymethylation of cytosine residues in B cells, which does not have a known mechanism of maintenance through mitosis. Although there is no evidence for active demethylation during B cell differentiation, the possibility of such a process cannot be ruled out.

Treatment of cells ex vivo with 5-azaC resulted in augmented plasma cell differentiation in a division-dependent manner, which suggested that loss of DNA methylation is limiting for plasma cell differentiation. This might be in contrast to observations that inhibition of DNA methylation reduces germinal-center formation. It is possible that DNA methylation functions to extend B cell activation at the cost of plasma cell differentiation. That is consistent with the greater DNA hypomethylation in plasma blasts that divided at least eight times than in B cells that also divided at least eight times. Such division-coupled epigenetic changes might also help explain why the number of divisions a B cell has undergone directly corresponds with the potential of that B cell to differentiate. Such epigenetic mechanisms might contribute to both the variability of the B cell response at the population level and the high correlation of the cell fates of sibling B cells. Our data do not preclude the possibility of other models of differentiation, such as the asymmetric division reported for T cells or a transcription-factor-centric model in which transcription factors drive or inhibit plasma cell formation. Instead, our data provide insight into an additional and critical layer of epigenetic regulation that influences B cell fate. Our results support a model in which DNA-methylation remodeling is coincident with gene expression and the cis-regulatory history of plasma cells and the epigenetic reprogramming events through cell division and differentiation.

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

Accession codes. GEO: gene-expression and RRBS data, GSE70294.

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

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AUTHOR CONTRIBUTIONS
B.G.B. contributed to experiment conception and design, performed the DNA-methylation analyses, mouse experiments, RNA-seq analysis and bioinformatics analyses and wrote the paper; C.D.S. contributed to experimental conception and design and performed RNA microarray analysis; A.P.R.B. provided technical expertise to mouse experiments; J.M.B. contributed to experimental conception and design and wrote the paper; and all authors provided editorial input.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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**ONLINE METHODS**

**Mice and LPS challenge.** C57BL/6J mice between 8 and 12 weeks of age were used for experiments except where otherwise specified. All animals were housed by the Emory Division of Animal Resources and all protocols were approved by the Emory Institutional Animal Care and Use Committee (IACUC). For animal studies a power analysis was performed and was included in our approved IACUC. Investigators were not blinded to sample identity, and no animals were excluded from analysis. Experiments were balanced such that similar numbers of mice were included in each group. LPS challenge was administered intravenously using 50 µg of *Salmonella* LPS (Enzo Life Sciences, ALX-581-008), and mice were analyzed 3 d after challenge. LPS-induced B cell differentiation was analyzed in two experiments with seven mice and eight mice, including eleven females and four males (Fig. 1). Microarray analysis and RRBS was performed on one experiment with three female mice. CD45.1+ mice were of strain B6.SJL-129Ssp-PecRd1BoyJ from Jackson Laboratories (#002014). B cell–deficient mice (µMT) were previously described and were also obtained from Jackson Laboratories (#002288, strain B6.129S2-Ighmtm1Cgn/J). For cell division assays, 20 × 10^6 CVT- or CFSE-labeled splenic CD45.1+B220+ B cells were adoptively transferred into µMT hosts and allowed to rest for 18–24 h before LPS challenge. Here, transferred splenic CD45.1+B220+ B cells were sex matched to hosts, and this was performed in three experiments that included three mice, six mice (Fig. 5a–d) and four mice (Fig. 5e–i) including three males and ten females.

**Cell Isolation and flow cytometry.** Splenic cell suspensions were made by mechanically forcing splenums through a 40 µm filter and lysing red blood cells with ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 30 s before quenching the reaction with four volumes RPMI 1640 media (Corning Cellgro) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 1% MEM non-essential amino acids, 100 µM sodium pyruvate (Sigma), 10 mM HEPES pH 7.3 (Sigma), 0.0035% β-mercaptoethanol (Sigma-Aldrich). Cells were washed and resuspended at 1 × 10^7 cells/ml in PBS with 1% BSA and 2% EDTA. Validation data for all antibodies used are available on the manufacturers’ websites. Prior to staining, cells were blocked with anti-Fc (anti-CD16/CD32) (Tonbo Biosciences, 2.4G2) at a final concentration of 0.25 µg per 1 × 10^6 cells for 15 min on ice. Staining panels included anti-CD11b (anti-CD11c (Tonbo Biosciences M1/70) and anti-CD11c (Tonbo Biosciences N418) conjugated to FITC or PerCP-Cy5.5 each at a concentration of 0.25 µg per 1 × 10^6 cells to remove autofluorescent macrophages. The following stains and antibody-fluorescein combinations were used to assess cellular phenotype: anti-B220-PerCP-Cy5.5 or -PE-Cy7 (Tonbo Biosciences, RA3-6B2) at 0.05 µg per 1 × 10^6 cells; anti-CD43-FITC (BD #553270) at 0.125 µg per 1 × 10^6 cells; anti-CD138-PE-BV421, or -BV711 (BD, 281-2) at 0.025 µg per 1 × 10^6 cells; anti-GL7-eFlour660 (eBioscience GL-7) at 0.025 µg per 1 × 10^6 cells; anti-CD45.1-APC-Cy7 (Tonbo Biosciences A20); Viability Violet Stain (Life Technologies L34955), CFSE (Tonbo #13-0850) and CTV (Life Technologies C34557). CFSE and CTV were both used at 10 mM per 1 × 10^6 cells/mL. Cells were stained for 30 min and fixed using 1% paraformaldehyde before analysis. Staining panels included fluorescence minus one (FMO) controls to ensure that correct compensation was applied, as well as isotype controls to assess non-specific staining. Flow cytometric analysis was collected on a Becton Dickinson (BD) LSRII and FCS files were exported using FACSDiva (v6.2). Analysis of flow cytometry data was conducted in R/Bioconductor (v3.2.2) using the ‘flowCore’ (v.1.36.9) package or FlowJo software (v9.7.6). Code is available upon request.

Naïve B cells were isolated using immunomagnetic negative selection for CD43, CD4, and Ter-119 (Miltenyi #130-090-862) following the manufacturer’s protocol. Purity was confirmed by flow cytometry. Plasmablasts and plasma cells were isolated by first enriching the CD138+ fraction of splenoocytes and/or bone marrow using a positive immunomagnetic enrichment on CD138 (Miltenyi #130-098-257) and then by flow cytometry using a BD FACSaria II at the Emory Flow Cytometry Core Laboratory. Adoptively transferred cells were isolated by labeling CD45.1+ cells with CD45.1-APC-Cy7 (Tonbo Biosciences A20) and immunomagnetic enrichment using anti-APC beads (Miltenyi #130-090-855) followed by flow cytometry using a BD FACSaria II at the Emory Flow Cytometry Core Laboratory.

**Ex vivo differentiation and 5-aza-cytidine treatment.** B cells were differentiated in software previously described, but with the incorporation of division-tracking dye. B cells were isolated using immunomagnetic separation as above, and stained with CVT or CFSE at a concentration of 20 × 10^6 cells/ml in PBS. Cells were differentiated at an initial concentration of 0.5 × 10^6 cells per ml with LPS (20 µg/ml; Sigma #L2630), IL-2 (20 ng/ml; eBioscience #14-8021), and IL-5 (5 ng/ml; eBioscience #14-8051). Half doses of LPS and cytokines were given on subsequent days. 5-azacytidine (Sigma-Aldrich #A2385) was added to cultures every day at concentrations ranging from 50 nM to 500 nM as indicated.

**Reverse-transcription quantitative PCR (RT-qPCR) analysis.** Gene expression was validated using 2 × 10^6 cells sorted into RLT lysis buffer, and total RNA was purified using the Quick-RNA MicroPrep Kit (Zymo Research). The entire RNA yield was reverse transcribed using Superscript II reverse transcriptase (Invitrogen), diluted before quantitative real-time PCR (qRT-PCR) on a CFX96 instrument (Bio-Rad) using SYBR Green incorporation. Expression is presented relative to that of 18S ribosomal RNA for each gene analyzed. A full list of qRT-PCR primers can be found in Supplementary Table 6.

**Code availability.** All code is available upon request.

**Microarray analysis.** Total RNA from each cell type was extracted using the RNeasy mini prep kit (Qiagen) and was used for microarray analysis on the MouseRef-8v2 BeadChip (Illumina). Gene expression data were quantile normalized using GenomeStudio v.2011.1 (Illumina) and exported for analysis. Quality control (QC) steps included mapping all probes to the mouse reference genome (mm9) using Bowtie49 (v.1.0.0) and removing probes that had multiple alignments, or did not align to a UCSC Known Gene exon50. The UCSC known gene database was obtained via the R/Bioconductor package ‘TxDb.Mmuculus.UCSC.mm9.knownGene’ (v3.2.2). This resulted in 22,907 of 25,697 probes passing quality control and coverage of 16,181 genes. Differential expression was identified using linear regression implemented in R/Bioconductor (v3.1.3)51. Multiple hypothesis testing was applied to probes with signal that was detected in at least one sample (N = 10,322) and with a change in expression of twofold or greater using a Benjamini-Hochberg false discovery rate (FDR)52. Those genes with a FDR of less than 0.01 were considered significant (Supplementary Table 1).

**RNA-seq analysis.** For RNA-seq analysis, 4 × 10^6 cells were sorted into RLT buffer (Qiagen) with 1% 2-mercaptoethanol (Sigma), vortexed and snap frozen. Prior to extraction 5 µl of 1:2,000 dilution of ERCC synthetic RNAs (ThermoFisher) were added to each sample. RNA was extract using RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. DNA was removed with RQ1 DNase (Promega) at 37 °C for 30 min and RNA was purified using 3× AMPPure XP clean up (Beckman Coulter). Stranded mRNA-seq libraries were constructed using KAPA Biosystems Stranded mRNA-Seq Kit following the manufacturer’s protocol (KAPA Biosystems). Equal molar amounts of mRNA-seq libraries were amplified for 11 PCR cycles and purified using a 1.5x ratio of AMPure XP beads (Beckman Coulter). Libraries were pooled in equal molar ratios based on the KAPA Library Quantification Kit (KAPA Biosystems) and sequenced on an Illumina HiSeq2500 with 50-base-pair paired-end reads.

**Mapping and quantification of RNA-seq data.** RNA-seq data was mapped back to the UCSC mouse genome mm9 using TopHat2 (ref. 53) (v2.0.13) with the following parameters “–p 8 –N2 –max-multihits 1 –read-gap-length 1” and the UCSC Known Genes50 mm9 transcript file as a guide. The 92 ERCC sequences were added to the mm9 genome as artificial chromosomes. PCR duplicates were determined using Picard (http://broadinstitute.github.io/picard/) and removed from subsequent analyses. Reads that uniquely overlapped mm9 exons were determined in R (v3.2.2) using the ‘summarizeOverlaps’ function in mode ‘IntersectionNotEmpty’ of the ‘GenomicAlignments’ package (v1.6.3). Reads per million (RPM) were calculated for each gene based on the number reads in all potential exons for a given gene and the total number of uniquely mappable reads per sample.
Fragments per kilobase per million (FPKM) were calculated based on RPM and the total size of non-overlapping exons for a gene. The number of mRNA molecules per cell was calculated with the following equation:

$$\text{mRNA}_{\text{geneA}} = \frac{\text{FPKM}_{\text{geneA}}}{\text{cells}} \times \frac{\text{molecules}}{\sum \text{FPKM}_{\text{ERCC}}}$$

**Differential analysis of RNA-seq data.** Differentially expressed genes (DEGs) were determined using EdgeR55 (v.3.12.0) based on both relative and absolute changes in expression. Gene counts were calculated using all reads mapping to exons of unique UCSC mm9 Known Genes56, determined as described above. For relative differences, normalization factors were determined using the EdgeR function “calcNormFactors”. For absolute differences, the normalization factors were determined as the sum of ERCC FPKM divided by the average ERCC FPKM across all samples. A minimum change in expression of twofold was imposed upon criteria for both relative and absolute differences. P values calculated by EdgeR were corrected for multiple hypothesis testing using Benjamini-Hochberg FDR correction52. In total, DEGs with an FDR of ≤0.01 with a change in expression of twofold or more by both relative criteria and absolute criteria were considered significant.

**Bioinformatics analysis of expression data.** Heat maps and hierarchical clustering of gene-expression data were used an ‘average’ or unweighted pair group method with arithmetic mean agglomeration method applied to the z-score-normalized probe signal (microarray) or average number of mRNAs/ cell (RNA-seq) using the R/Bioconductor functions ‘hclust’ and ‘image’ in a method very similar to that employed by the ‘heat map’ function51 (R code available upon request). PCA was done using the R/Bioconductor function ‘prcomp’ also applied to Z-score normalized expression data.

Gene ontology analysis was conducted on differentially expressed genes (DEGs) using the R/Bioconductor package GOstats (v2.32.0)56. For microarray data, all genes with probes that passed QC were used as background. For RNA-seq data, all UCSC mm9 Known Genes50 were used as background. Gene Set Enrichment Analysis (GSEA v2.1.0) was performed using the pre-ranked list option. For microarray data, the rank was determined by the average t-statistic for all probes mapping to a given gene. Only probes that were detected in at least one sample were used. For RNA-seq data the rank was determined by the $-\log_{10}(\text{FDR}) \times \text{sign}(\text{fold change in expression})$. Here, the FDR is the average FDR determined using both absolute and criteria described above.

**DNA-methylation assay.** DNA was isolated from cells digested overnight with proteinase K and RNase at 67 °C and was extracted using phenol-chloroform-isoamylalcohol and ethanol precipitation. RRBS libraries were made from 10 to 500 ng of DNA and were digested overnight with 20 U MspI (New England Biolabs) following the manufacturer’s protocol. RRBS libraries for B cell divisions were also digested with TaqI in separate reactions (New England Biolabs). Digested DNA was purified using a 1.8× Solid Phase Reversible Immobilization (SPRI) clean-up with Agencourt AMPure XP beads (Beckman Coulter). Illumina compatible sequencing adapters were used and contained fully methylated cytosine residues and were either NEXTflex Bisulfite-Seq Barcodes (BIOO Scientific) or were designed similar to those previously described57 and synthesized by Integrated DNA Technologies. DNA was end-repaired and A-tailed and sequencing adaptors were ligated using the Hyper Prep Kit (KAPA Biosystems) following the manufacturer’s protocol. Adaptor-ligated DNA was bisulfite treated using the Epitect Bisulfite Kit (Qiagen), modifying the manufacturer’s protocol by extending the denaturation thermocycler step from 5 min to 10 min at 99 °C. Adaptor-ligated bisulfite treated libraries were amplified 10–15 times using HiFi Urevl+ Polymerase (KAPA Biosystems) and library concentration was estimated using the KAPA quantification kit (KAPA Biosystems). Size was estimated using a high sensitivity DNA chip (Agilent Technologies). Libraries were sequenced using 50-base-pair single-end or paired-end reads on a HiSeq2500 by the Genome Technology Center at New York University (NYU).

Combined Bisulfite Restriction Analysis (COBRA) was performed similar to that previously described25. Briefly, high molecular weight DNA was bisulfite treated (see above) and 1 ng of bisulfite converted DNA was amplified 35–40 times using JumpStart Taq polymerase (Sigma) and bisulfite primers (Supplementary Table 6). Half of the amplified product was digested with TaqI (NEB) or BstUI (NEB) and the other half was mock digested as a control, before visualization on 1.5–2% agarose gels. DNA methylation was also quantitated by a qPCR approach where genomic DNA was aliquoted into three equal portions: one was mock digested to quantitate the total amount of DNA, one was digested with the methyl-sensitive restriction enzyme HpaII to quantitate unmethylated DNA, and the final aliquot was digested with the methyl-insensitive isoschizomer MspI as a negative control. Equal portions of each aliquot were subjected to qPCR, and DNA methylation levels were quantitated as the ratio of HpaII-digested material to mock-digested material. Quantitation was based upon a standard curve of genomic DNA and all primers (Supplementary Table 6) were between 90% and 100% efficient.

**DNA-methylation bioinformatics analysis.** RRBS data were aligned to the in silico bisulfite converted genome (mm9) using Bismark58 (v.0.13.1) with the Bowtie2 (ref. 59) option. Binary alignment map (BAM) files were parsed to derive DNA methylation calls that were collapsed to the CpG level using custom R scripts that made use of the ‘Rsamtools’ (v.1.2.2.0) and ‘data.table’ (v.1.9.6) packages (code available upon request). Data were compiled into data sets that included a sample specific coverage (minimum 10X coverage per sample) and a group specific coverage (minimum 10X coverage per group). Hierarchical clustering and PCA were performed on sample specific data sets in a manner analogous to that described for the gene expression analysis except no normalization was performed and heat maps were ordered by increasing DNA methylation from top to bottom. The distribution of DNA methylation values were assessed using the ‘density’ function in R/Bioconductor and represents the probability density function across sample specific coverage. Average methylation was determined based on sample coverage and differences were determined with Welch’s t-test. B cell, plasmablast and plasma cell differentially methylated CpG loci (DML) were identified using Dispersion Shrinkage for Sequencing (DSS)19 and division specific DML were determined using the general experimental design version of DSS60. DSS was applied to group level data and CpG loci that had an FDR of 50.01 with a minimum change of 20% in DNA methylation were considered significant. Contiguous DML were defined as two or more DML that were located adjacent to each other on the genome relative to assay group coverage. Significance of DML contiguity was assessed by permutation analysis. This involved randomly permuting DML 1,000 times and calculating the percent of permutated DML that occurred in contiguous regions for each permutation. The P value was determined by the number of times that the permuted value was equal to or greater than the actual value.

Overlap of DML with enhancer elements (methods described in the Meta-analysis subsection) was assessed using Fisher’s exact test61 implemented in R/Bioconductor. Transcription factor motifs enriched within 50 bp of DML were determined with HOMER software33 (v.4.7.2) relative to RRBS assay coverage. Results with an FDR ≤0.05 were considered significant. Motif position weight matrices were clustered using the “PWMSimilarity” function of the “TFBSTools” package62 (v.1.8.2) with a minimum overlap of 6 nucleotides. Data were clustered using hierarchical clustering as described above. Code is available upon request.

**Correlation of gene expression and DNA methylation.** To analyze DNA methylation and gene expression correlation, CpG loci were annotated to the closest UCSC mm9 Known Gene34 transcript using custom R/Bioconductor code (available on request). CpG loci that were within 100 kb of a transcript were assigned to the closest gene. Subsequently, the change in expression (‘fold’ value) for each gene was plotted by the change in absolute DNA methylation. Significance of inverse correlation was assessed using Fisher’s exact test34,61 (implemented in R) to determine if more DML-DEG correlations were negatively associated than expected by chance. The correlation of gene expression and DNA methylation was assessed with the Spearman’s rank correlation coefficient. The level of association was determined using the Mantel test62 (implemented in R) with a 95% confidence interval.

**Meta-analysis.** Analysis of histone modifications used previously described chromatin immunoprecipitation sequencing (ChIP-seq) experiments performed on primary B cells31–33,63,64. ChIP-seq data were obtained from Gene Expression Omnibus experiments GSE30859 (ref. 33), GSE38046 (ref. 64), GSE51336 (ref. 31), GSE42706 (ref. 63) and GSE51011 (ref. 32); specific data...
sets are listed in Supplementary Table 7. Data were uniformly aligned and processed to the mouse genome (mm9) using Bowtie2 (ref. 59) (v.2.1.0). ChIP-seq fragment size for each data set was calculated using the "chip-seq" (v.1.20.0) package in R/Bioconductor based on the SISSEr method previously described. Enriched regions for those published by the ENCODE project were downloaded from the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgFileSearch?db=mm9). Enriched regions for other studies were determined using MACS software (v1.4). Enhancers were determined for spleen, CH12 cells, thymus, and whole brain H3K4me1, H3K27ac, and H3K4me3 data by taking overlapping H3K4me1 and H3K27ac regions that did not overlap a region enriched for H3K4me3. Odds ratio and significance of overlap with DML were determined using Fisher’s exact test.

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